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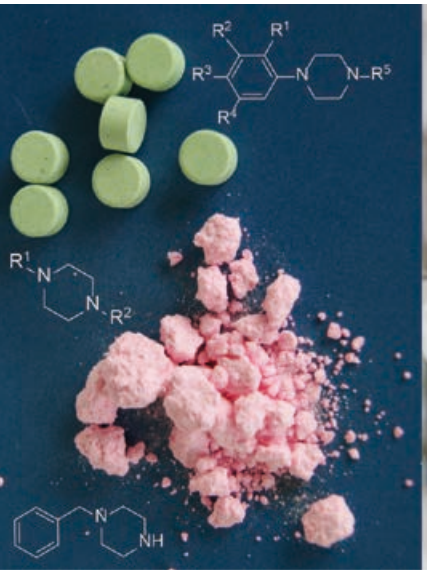

Recommended methods for the **Identification and Analysis of Piperazines in Seized Materials**

MANUAL FOR USE BY NATIONAL DRUG ANALYSIS LABORATORIES

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Laboratory and Scientific Section
UNITED NATIONS OFFICE ON DRUGS AND CRIME
Vienna

Recommended Methods for the Identification and Analysis of Piperazines in Seized Materials

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UNITED NATIONS
New York, 2013

Note

Operating and experimental conditions are reproduced from the original reference materials, including unpublished methods, validated and used in selected national laboratories as per the list of references. A number of alternative conditions and substitution of named commercial products may provide comparable results in many cases, but any modification has to be validated before it is integrated into laboratory routines.

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Contents

1. Introduction

1.1 Background

Piperazine, a heterocyclic six-membered ring compound which contains two nitrogens in the 1 and 4 positions, is a cyclic member of the ethylenediamine group of molecules [1, 2]. The abuse of substituted derivatives of piperazine was first reported in the United States in 1996 and has, since then, spread to a number of countries worldwide [3]. The large scale use of synthetic derivatives of piperazine as substitutes or mimics of "ecstasy" started in New Zealand in the early 2000s and became common in Europe after 2004 [4].

The first piperazine derivative encountered was 1-benzylpiperazine (BZP), one of a group of phenyl and benzyl substituted piperazines that have become prevalent worldwide, especially in traditional 3,4-methylenedioxymethamphetamine (MDMA) markets. Other widely used piperazines include 1-(3-chlorophenyl)piperazine, (*m*CPP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), the latter of which is commonly found in combination with BZP.

BZP itself is a central nervous system stimulant with a potency of 10% that of d-amfetamine [4]. It has been reported to stimulate the release of dopamine, noradrenaline, and serotonin, and also inhibit their reuptake. The substances are thus amfetamine mimics and predominantly found in tablet form either alone, in combination with other piperazines or with amfetamine, cocaine, ketamine or MDMA.

Neither BZP nor any other substituted piperazine are listed in the Schedules of the United Nations 1971 Convention on Psychotropic Substances. However, in 2007, the International Narcotics Control Board (INCB) requested the World Health Organization (WHO) to consider reviewing piperazine derived compounds for possible scheduling under the 1971 Convention. Independently, many countries have introduced legislation controlling the use of BZP. This includes the United States and the countries of the European Union (EU), which submitted BZP for EU control in 2008 following a risk assessment using the early warning system of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [5].

1.2 Purpose and use of the *Manual*

The present *Manual* is one in a series of similar publications dealing with the identification and analysis of various classes of drugs under international control. These manuals are the outcome of a programme pursued by UNODC since the early 1980s, aimed at the harmonization and establishment of recommended methods of analysis for national drug analysis laboratories.

This *Manual* was prepared taking into account the Commission on Narcotic Drugs 2012 resolution: 55/1 "Promoting international cooperation in responding to the challenges posed by new psychoactive substances", which encourages the United Nations Office on Drugs and Crime and other relevant international organizations, upon request, to provide Member States with technical assistance, including by supporting forensic and toxicological capability, to respond to the challenges posed by new psychoactive substances.

In accordance with the overall objective of the series, the present manual suggests approaches that may assist drug analysts in the selection of methods appropriate for the sample under examination and provide data suitable for the purpose at hand, leaving room also for adaptation to the level of sophistication of different laboratories and various legal requirements. The majority of methods included in this manual are validated, methods which have been used for a number of years in reputable laboratories and as part of inter-laboratory studies, collaborative exercises and proficiency tests. The reader should be aware, however, that there are a number of other methods, including those published in the forensic science literature, which may also produce acceptable results. **Any new method that is to be used in the reader's laboratory must be validated and/or verified prior to routine use.**

In addition, there are a number of more sophisticated approaches, but they may not be necessary for routine operational applications. Therefore, the methods described here should be understood as guidance, that is, minor modifications to suit local circumstances should not affect the validity of the results. The choice of the methodology and approach to analysis, as well as the decision whether or not additional methods are required, remain with the analyst and may also be dependent on the availability of appropriate instrumentation and the level of legally acceptable proof in the jurisdiction within which the analyst works.

Attention is also drawn to the vital importance of the availability to drug analysts of reference materials and books on drugs of abuse and the analytical techniques used for their identification. Moreover, the analyst must of necessity keep abreast of current trends in drug analysis, consistently following current analytical and forensic science literature.

UNODC's Laboratory and Scientific Section would welcome observations on the contents and usefulness of the present *Manual*. Comments and suggestions may be addressed to:

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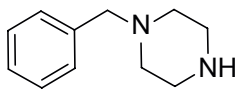
All manuals, as well as guidelines and other scientific-technical publications, may be requested by contacting the address above.

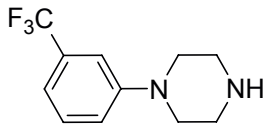
2. General aspects

2.1 Description of the pure compounds.

The following table presents the structures and selected data for the three most commonly encountered piperazines. A comprehensive list of piperazines is provided in table 2.

Table 1. Description of most common piperazines

1-Benzylpiperazine (BZP) 	Empirical formula:	$C_{11}H_{16}N_2$
	CAS No.:	2759-28-6
	Molecular weight:	176.26 g/mol
	Refractive index:	1.5470
	Density:	1.014 g/ml
	Physical appearance:	clear to yellowish liquid

1-(3-Trifluoromethylphenyl) piperazine (TFMPP) 	Empirical formula:	$C_{11}H_{13}F_3N_2$
	CAS No.:	15532-75-9
	Molecular weight:	230.23 g/mol
	Refractive index:	1.521
	Density:	1.226 g/ml
	Physical appearance:	white powder

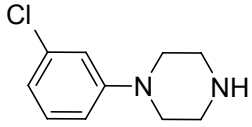
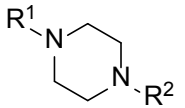
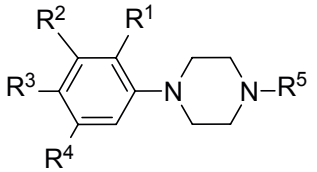
1-(3-Chlorophenyl)piperazine (*m*CPP) 	Empirical formula:	$C_{10}H_{13}ClN_2$
	CAS No.:	6640-24-0
	Molecular weight:	196.68 g/mol
	Refractive index:	1.598-1.600
	Density:	1.19 - 1.195 g/ml
	Physical appearance:	clear to yellowish liquid

Table 2. Chemical structures and description of selected piperazines



Common name	*Abbreviation*	*CAS number*	R_1	R_2
Piperazine		110-85-0	H	H
1-Benzylpiperazine	BZP	2759-28-6	Ph-CH_2	H
1-Benzyl-4-methylpiperazine	MBZP	374898-00-7	Ph-CH_2	CH_3
1,4-Dibenzylpiperazine	DBZP	1034-11-3	Ph-CH_2	C_7H_7
1-(3-Thienylmethyl)piperazine	3-TMP	130288-91-4	C_5H_5S	H
1-(2-Phenylethyl)piperazine	2-PEP	5321-49-3	Ph-CH_2-CH_2	H
1-(3,4-Methylenedioxyphenyl)piperazine	MDBZP	55827-51-5	3,4-methylenedioxybenzyl	H



Common name	*Abbreviation*	*CAS number*	R_1	R_2	R_3	R_4	R_5
1-(2-Methoxyphenyl)piperazine	2-MeOPP / *o*MeOPP	35386-24-4	OCH_3	H	H	H	H
1-(3-Methoxyphenyl)piperazine	3-MeOPP / *m*MeOPP	16015-71-7	H	OCH_3	H	H	H

1-(4-Methoxyphenyl)piperazine	4-MeOPP / *p*MeOPP	38212-30-5	H	H	OCH_3	H	H
1-(2-Trifluoromethylphenyl)piperazine	*o*TFMPP	3854-31-9	CF_3	H	H	H	H
1-(3-Trifluoromethylphenyl)piperazine	TFMPP / *m*TFMPP	15532-75-9	H	CF_3	H	H	H
1-(4-Trifluoromethylphenyl)piperazine	*p*TFMPP	30459-17-7	H	H	CF_3	H	H
2-Methylphenylpiperazine	2-MePP / *o*MePP	39512-51-1	CH_3	H	H	H	H
3-Methylphenylpiperazine	3-MePP / *m*MePP	41186-03-2	H	CH_3	H	H	H
4-Methylphenylpiperazine	4-MePP / *p*MePP	39593-08-3	H	H	CH_3	H	H
1-(4-Bromo-2,5-dimethoxybenzyl) piperazine	2C-B BZP	1094424-37-9	OCH_3	H	Br	OCH_3	H
1-(2-Chlorophenyl)piperazine	2CPP / *o*CPP	41202-32-8	Cl	H	H	H	H
1-(3-Chlorophenyl)piperazine	*m*CPP	6640-24-0	H	Cl	H	H	H
1-(4-Chlorophenyl)piperazine	4-CPP / *p*CPP	38212-33-8	H	H	Cl	H	H
1-(2-Fluorophenyl)piperazine	2-FPP / *o*FPP	1011-15-0	F	H	H	H	H
1-(4-Fluorophenyl)piperazine	4-FPP / *p*FPP	2252-63-3	H	H	F	H	H
1-(2,3-Dimethylphenyl)piperazine	2,3-XP	1013-22-5	CH_3	CH_3	H	H	H
1-(3,4-Dimethylphenyl)piperazine	3,4-XP	1014-05-7	H	CH_3	CH_3	H	H
1-(2,5-Dimethylphenyl)piperazine	2,5-XP	1013-25-8	CH_3	H	H	CH_3	H
1-(2,4-Dimethylphenyl)piperazine	2,4-XP	1013-76-9	CH_3	H	CH_3	H	H
1-(3-Chlorophenyl)-4-(3-chloropropyl) piperazine	*m*CPCPP	39577-43-0	H	Cl	H	H	C_3H_6Cl

2.2 Licit uses

1-Benzylpiperazine (BZP) and the other substituted piperazines listed in tables 1 and 2 have no current human or veterinary pharmaceutical use in any country, although piperazine itself is used as an anthelmintic drug. Piperazine derivatives serve as precursors or intermediates in the synthesis of many pharmaceutically active compounds, including ciproflaxin, the quinolone antibiotics, phenothiazines, sildenafil, tadalafil and antihelminthics [6, 7, 8, 9].

Of the substituted piperazines that have been used illicitly, *m*CPP is an synthetic precursor in the production, and an active metabolite, of the anti-depressants trazodone, nefazodone and etoperidon [10, 11]. 1-(3,4-methylenedioxyphenyl)piperazine (MDBZP) is a metabolite of the withdrawn nootropic drug fipexide and 1-(4-methoxyphenyl)piperazine (MeOPP) is a known metabolite of a number of prescribed drugs including enciprazione, milipertine and urapidil [11].

2.3 Control status

None of the benzyl or phenyl substituted piperazines covered in this *Manual* are listed in the Schedules of the United Nations 1971 Convention on Psychotropic Substances. However, many countries have introduced national control measures for some piperazines. For example, BZP was classified as a schedule 1 controlled substance in the United States in 2002, while the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) submitted BZP for EU control following completion of a risk assessment in 2008. 1-(3-Chlorophenyl)piperazine (*m*CPP) is not controlled internationally because it is used in drug synthesis, and has also not been submitted for risk assessment under the EU system, although a number of European countries have independently implemented measures for its control [12]. During a recent meeting of the WHO Expert Committee on Drug Dependence, several members of the piperazine family were pre-reviewed (BZP, TFMPP, *m*CPP, MeOPP and MDBZP) [11].

2.4 Illicit products/use

The bulk powders used in formulations of piperazines are readily available from commercial suppliers in both China and India. The bulk material is then cut with sugars and/or other drugs prior to processing into capsules and tablets, which are similarly priced to ecstasy. BZP is most often encountered as off-white or coloured tablets, which often bear imprints similar to those seen on MDMA tablets, and indeed the tablets are often sold as “ecstasy”. Typical concentrations of BZP in these formulations range from 50-200 mg. The concentration *m*CPP in seizures of tablets has been reported to be in the range 90-110 mg [13]. Seizures are often found to contain a mixture of substituted piperazines cut with caffeine, and often contain controlled substances such as MDMA, ketamine or amfetamine [4,14].

The piperazines are usually ingested as tablets or capsules. However with prolonged use, a more rapid drug response is often desired and this is usually achieved by smoking, snorting, or more rarely by injection. Snorting and injection have unpleasant side effects such as burning of the nasal passages with the former and a burning sensation with the latter route. These effects are a result of the typically very caustic nature of the piperazine formulations (pH of 12). For this reason, alcohol or some other drug is commonly used in conjunction with the piperazine to minimize these adverse effects. This class of drugs seems to attract a significant population of new drug users and this may be due to the perception that it is a safe/legal drug choice. Users of substituted piperazines seem to be more apt to use multi-drug cocktails than MDMA users, with alcohol, cannabis and synthetic cannabinoids being the most commonly reported drugs used in combination [8, 15, 16, 17].

2.5 Pharmacology

The majority of pharmacological studies of piperazines have focused on BZP and have indicated that it mimics the behaviour of d-amfetamine, with 10% of its potency. BZP has been reported to exhibit a potential for abuse and dependence similar to that of amphetamine, and causes a stimulus-like effect, increasing heart rate and systolic blood pressure. Furthermore, results from animal studies demonstrate that this compound stimulates the release, and inhibits the reuptake, of dopamine, serotonin (5-HT) and noradrenaline [17].

Research on mixtures of BZP and TFMPP (conducted because they are frequently found in combination) showed the release of both dopamine and serotonin via mechanisms dependent on their transporters [18]. Combinations of BZP and TFMPP, in proportions ranging from 2:1 to 10:1, have been reported to mimic the molecular mechanism of MDMA, causing similar entactogenic body effects and therefore making it a popular MDMA substitute [17, 19, 20].

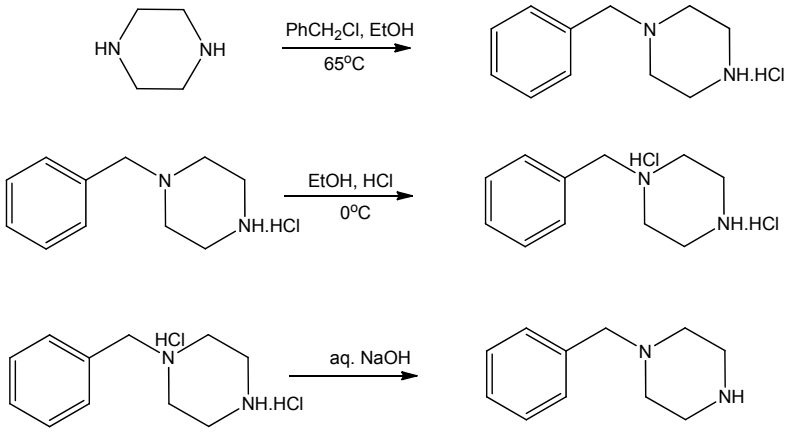
While there are a lack of detailed studies concerning many of the piperazine derivatives, there has been some research on *m*CPP, MDBZP and *p*MeOPP, though mostly on metabolism rather than toxicological effects [21]. It has been reported that serotonin syndrome, which induces symptoms such as anxiety, dizziness, confusion, shivering and sensitivity to light and noise, can develop following mCPP consumption [4]. In animal studies, high doses of BZP/TFMPP have been observed to cause seizures in rats. In humans, a high rate of adverse reactions, including severe toxicity and seizures, to the consumption of BZP/TFMPP pills within the recreational use range, with and without simultaneous alcohol intake have been reported [22, 23, 24, 25].

3. Illicit manufacture of piperazines

3.1 Illicit manufacture

The synthesis of BZP involves the reaction of piperazine and benzyl chloride, however, if piperazine as its free base is used, the reaction produces 1,4-dibenzylpiperazine (DBZP) as a by-product. The procedure shown in figure I utilizing a mixture of piperazine. HCl and piperazine hexahydrate proceeds with no formation of the dibenzylated compound [26]. The reaction at 65ºC produces the monohydrochloride salt, which upon cooling and treatment with HCl forms the dihydrochloride salt. The free base can be isolated by increasing the pH (> 12) and extracting with chloroform. The synthesis is simple and rapid with a very high yield (84-85%). The yield of the reaction can be increased to 95-96% and the reaction side products, including 1,4-dibenzylpiperazine (DBZP) reduced by using a microwave method, in which the transformation of the microwave energy into heat leads to increased reaction rates and higher yields [27].

Figure I. Synthesis of 1-benzylpiperazine (BZP).



There are several synthetic routes for 1-(3-chlorophenyl)piperazine (*m*CPP), the most common of which is the reaction of diethanolamine with *m*-chloroaniline. Other methods involve the reaction of *m*-chloroaniline with bis(2-chloroethyl)amine or the reaction of piperazine with *m*-dichlorobenzene. As with BZP, conventional synthesis routes are simple and produce high yields (84-86%) [2, 28, 29, 30]. However, it is unlikely that the BZP, TFMPP or ICPP found in illicit products have been synthesized in a clandestine laboratory as these compounds and their precursors are readily available commercially. Indeed, few piperazine clandestine laboratories have ever been encountered with the most recently recorded in the literature being in 2008 in Colorado, United States [29].

4. Qualitative and quantitative analysis of materials containing piperazines

Generally, in attempting to establish the identity of a controlled drug in suspect material, the analytical approach must entail the determination of at least two uncorrelated parameters, one of which should provide information on the chemical structure of the analyte (for example, IR, MS; or tandem methods such as GC-MS).

It is recognized that the selection of these parameters in any particular case would take into account the drug involved and the laboratory resources available to the analyst. It is also accepted that unique requirements in different jurisdictions may dictate the actual practices followed by a particular laboratory.

4.1 Sampling

The principal reason for a sampling procedure is to permit an accurate and meaningful chemical analysis. Because most qualitative and quantitative methods used in forensic drug analysis laboratories require very small aliquots of material, it is vital that these small aliquots be representative of the bulk from which they have been drawn. Sampling should conform to the principles of analytical chemistry, as laid down, for example, in national pharmacopoeias or by regional or international organizations. For general aspects of qualitative sampling of multi-unit samples, refer to the *Guidelines on Representative Drug Sampling* manual.

http://www.unodc.org/unodc/en/scientists/publications_manuals.html.

For seized material with obviously different external characteristics, a sampling method based on the Bayes' model may be preferred over the hypergeometric approach.

4.2 Solubility

The solubility properties provided in table 3 below can be utilized to separate piperazines from diluents and adulterants [31]. For example, ether or acetone may

be used to separate BZP from 3-MeOPP and 2-MeOPP since neither of those two compounds are very soluble in ether or acetone. BZP is also insoluble in water and this property could be utilized to separate BZP from hydrochloride salts.

Compound:	Acetone	Chloroform	Ether	Hexane	Methanol	Water
BZP	VS	PS	FS	VSS	S	I
TFMPP.HCl	SS	S	VSS	I	FS	VS
2-MeOPP.HCl	I	FS	VSS	I	FS	VS
3-MeOPP.2HCl	I	VSS	VSS	I	S	VS
4-MeOPP.2HCl	VSS	FS	I	I	FS	FS

Table 3. Solubility of selected piperazines [32]

Descriptive term	Parts of solvent required for 1 part of solute
Very soluble (VS)	Less than 1
Freely soluble (FS)	From 1 to 10
Soluble (S)	From 10 to 30
Sparingly soluble (PS)	From 30 to 100
Slightly soluble (SS)	From 100 to 1000
Very slightly soluble (VSS)	From 1000 to 10,000
Insoluble (I)	More than 10,000

4.3 Screening tests

A screening test is a preliminary test which is used to indicate or eliminate a class or group of drugs. It also has the function of narrowing the scope and focusing the direction of the analysis. By evaluating the results, further tests are indicated which can lead to the confirmation of the identity of the unknown substance.

4.3.1 Colour tests

Colour or chemical spot tests are used in forensic drug analysis as a quick method to give a presumptive indication of the possible presence or absence of a specific drug or class of drugs in a questioned sample. The colour obtained in any particular test may vary depending on the conditions of the test, amount of substance present, and extraneous material in the test sample. Colour tests are conducted by placing a small amount of a sample into a spot plate cavity. A small amount of the particular reagent is then added, and any resulting colour change is observed. Colour test reagents must be checked with known substances when prepared, and a blank should

be run next to the sample to preclude false positive results.

Colour tests are often non-specific in nature and serve to include (or exclude) the presence of a broad range of compounds. However, other colour reactions can be more specific and demonstrate the presence or absence of certain functional groups. By applying a series of different colour tests to the unknown sample, the analyst can narrow down the possible identity of the compound(s) present. It is mandatory for analysts to confirm such results by the use of alternative techniques. Information on the preparation of the various reagents is shown below and the subsequent table presents the observed colour changes with various amounts of the different piperazines tested.

(a) Marquis reagent [33]:

Reagent A:	40 % Formaldehyde solution
Reagent B:	Sulphuric acid (conc.)

Method
Mix 1 drop of formaldehyde solution with 1ml of concentrated sulphuric acid. Place the test sample in a spot plate depression and add 3 drops of the mixed reagents.

(b) Simon's reagent [33]:

Reagent A:	20% aqueous sodium carbonate solution
Reagent B:	50% ethanolic acetaldehyde solution
Reagent C:	1% aqueous sodium nitroprusside

Method
Prepared reagents should be stored in separate containers and refrigerated. Place the test sample in a spot plate depression and add 1 drop of reagent A, followed by equivalent amounts of reagent B, then reagent C.

(c) Dragendorff reagent [33]:

Reagent A:	Bismuth subnitrate (1g)
Reagent B:	Hydrochloric acid (conc.)
Reagent C:	Ammonia (25%, aq)
Reagent D:	Potassium iodide (3g)
Reagent E:	Acetic acid (70%, aq)

Method
Dissolve 1g of bismuth subnitrate in a small amount of concentrated HCl. Add 25% aqueous ammonia drop-wise until no more precipitate forms. Filter and preserve the precipitate, wash it with water and then dissolve the precipitate in 1 ml of concentrated HCl. Prepare a solution of 3 g potassium iodide in 1 ml water. Add this to the precipitate solution. To the resulting solution, add 48 ml

of 70% aqueous acetic acid. Place the test sample in a spot plate depression and add 3 drops of the reagent.

Interpretation of colour tests

When interpreting the results of a colour test, the analyst must keep two things in mind:

1. Is a colour observed ?
2. Of what significance is the colour (or lack of colour) ?

Table 4. Piperazine colour test results. [33, 34]

Compound	*Marquis*		*Conc. H_2SO_4*	*Simon's*		*Dragendorff*	
Sample	*3 mg*	*10 mg*	*10 mg*	*3 mg*	*10 mg*	*3 mg*	*10 mg*
BZP	White to brown-green precipitate with fumes	White to brown-green precipitate with fumes	White to dark green precipitate with fumes	Pale blue	Strong Blue	Red precipitate	Red precipitate
2-MePP	No reaction	Not tested	Not tested	Blue	Blue	Red precipitate	Red precipitate
3-MePP	No reaction	Not tested	Not tested	No reaction	No reaction	Not tested	Not tested
4-MePP	No reaction	Not tested	Not tested	No reaction	No reaction	Not tested	Not tested
2-MeOPP	No reaction	Gradual pink colour	Gradual pink colour	Pale blue	Blue	Red precipitate	Red precipitate
4-MeOPP	No reaction	Fizz no colour change	Fizz no colour change	Pale blue	Blue	Red precipitate	Red precipitate
3-CPP/ *m*CPP	No reaction	Fizz no colour change	Fizz no colour change	No reaction	No reaction	Red precipitate	Red precipitate
3-CPP. HCl / *m*CPP.HCl	Fizz no colour change	Fizz no colour change	Fizz no colour change	Slight purple to blue	Blue, slow to yellow	Red precipitate	Red precipitate

Table 4. (cont.)

4-CPP HCl	Fizz no colour change	Fizz no colour change	Fizz no colour change	Slight purple to blue	Blue, slow to yellow	Red precipitate	Red precipitate
3-TFMPP	White to pale brown precipitate	White to pale brown precipitate	White precipitate, with fumes	No reaction	Blue	Red precipitate	Red precipitate
2-TFMPP	White to pale brown precipitate	White to pale brown precipitate	White precipitate	No reaction	Blue	Red precipitate	Red precipitate
4-TFMPP	Gradual brownish-red colour	Gradual brownish-red colour	Fizz no colour change	No reaction	Blue	Red precipitate	Red precipitate
2-FPP	Fizz no colour change	Fizz no colour change	Fizz no colour change	Purple to blue	Blue, slow to yellow	Red precipitate	Red precipitate
4-FPP	Fizz no colour change	Fizz no colour change	Fizz no colour change	Blue	Blue, slow to yellow	Red precipitate	Red precipitate
Methafetamine HCl	Orange-brown	Not tested	Not tested	Blue	Not tested	Red precipitate	Not tested
MDMA HCl	Black	Not tested	Not tested	Blue	Not tested	Red precipitate	Not tested
Dimethylamfetamine. HCl	Brown	Not tested	Not tested	No reaction	Not tested	Red precipitate	Not tested

Analytical Notes

Marquis reagent

This produces colour changes with a large number of heterocyclic compounds. However, as the sulphuric acid component of this reagent produces colour changes when used alone, it is therefore essential to use sulphuric acid (3 drops) in the testing as a control.

For BZP-like compounds, the Marquis reagent showed negative results or faint colouration. For most of the compounds the results are very similar to that of the sulphuric acid control. For the purpose of comparison, the reagent produces a strong red-orange colour with amphetamines, while MDMA-type compounds produce a blue-black colour.

Simon's reagent

A blue colour indicates the presence of a secondary amine and for some piperazines the colour changes gradually from blue to yellow. Simon's reagent is less sensitive to BZP-like compounds than drugs such as methamfetamine or MDMA, therefore the result will be masked if these substances are also present.

The use of Simon's reagent alone will do little to distinguish methamphetamine or MDMA from piperazines, however, the combination of the Marquis reagent with the Simon's reagent may be effective in distinguishing some piperazines from methamfetamine or MDMA while in the field.

Dragendorff reagent

An orange, red-orange, or brown-orange precipitate suggests the presence of an alkaloidal base and tertiary amines often show a strong positive result. The results with the piperazines, while positive is not as strong as the result with dimethylamfetamine.

4.4 Microcrystalline tests

Microcrystalline tests are chemical-precipitation tests that are quick, simple, extremely sensitive, and require only a small amount of sample. They are used to indicate the identity of a compound, or to determine its optical isomer.

These tests involve the formation of crystals from the reaction of the target compound with a reagent. The resulting crystals are analysed by means of a polarizing microscope and comparison with reference material. Occasionally, it can be difficult to obtain an exact match between the sample and reference material if, for example, other materials that may cause the deformation of crystals are present.

Microcrystalline tests can be performed in the following ways:

1. *Direct addition:* A portion of sample powder is placed on a microscope slide and a drop of reagent is placed near it on the slide. The two are then drawn together using a glass rod.

   Example: Test for caffeine using 5% gold chloride in dilute phosphoric acid.

2. *Solution mixing:* A portion of sample powder is first dissolved in a solvent (often directly on the microscope slide itself). A drop of reagent is placed beside it and slowly drawn into the solution using a glass rod.

   Example: Dissolution of a small quantity of a cocaine sample directly in 20% acetic acid followed by the addition of 5% gold chloride.

3. *Volatility or hanging drop tests:* This technique is dependent upon the volatility of the compound being tested and is most frequently applied to the determination of optical isomers of amines, particularly amfetamine and methamfetamine.

   Example: A small amount of sample is placed in a spot plate and a drop of base added onto the sample. A drop of the test reagent is then placed onto a cover slip and is inverted over the depression. After standing for 5-10 minutes the resultant crystals can be observed.

4.4.1 Piperazine microcrystalline test (platinic bromide in sulphuric acid)

Reagent

Dissolve 1 g platinic chloride ($H_2PtCl_6 \cdot 6H_2O$) in 1.7 ml HBr (40%). Dilute to 20 ml with 2 parts concentrated sulphuric acid and 3 parts water.

Method

Add reagent to an aqueous drop of test solution and evaporate.

Table 5. Results of piperazine microcrystalline tests using platinic bromide in sulphuric acid [31]

Compound	*Crystal Produced*
BZP	Rectangles with indented ends
TFMPP	Oils, then clusters of rods from centre core (bundles of rods), (overgrown bow ties)
2-MeOPP	Clusters of rods (wide blades/rods from core)
3-MeOPP	Crosses with comb edges
4-MeOPP	Rhomboid type crystals (rods/plates with rough edges)

4.4.2 Piperazine microcrystalline tests (mercury chloride)

Reagent

Aqueous solution of mercury chloride (10 g/L),

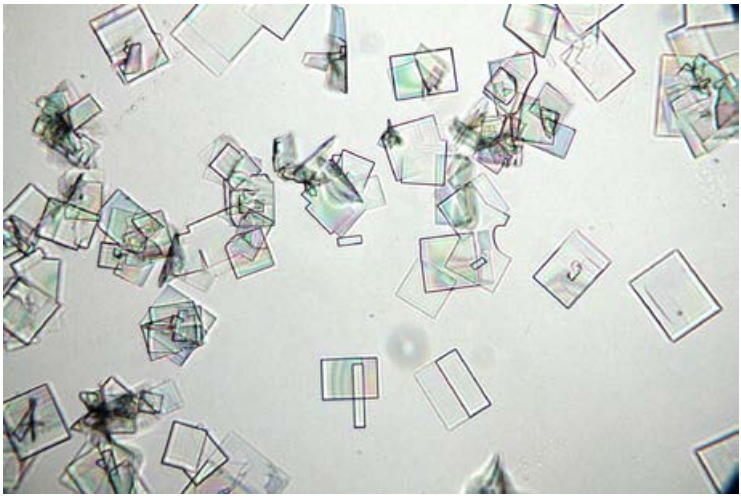
Method

An aliquot (10 µl) of the test solution (1 g/L) is mixed with 10 µl of the reagent on a glass slide. A plastic pipette is used to aid nucleation and crystal formation [35].

Results

For BZP, transparent flat square plates were produced as shown in figure II, while for TFMPP, a white precipitate was produced with no formation of crystals.

Figure II. BZP mercuric chloride microcrystalline test [36]



4.5 Thin-layer chromatography (TLC)

TLC is a commonly used technique for the separation and identification of illicit drugs. It is inexpensive, rapid, sensitive (sub-milligram quantities of analyte required), flexible in the selection of both the stationary and mobile phase, and amenable to a wide variety of substances, in base and salt form, ranging from the most polar to non-polar materials.

TLC plates (stationary phases)

Coating: Silica gel with layer thickness of 0.25 mm and containing an inert indicator, which fluoresces under UV light of wavelength 254 nm (Silica gel GF254).

Typical plate sizes: 20x20 cm; 20x10 cm; 10x5 cm (the latter should be used with the 10 cm side vertical with the TLC tank).

Plates prepared by the analyst must be activated before use by placing them into an oven at 120°C for at least 10 to 30 minutes. Plates are then stored in a grease-free desiccator over silica gel. Heat activation is not required for commercially available coated plates.

Solvent systems

Prepare developing solvent system (system A, B, C, D or E as shown in table 7) as accurately as possible by use of pipettes, dispensers and measuring cylinders [33]. Leave the solvent system in the TLC tank for sufficient time to allow vapour phase saturation to be achieved prior to the analysis (with adsorbent paper-lined tanks, this takes approximately 5 minutes).

Table 6. Solvent systems and visualization methods for TLC analysis of piperazines [33]

System	*Solvents*	*Solvent proportions (by ratio)*	*Visualisation method*
A	2-butanone dimethylformamide aqueous ammonia (25%)	13 0.9 0.1	UV light
B	2-propanol aqueous ammonia (25%)	95 5	Dragendorff reagent
C	acetone toluene aqueous ammonia (25%)	20 10 1	Simon's reagent
D	methanol aqueous ammonia (25%)	100 1.5	Iodoplatinate reagent
E	1-butanol acetic acid water	2 1 1	1% Iodine-methanol

Visualization methods

A. *UV light*

B. *Dragendorff reagent*

Prepare as described in section 4.3.1.c.

C. *Simon's reagent* (modification of reagent used in section 4.3.1.b).

Prepare solutions A (20% aqueous sodium carbonate solution) and B (1% aqueous sodium nitroprusside). Mix equal volumes of A and B and spray the plate. After spraying the plates, expose them to acetaldehyde gas.

D. *Iodoplatinate reagent*

Solution A: aqueous 10% hydrogen hexachloroplatinate hexahydrate solution.

Solution B: aqueous 4% potassium iodide solution.

Mix solutions A, B and water in the ratio of 1 : 25 : 24 by volume.

E. *1% w/v Iodine-methanol solution*

Spotting and developing

Apply as separate spots 1 µL and 5 µL aliquots of sample solution, 2 µL of the standard solutions and 2 µL of solvent (as a negative control) on the TLC plate. Spotting must be done carefully to avoid damaging the surface of the plate.

Analytical notes

- The starting point of the run, i.e. the "spotting line" should be at least 2 cm from the bottom of the plate.
- The spacing between applications of sample (spotting points) should be at least 1 cm and spots should not be placed closer than 1.5 cm to the side edge of the plate.
- To avoid diffuse spots during development, the size of the sample spot should be as small as possible (2 mm) by applying solutions in aliquots rather than a single discharge.
- Allow spots to dry and place plate into solvent-saturated tank (saturation of the vapour phase is achieved by using solvent-saturated pads or filter paper as lining of the tank).
- Remove plate from the development tank as soon as possible after the solvent reaches the development line (10 cm from starting line) marked beforehand; otherwise, diffused spots will occur.

Visualization/detection

The plates must be dried prior to visualization. The solvent can be allowed to evaporate at room temperature or with a hot air blower. In the later case, care must be exercised that no component of interest is thermally labile. It is important for proper colour development that all traces of ammonia or other bases are removed from the plate.

Interpretation

After visualization, mark spots (e.g. by pencil) and calculate retardation factor (R_f) values.

$$R_f = \frac{\text{Migration distance: from origin to centre of spot}}{\text{Development distance: from origin to solvent front}}$$

Table 7. Piperazine TLC Data [33]

Compound	*Developing System (R_f)*				
	A	*B*	*C*	*D*	*E*
BZP	0.03	0.15	0.13	0.25	0.66
2-TFMPP	0.11	0.41	0.36	0.33	0.8
*m*TFMPP	0.11	0.37	0.36	0.38	0.78
4-TFMPP	0.11	0.37	0.36	0.33	0.77
2-MeOPP	0.05	0.26	0.18	0.28	0.74
4-MeOPP	0.05	0.25	0.2	0.28	0.72
*m*CPP/3-CPP	0.11	0.38	0.37	0.32	0.77
4-CPP	0.07	0.3	0.3	0.27	0.77
2-FPP	0.12	0.4	0.36	0.28	0.74
4-FPP	0.07	0.3	0.25	0.24	0.74
Methamphetamine	0.09	0.37	0.32	0.21	0.76
Dimethylamphetamine	0.25	0.42	0.51	0.28	0.7
MDMA	0.09	0.36	0.32	0.21	0.74

Analytical notes

- R_f values are not always reproducible due to small changes in plate composition and activation in solvent systems, tank saturation or development distance. Therefore, the R_f values provided are indications of the chromatographic behaviour of the substances listed.
- It is essential that reference standards be run simultaneously on the same plate.
- For identification purposes, both the R_f value and the colour of the spots after spraying with the appropriate visualization reagents should always be considered.

4.6 Gas chromatography (GC) with flame ionization detection (GC-FID)

The GC instrument of choice for routine analytical work is the narrow bore capillary gas chromatograph, using columns with internal diameter between 0.2 and 0.32 mm.

4.6.1 Qualitative GC-FID method

GC oven conditions:	Column temperature initially set at 100°C and held isothermal for 1 min., the temperature was then ramped to 280°C at 25°C/min and held isothermal for 3 mins.
Column:	5% phenyl / 95% methyl silicone column, 10 m length, 0.32 mm ID, 0.52 µm film thickness
Injection parameters:	Mode: Split (50:1), 280°C, 1 µL injected
Carrier gas:	Hydrogen 1.8 ml/min
Detector:	FID, Detector temp: 280°C

Table 8. Relative retention times (RRT) for samples dissolved in methanol

Compound	*Relative retention time (RRT)*
Dimethyl sulfone	0.277
Methamfetamine	0.615
Dimethylphthalate	0.947
BZP	**1.00 (4.212 min)**
TFMPP	1.039
MDMA	1.043
2-MeOPP	1.155
4-MeOPP	1.287
3-MeOPP	1.303
Caffeine	1.362

4.6.2 Quantitative GC method

Internal standard stock solution

Prepare a solution containing 0.25 mg/ml dimethylphthalate in methanol.

Standard solution preparation

Prepare a solution by dissolving approximately 1.0 mg/ml of the piperazine to be analysed in the internal standard stock solution.

Sample preparation

Accurately weigh an amount of the sample to be tested into a volumetric flask and fill to the mark with the internal standard stock solution. If necessary, dilute the sample so the final concentration is approximately that of the standard solution concentration.

GC oven conditions:	Column temperature initially set at 130°C and held isothermal for 1 min, the temperature was then increased to 200°C at 25°C/min and held isothermal for 3 mins.
Column:	10 m x 0.32 mm x 0.52 µm film thickness
Phase:	5% phenyl/95% methyl silicone
Carrier gas:	Hydrogen at 1 ml/minute
Injection Parameters:	Split (50:1), 280°C, 1 µL injection
Detector:	FID

Results

Linear range: 0.050-1.206 mg/ml

Repeatability: RSD less than 0.5%

Correlation coefficient: 0.999

Accuracy: Error less than 5%

Table 9. RRT for samples dissolved in internal standard stock solution

Compound	*Relative retention time (RRT)*
Methamphetamine	0.472
2-MeOPP	1.279
BZP	**1.00 (2.23)**
TFMPP	1.073

3-MeOPP	1.506
Dimethylphthalate	0.917
Caffeine	1.969
4-MeOPP	1.547

4.7 Gas chromatography-mass spectrometry (GC-MS)

GC-MS is one of the most commonly used techniques for the identification of forensic drug samples. As a hyphenated technique, it unifies the separation power and sensitivity of a GC with the analyte specificity of a spectroscopic technique. It can provide highly specific spectral data on individual compounds in a complex mixture without prior isolation.

4.7.1 GC-MS method 1 [37]

GC oven conditions:	Column temperature initially set at 100°C and held isothermal for 5 mins., the temperature was then ramped to 290°C at 10°C/min and held isothermal for 20 mins.
Column:	5% phenyl/95% methyl silicone column, 30 m length, 0.25 mm ID, 0.25 µm film thickness
Injection parameters:	Splitless, 1 µL injected
	Injector temp: 250°C
Carrier gas:	Helium, 1.1 ml/min
Detector:	Ionization mode: EI mode, 70 eV
	Transfer line temp: 290°C
	Ion source temperature: 200°C
MS parameters	Scan parameters: TIC
	Scan range: 30-350 amu

Table 10. GC RT and RRT for samples dissolved in methanol

Compound	*GC RT*	*GC RRT*
4-FPP	13.37	0.97
2-MeOPP	14.85	1.08
1-Phenylpiperazine	**13.75**	**1.00**
3-MeOPP	16.47	1.20
3-FPP	13.71	1.00
4-MeOPP	16.15	1.17
2-FPP	12.77	0.93
2-CPP	14.64	1.06
3-CPP/*m*CPP	15.99	1.16
4-CPP	16.04	1.17
2,3-XP	15.26	1.11
3,4-XP	16.25	1.18
*m*TFMPP	14.65	1.07
2,5-XP	14.81	1.08
2,4-XP	14.87	1.08
2-TFMPP	13.53	0.98
3-TMP	13.32	0.97
MBZP	13.05	0.95
BZP	13.10	0.95
MDBZP	17.32	1.26
2-PEP	15.00	1.09

Note: Relative retention times were calculated from data in table 3 in reference 37

4.7.2 GC-MS method 2 [38]

GC oven conditions:	Column temperature initially set at 80°C and held isothermal for 4 mins., the temperature was then ramped to 280°C at 20°C/min, held isothermal for 8 mins. The temperature was then increased to 280°C at 20°C/min and held isothermal for 11.5 minutes.
Column:	Shimadzu QP2010 GC/MS with a HP5MS column (30 m x 0.25 mm, 0.50 µm)
Injection parameters:	Mode: Splitless, injection temperature (225°C) 1 µL injected
Carrier gas:	Helium, 1 ml/minute. Pressure (9.5 psi)
Detector:	Ionization mode: EI mode, 70 eV
	Transfer line temp: 300°C
	Ion source temperature: 230°C
MS parameters:	Solvent delay: 3 mins.
	Scan parameters: TIC
	Scan range: 40-450 amu at 1 scan/sec

Table 11. RT and RRT for samples dissolved in methanol

Compound	*RT (mins)*	*RRT (mins)*
Quinoline	9.049	0.82
BZP	**10.989**	**1.00**
4-FPP	11.132	1.01
TFMPP	11.185	1.02
3-MePP	11.800	1.07
4-MePP	11.800	1.07
2-CPP	11.867	1.08
2-MeOPP	11.861	1.08
*m*CPP	12.600	1.15
4-MeOPP	12.639	1.15
4-CPP	12.639	1.15
Pyribenzamine	13.941	1.27
DBZP	14.919	1.36

4.7.3 GC-MS method 3 [39, 40]

Sample preparation: Samples dissolved in acetonitrile.

GC oven conditions:	Column temperature initially set at 100°C and held isothermal for 1 min., the temperature was then ramped to 180°C at 12°C/min. and held isothermal for 2 mins. The temperature was then increased to 200°C at 10°C/min and held isothermal for 5 mins.
Column:	Agilent 7890A GC/MS with a 100% trifluoropropyl methyl polysiloxane (Rtx-200) capillary column (30 m x 0.25 mm, 0.50 µm)
Injection parameters:	Mode: Splitless, 250°C, 1 µL injected
Carrier gas:	Helium, 0.7 ml/minute. Pressure (10 psi)
Detector:	Ionization mode: EI mode, 70 eV
	Transfer line temp: 280°C
	Ion source temp: 230°C
	Interface temp: 250°C

4.8 Gas chromatography-infrared detection (GC-IRD) [39, 40]

Recent advances in Fourier transform infrared (FTIR) spectroscopy and capillary gas chromatography have made it possible to produce hyphenated GC-FTIR instruments. This technique uses the properties of capillary gas chromatography to vaporize and separate the individual components of a sample followed by the detection of vapour phase infra red spectra of each component.

Sample preparation: Samples dissolved in acetonitrile.

GC-IRD operating conditions

GC oven conditions:	Column temperature initially set at 100°C and held isothermal for 1 min, the temperature was then ramped to 230°C at 20°C/min and held isothermal for 15 mins.
Column:	Hewlett–Packard 5890 Series II GC with a 50% phenyl–50% methyl polysiloxane (Rxi-50) capillary column (30 m x 0.25 mm (i.d.), 0.5 µm
Injection parameters:	Mode: Splitless, 1µL

Carrier gas:	Helium at 0.7 mL/min., pressure (10 psi)
IR Range	4000-650 cm^{-1}
Resolution	8 cm^{-1}
Detection: scan rate	Scan rate: 1.5 scans/sec
IRD flow cell: 280°C	
Transfer line temp: 280°C	

4.9 High pressure liquid chromatography (HPLC)

In addition to GC, HPLC is another major separation technique used in forensic drug analysis. Reverse phase chromatography is most commonly used for the analysis of drugs in seized materials and the most universal and versatile column is a bonded octadecyl silica column (C18). Column length, diameter, particle size, pore size and carbon load should be considered before final selection of the column. As there are a large variety of stationary and mobile phases available to the analyst, all methods must be properly validated and/or verified prior to routine use.

4.9.1 HPLC method 1 (qualitative) [37]

Sample preparation

Dissolve approximately 10 mg of sample in 10 ml of 20 mM HCl:methanol, 1:1 solution. Dilute 1 ml of the solution with methanol to a total volume of 10 ml. Filter with a 0.45 µm membrane filter.

Column:	4.6 mm (ID) x 250 mm, 5 µm, C18 thermostated at 40°C.
Mobile phase:	(A) Acetonitrile
	(B) 5mM Heptafluorobutyric acid
	A gradient program was utilized
	0 mins 18:82, A:B
	10 mins 18:82, A:B
	25 mins 28:72, A:B
	50 mins 30:70, A:B
Flow rate:	1 ml/min
Injection volume:	10 µL
Detection:	Photo diode array (PDA) 199-360nm

Table 12. RT and RRT to 1-phenylpiperazine for selected piperazines

Compound	*Relative time (RT)*	*RRT*
4-FPP	4.20	0.68
2-MeOPP	8.02	1.29
1-Phenylpiperazine	**6.21**	**1.00**
3-MeOPP	9.50	1.53
3-FPP	10.82	1.74
4-MeOPP	8.02	1.29
2-FPP	9.42	1.52
2-CPP	16.15	2.60
3-CPP/*m*CPP	19.03	3.06
4-CPP	19.38	3.12
2,3-XP	26.63	4.29
3,4-XP	22.26	3.58
4-TFMPP	31.66	5.10
2,5-XP	29.41	4.74
2,4-XP	30.83	4.96
TFMPP	30.06	4.84
3-TMP	5.73	0.92
MBZP	6.80	1.09
BZP	6.60	1.06
MDBZP	7.05	1.14
2-PEP	8.12	1.31

Note: RT and RRT values calculated from data in Table 3 in reference 37

4.9.2 HPLC method 3 (qualitative and quantitative) [31]

Sample preparation

Accurately weigh an amount of sample into a volumetric flask and dilute with 0.01M HCl. If necessary, dilute the sample so the final concentration approximates the standard concentration.

HPLC operating conditions

Column:	4.6 mm x 250 mm, 10 µm, C18
Mobile phase:	86% Sodium hexylammonium phosphate (NaHAP) Buffer : 14 % acetonitrile.
	Buffer preparation (4000 ml distilled water, 10 g sodium hydroxide, 30 ml phosphoric acid and 8 ml hexylamine)
Flow rate:	1 ml/min
Injection volume:	3 µL
Detection:	UV, 210 nm

Results

Linear range: 0.051-0.508 mg/ml

Repeatability: RSD less than 3%

Correlation coefficient: 0.9993

Accuracy: error less than 5%

Table 13. Relative retention times of selected piperazines

Compound	*RRT*
2-MeOPP	**1.0 (5.13min)**
BZP	0.45
3-MeOPP	1.10
4-MeOPP	0.87
TFMPP	5.11

4.10 Capillary electrophoresis (CE)

Capillary electrophoresis is an analytical technique involving the separation of charged species based on their migration under the influence of an applied electric field through a fused silica capillary. The following section presents a method for both the qualitative and quantitative analysis of selected piperazines using capillary electrophoresis (CE).

4.10.1 CE method (qualitative and quantitative) [31]

Internal standard stock solution

Prepare a solution of thiamine hydrochloride in water at a concentration of 0.2 mg/ml.

Standard solution preparation

Prepare a standard solution at approximately 0.4 mg/ml dissolving in the internal standard stock solution.

Sample preparation

Accurately weigh an amount of sample and dissolve with internal standard stock solution. The sample should then be diluted with internal stock solution to a concentration approximately equal to that of the standard.

Mode:	Free zone
Capillary:	34 cm x 50 µm fused silica capillary
Run buffer:	100 mM lithium phosphate buffer at pH 2.3
Detector:	UV, 210 nm
Voltage:	20 kV
Temperature:	20°C air cooled
Injection:	Hydrodynamic, 50 mbar for 2.5 s
Run time:	6 mins.
Rinse time:	1 min.

Results

Linear range: 0.05-1.2 mg/ml

Repeatability: RSD less than 3%

Correlation coefficient: 0.999

Accuracy: error less than 5%

Table 14. Relative migration times (RMT)

Compound	*RMT*
Thiamine	0.892
BZP	**1.0 (3.525 mins)**
TFMPP	1.417
2-MeOPP	1.337
3-MeOPP	1.349
4-MeOPP	1.296

4.11 Fourier transform infrared (FTIR) spectroscopy

The confirmation of the identity of a substance can be achieved by FTIR. Unequivocal identification of a particular piperazine is thus possible from each unique spectrum. For powders, considered from prior chromatographic analysis to be reasonably pure, the infrared spectrum of the powder may be run directly in a KBr disc for comparison with those of the free base or HCl salt of a particular piperazine. For tablets, capsules and mixtures of powders, an extraction procedure would be required to liberate the free base in pure form.

Analytical notes

- The KBr disc method consists of grinding a dry sample to a very fine powder, then mixing about 2 mg of homogenized sample powder with 200 mg of carefully dried and ground KBr. After grinding, the mixture is pressed into a thin transparent disk.
- KBr should be "IR Grade" and dried at 105°C for a minimum of one hour. It can be stored in a desiccator containing a strong desiccant (silica gel) or left in the oven and removed when required.

Table 15. Characteristic IR spectral bands for selected piperazines (liquid samples were analysed as thin films between NaCl plates and solids were analysed as KBr discs, scan range 600 cm^{-1}-4000 cm^{-1}) [33].

Substance	*Characteristic IR bands (wavenumber, cm^{-1})*
BZP	698, 739, 1142, 1319, 1454
TFMPP	1120, 1163, 1319, 1354, 1450
2-TFMPP	1036, 1109, 1136, 1315, 1454
4-TFMPP	1068, 1109, 1244, 1325, 1614
2-MeOPP	748, 1028, 1240, 1450, 1500
BZP.2HCl	702, 748, 957, 1074, 1431
TFMPP.HCl	1120, 1165, 1321, 1352, 1589
4-MeOPP.2HCl	835, 1018, 1255, 1444, 1518
4-CPP.HCl	818, 1147, 1253, 1454, 1497
4-FPP.2HCl	845, 1165, 1228, 1423, 1512
2-FPP.HCl	764, 1149, 1209, 1252, 1500
3-CPP/*m*CPP.HCl	750, 945, 1253, 1489, 1595

5. Library information

There are few libraries available for the identification of the piperazine compounds and if available are costly. Takahashi *et al*. outlines a method for the creation of such a library [37].

5.1 Ultraviolet (UV) spectrophotometry

Selected piperazines in aqueous acid have absorbance maxima at the following wavelengths.

Table 16. UV Spectroscopy data for selected piperazines [31]

Compound	*Maximum Absorbance (nm)*
BZP	193
2-MeOPP	206
3-MeOPP	210
4-MeOPP	196
TFMPP	202

5.2 GC-MS data for selected piperazines

The fragmentation patterns of selected piperazines were obtained using electron ionization (EI) at an energy of 70 eV. The ions are listed in decreasing order of peak intensity under the experimental conditions used.

Table 17. Characteristic ions of the EI mass spectra for selected piperazines [33]

Substance	*Characteristic ions (m/z)*
BZP	91, 134, 56, 120, 176 (M^+)
*m*TFMPP	188, 145, 172, 56, 230 (M^+)
*o*TFMPP	188, 145, 172, 56, 230 (M^+)
*p*TFMPP	188, 145, 172, 56, 230 (M^+)
*o*MeOPP	150, 135, 120, 192 (M^+)
*p*MeOPP	150, 135, 120, 192 (M^+)
*m*CPP	154, 56, 196 (M^+)
*p*CPP	154, 56, 196 (M^+)
*o*FPP	138, 122, 56, 180 (M^+)
*p*FPP	138, 122, 56, 180 (M^+)

Additional reading

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