UNITED NATIONS
Office on Drugs and Crime

RECOMMENDED METHODS FOR
THE IDENTIFICATION AND ANALYSIS OF

AMPHETAMINE, METHAMPHETAMINE AND THEIR RING-SUBSTITUTED ANALOGUES IN SEIZED MATERIALS

(revised and updated)

MANUAL FOR USE BY NATIONAL DRUG TESTING LABORATORIES

Laboratory and Scientific Section
United Nations Office on Drugs and Crime
Vienna

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Contents

I. INTRODUCTION

International attention is focusing more and more on the growing issue of amphetamine-type stimulants (ATS). Particularly over the last 10 to 15 years, abuse of ATS, involving amphetamines (amphetamine and methamphetamine) and substances of the "ecstasy"-group (MDMA, MDA, MDEA, etc.), has become a global problem. There are regional differences, but today no country is spared one of the many facets of ATS manufacture, trafficking or abuse.

This new situation, involving often new and unfamiliar ATS, or combinations, and trafficking trends, presents a challenge both to national law enforcement authorities and to the scientific staff of forensic laboratories.

Today, analysts must be able to analyse a wide range of substances and preparations, and use faster, more accurate and more specific methods for identification and analysis in order to cope with the increased analysis turnover and the requirements of stiffer national drug laws. In addition, the international character of drug trafficking requires the timely exchange of analytical data between laboratories and law enforcement authorities at the national, regional and international levels. For these reasons, UNODC's Laboratory and Scientific Section has since the early 1980s pursued a programme of harmonization and establishment of recommended methods of testing for national drug testing laboratories.

A consultative meeting comprised of 13 experts was convened in September 1998 in London by UNODC's Laboratory and Scientific Section in cooperation with the Forensic Science Service of the United Kingdom to review methods for the identification and analysis of amphetamine-type stimulants (ATS) and their ring-substituted analogues in seized material. This manual reflects the conclusions of that meeting, reviewed and up-dated again in 2004/05. It provides practical assistance to national authorities by describing recommended methods to be used in drug testing laboratories for the identification and analysis of amphetamine-type stimulants (ATS) and their ring-substituted analogues.

This manual is one in a series of similar publications dealing with the identification and analysis of various groups of drugs under international control. It combines and replaces previously published manuals on Recommended Methods for Testing Amphetamine and Methamphetamine (ST/NAR/9, 1987) and Recommended Methods for Testing Illicit Ring-Substituted Amphetamine Derivatives (ST/NAR/12, 1988).

The present and previous manuals suggest approaches that may assist drug analysts in the selection of methods appropriate to the sample under examination, leaving room also for adaptation to the level of sophistication of different laboratories. For the first time in this series of publications, the present manual has

also annexed selected validated methods. Most methods described are published in the scientific literature, and have been used for a number of years in reputable laboratories. In identifying those methods, the consultative meeting was aware that a number of other published methods in the forensic science literature also produce acceptable results.

The present manual is limited to analytical methods for ATS. A separate manual on analytical techniques more generally, and their characteristics and practical use for drug analysis, complements this series of manuals on recommended methods.

II. USE OF THE MANUAL

Not all methods described in this manual need to be applied to all samples suspected to consist of or contain amphetamine, methamphetamine or other ATS. The choice of the methodology and approach to their analysis remains within the discretion of the analyst and depends on the type of drug involved, the availability of appropriate instrumentation and of reference materials as well as on the level of legally acceptable proof in the jurisdiction within which the analyst works.

While it is therefore recognized that unique requirements in different jurisdictions may dictate the actual practices followed by a particular laboratory, good laboratory practice (GLP) requires that an analytical approach to establish the identity of a controlled drug in suspected material must, as a minimum, entail the determination of at least two uncorrelated parameters. The selection of these parameters in any particular case would have to take into account the drug involved and the laboratory resources available to the analyst. When possible, three entirely different analytical techniques should be used, for example: colour tests, chromatography (e.g., TLC, GC or HPLC) and spectroscopy (e.g., IR or UV). Hyphenated techniques, such as GC-MS, count as two parameters, provided the information from both techniques is used (i.e. retention time and mass spectral characteristics).

Attention is also drawn to the vital importance of the availability to drug analysts of reference books on drugs of abuse and analytical techniques. Moreover, the analyst must of necessity keep abreast of current trends in drug analysis, consistently following current analytical and forensic science literature. UNODC assists laboratories in this regard by providing, upon request, selected articles from the scientific 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.

III. CLASSIFICATION/DEFINITIONS

Amphetamine-type stimulants (ATS) are a group of substances, mostly synthetic in origin, that are structurally derived from β-phenethylamine (β-PEA, figure I). ATS generally stimulate the central nervous system (CNS). Therefore, to varying degrees, they are considered as prototypes of central nervous system stimulants with a potential of psychotic toxicity when overdosed or abused for long periods of time. ATS may produce one or more dose-related symptoms, including increased alertness and euphoria, increased heart rate, blood pressure, respiration and body temperature. Agitation, tremors, hypertension, memory loss, hallucinations, psychotic episodes, paranoid delusions, and violent behaviour can result from chronic abuse. Withdrawal from high doses of ATS could result in severe depression. ATS are illegally produced in a variety of preparations (powder, tablets, or capsules), and they may be injected, ingested orally, snorted, or smoked.

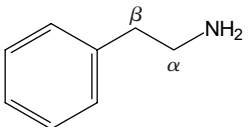

Figure I

Chemical modification at the positions R1 to R9* (figure II) results in a practically unlimited number of pharmacologically active compounds, some of which are more potent stimulants than others. Although there are several possibilities for side chain modification, substitution on the aromatic ring contributes the most to substantial qualitative differences in pharmacological effects.

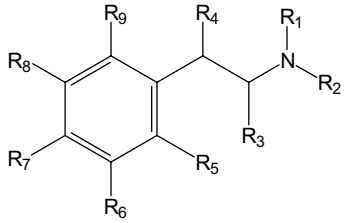

Figure II

In terms of structural characteristics, ATS can be divided into three major sub-groups, which largely correspond to the following substitution patterns on the aromatic ring:

(a) No substitution on aromatic ring (e.g. amphetamine, methamphetamine, fenetylline).

(b) Methylenedioxy-substitution on aromatic ring (e.g. MDA, MDMA, MBDB).

(c) Other substitution patterns, usually including one or more alkyloxy group (e.g. 2C-B, STP/DOM).

*All other substituents required to saturate valences are not shown, as they are usually hydrogen (H).

For practical reasons, this manual provides specific data only for a selection of the most common ATS. In particular, it includes ATS under international control and selected ATS under national control. The analyst should be aware that other closely related analogues may be encountered. In most cases, the methodology presented will be applicable to those analogues as well.

The chemical structures of selected ATS, together with common names and IUPAC nomenclature, are given in annex I.

IV. DESCRIPTION OF PURE COMPOUNDS

Seized ATS are commonly encountered in the form of salts, in particular as hydrochloride, sulphate, phosphate, or bromide salt. However, it is not uncommon in clandestine laboratory investigations to find those compounds in base form as well (usually a brownish oily liquid). Salts are crystalline or powdered substances, which vary in colour from white (similar to pharmaceutical grade products) to pink, yellow or brown. They are often damp with a characteristic smell, owing to the presence of solvent and/or precursor residues.

ATS can be also found in the form of tablets. In addition to the active ATS, tablets often contain different adulterants, cutting agents, common food colours and/or different excipients and binding agents.

Amphetamine: Illicit amphetamine is frequently encountered as the sulphate salt in powder form, and rarely as tablet. Amphetamine base may be seized in clandestine laboratories, typically as a dark brown oily liquid with a characteristic unpleasant smell of 1-phenyl-2-propanone (P-2-P) and/or solvent residues.

Methamphetamine: Illicitly manufactured methamphetamine is available in different forms, depending on the geographical region. Forms include powder, crystals (commonly known as "Cristal", "Ice" or "Shabu") and tablets (commonly known as "Yaba"). The most frequently encountered salt form is the hydrochloride.

Methylenedioxy ring-substituted ATS: MDMA, MDA, and MDEA are usually found as tablets which may or may not bear one of a logo. Powders are only occasionally found, but typically contain high concentration of active substances. Tablets are frequently brightly coloured; they often vary in size. The drug content usually ranges from 40-140 mg. Regional differences in drug content, and changes over time, are known. In Europe, for example, the average MDMA content in ecstasy tablets has dropped to about 60-70 mg (compared to around 100 mg in the mid-1990s).

The most commonly encountered salt form of the methylenedioxy-type ATS is the hydrochloride, but phosphate and bromide salts are also seen.

A. STEREOCHEMISTRY

Most ATS have at least one chiral centre and can therefore be found as a racemic mixture or as individual enantiomers.* In illicit markets, most ATS are encountered

*The terms (d) or (+), (l) or (-) and (d,l) or (±) are typically used to designate the optical rotation of chiral substances. (R) and (S) designations describe the absolute steric configuration of substituents at individual chiral centres, and are preferred, especially in the case of diastereomers.

in a typical stereochemical make-up. Amphetamine, for example, and most ring-substituted ATS, are typically encountered as the racemate, while methamphetamine is frequently seen as S-, or dextro, enantiomer (also known as "Ice", or "Shabu"), in addition to the racemate. The analysis of optical isomers is described in chapter VI.G. below.

B. PHYSICAL CHARACTERISTICS

Melting/boiling points: The melting and/or boiling points are available for the most commonly encountered ATS. The analyst should be aware, however, that such data refer to pure substances.* Except for high purity ATS, such as crystalline methamphetamine ("Ice"), melting points should therefore only be used as a presumptive test (for the use of melting points for the differentiation of isomers, see chapter VI.G.1, below).

Solubilities: The solubilities of selected ATS and their salts are provided in the section on anion tests (see p. 21 below). Selective re-crystallization based on differences in solubilities can be used for the separation of some ATS salts (see Chapter VI.F. on FTIR, below).

Spectroscopic data: Mass spectral (MS), infra red (IR) and nuclear magnetic resonance (NMR) data of the most common ATS are available in the earlier edition of the two UN manuals related to the analysis of ATS, namely, "Recommended methods for testing amphetamine and methamphetamine" (ST/NAR/9), and "Recommended methods for testing illicit ring-substituted amphetamine derivatives" (ST/NAR/12). Data can also be accessed at the Laboratory and Scientific Section's web page.

*The analyst should also be aware that melting points for some ATS may also vary depending on the solvent used for crystallization.

V. ILLICIT ATS MANUFACTURE

Knowledge of illicit manufacturing routes of drugs of abuse can play an important role in the interpretation of analytical results, especially in those cases where more in-depth analyses of impurities and manufacturing by-products, so-called impurity profiling studies, are carried out.

Use of illicitly obtained or published methods ("underground literature" or internet) for synthesis, inexperienced clandestine "chemists", inappropriate laboratory equipment and lack of laboratory quality control often result in impure and inferior products, and variability in quality and potency. As a consequence, illicitly manufactured drugs often contain by-products and intermediates stemming from impure starting materials, incomplete reactions, and inadequate purification of intermediates and the final synthetic product. The types and quantities of impurities present in illicit ATS samples (the "impurity profile") largely depend on the method of synthesis, the proportions, source and purity of starting materials, the reaction conditions, and the purification procedures, if any.

The presence or absence of specific impurities (markers) can be useful in determining the synthetic route employed, and the starting materials (precursors) used. Solvent analysis can further add to the body of information, and thus can be a useful tool for ATS sample comparison and characterization.

While impurity profiling studies are not the subject of this manual, some of the methods described can be adapted for such purposes.*

Several synthesis routes for ATS are described in the literature and used by illegal/clandestine manufacturers. Most commonly used synthetic methods for the illicit manufacture of amphetamine can be also altered to produce methamphetamine or ring-substituted amphetamines. This is most often accomplished by substituting the amine source or the source of the aromatic ring, respectively, during the reaction process. In general, the availability of precursors greatly determines the choice of synthesis route used in illicit operations.

Brief descriptions of the most commonly employed synthetic routes for amphetamine, methamphetamine and MDMA are presented below.**

Synthesis routes are classified on the basis of the reduction species used in the reaction and the reduction mechanism. In practice, many of those reactions

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*For a general introduction to the subject, the reader is referred to the United Nations manual "Drug characterization/impurity profiling: Background and concepts" (ST/NAR/32/Rev.1, 2001); for more specific methods and approaches for the impurity profiling of methamphetamine see also UNODC Scientific and Technical Publication No.17 (SCITEC/17), 2000.

**For additional details, the reader is referred to the United Nations manual "Clandestine manufacture of substances under international control" (ST/NAR/10/Rev.2, 1998)

are known by popular names such as "Leuckart" method, hydriodic acid/red phosphorus, oxime, nitrostyrene, Birch or "Emde" method. Those popular names are based on the chemist who first described the method, or on characteristic reagents or important intermediates. Popular names are included whenever possible.

A. AMPHETAMINE SYNTHESIS

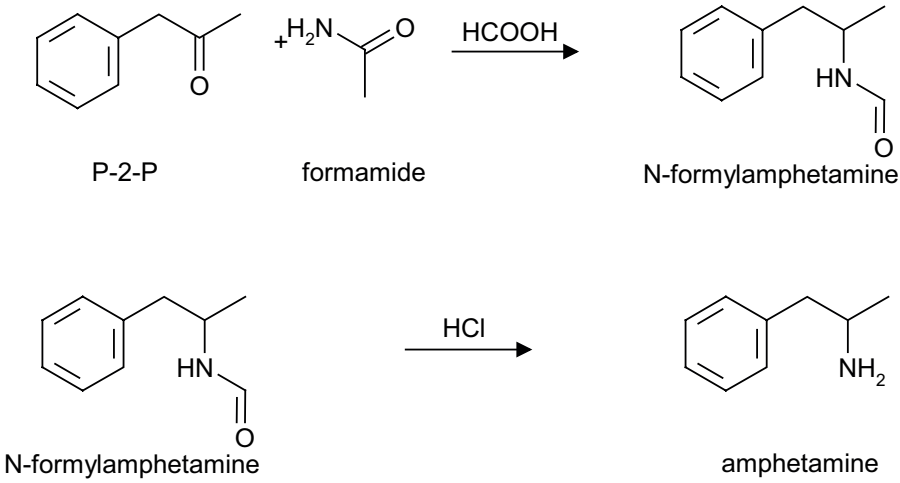
The central reaction of all methods used for the synthesis of amphetamine is based on the catalytic reduction of 1-phenyl-2-propanone (P-2-P, benzyl methyl ketone, BMK, phenylacetone) in the presence of ammonia or methylamine. The most popular reduction methods today are the Leuckart method (non-metal reduction) and the catalytic metal reduction (reductive amination, catalytic hydrogenation or hydrogenolysis).

Leuckart reaction (non-metal reduction)

Due to its simplicity, the "Leuckart" reaction continues to be one of the most popular synthetic routes employed for the illicit manufacture of amphetamines. The Leuckart synthesis is a non-metal reduction usually carried out in three steps.

For amphetamine synthesis, a mixture of P-2-P and formamide (sometimes in the presence of formic acid), or ammonium formate, is heated until a condensation reaction results in the intermediate product N-formylamphetamine. In the second step, N-formylamphetamine is hydrolysed typically using hydrochloric acid (see figure III). The reaction mixture is then basified, isolated, and (steam) distilled. In the final step, the product is precipitated out of the solution, typically as the sulphate salt. Amphetamine base is an oily liquid with a characteristic "fishy-amine" odour.

Figure III. Leuckart reaction used in illicit amphetamine manufacture



The Leuckart method is one of the most studied methods. Several route-specific impurities were identified and described in the literature. The most prominent impurities are the intermediate N-formylamphetamine (usually carried over into the final product) and 4-methyl-5-phenyl pyrimidine. Other synthetic routes do not give as many route-specific impurities as the Leuckart method.

Reductive amination (catalytic metal reduction)

Reductive amination is a process of catalytic or chemical reduction of aldehydes and ketones in the presence of ammonia, or a primary or secondary amine, resulting in the related amine of higher order. The reaction mechanism proceeds through the formation of an imine or iminium intermediate upon reaction of a carbonyl compound with an appropriate amino compound, followed by reduction. Synthesis of amphetamine using reductive amination methods utilizes P-2-P and a catalyst of choice. The most frequently used methods can be divided into three different types based on the reducing species:

(a) Heterogeneous catalytic reduction using platinum oxide, palladium or Raney nickel

(b) Dissolving metal reduction using aluminum, zinc or magnesium amalgams

(c) Metal hydride reduction using lithium aluminum hydride ($LiAlH_4$), sodium borohydride ($NaBH_4$) or, less frequently, sodium cyanoborohydride ($NaCNBH_3$).

Heterogeneous catalytic reduction is usually achieved by using a mixture of P-2-P and ammonia gas charged with hydrogen in the presence of a selected catalyst. Palladium on charcoal (Pd/C) or platinum oxide are the most commonly used catalysts, followed by Raney nickel. Reductions are typically achieved at low pressure and low temperature. In rare occasions, high-pressure aminations in a Parr pressure reaction apparatus ("pressure" or "pipe bomb") have also been encountered.

Dissolving metal reduction using aluminum, zinc or magnesium amalgams is the one of most commonly used reductive amination method. The most popular procedure uses aluminum-mercury amalgam (Al-Hg). The mechanism of the amalgam reduction proceeds via the reduction of a Schiff base adduct of P-2-P and the appropriate amine. In crude clandestine conditions, this method utilizes aluminum foil or grit, and mercuric chloride ($HgCl_2$).

The most characteristic impurities from reductive aminations are Schiff bases, postulated as being formed by the condensation of P-2-P and amphetamine, however they are not route-specific impurities and may be present in any synthetic procedure involving P-2-P. P-2-P and imine type compounds may be also found as impurities. Inorganic impurities arising from the use of a particular catalyst may serve as markers.

Other, less frequently used reductive amination methods include metal hydride reductions, such as the "nitropropane" route and the "oxime" route, named after characteristic intermediates (phenyl-nitropropene and oxime, respectively), which are formed during the reaction.

The oxime route is a reaction of P-2-P with hydroxylamine. The oxime intermediate is subsequently hydrogenated to yield amphetamine. An oxime intermediate is usually hydrolysed by metal reduction using Pd/H_2, or by metal hydride reduction using $LiAlH_4$.

The nitropropene route involves the condensation of benzaldehyde with nitroethane, which yields 1-phenyl-2-nitropropene. Subsequent hydrogenation of the double bond and reduction of the nitro-group results in amphetamine. The reduction phase is usually completed using Pd/H_2 or $LiAlH_4$.

B. METHAMPHETAMINE SYNTHESIS

Methamphetamine can be also synthesized using the above methods by replacing ammonia with methylamine.

However, the most popular methamphetamine synthetic routes employ ephedrine or pseudoephedrine as a precursor instead of P-2-P. The reactions are usually done by one of the following reactions (see figure IV):

(a) non-metal reductions such as the "hydriodic acid-red phosphorus" method,

(b) dissolving metal reduction such as the "Birch" reduction, or

(c) heterogeneous catalytic reduction using thionylchloride and palladium or platinum oxide as a catalyst ("Emde" method).

Figure IV. Common routes for illicit methamphetamine manufacture

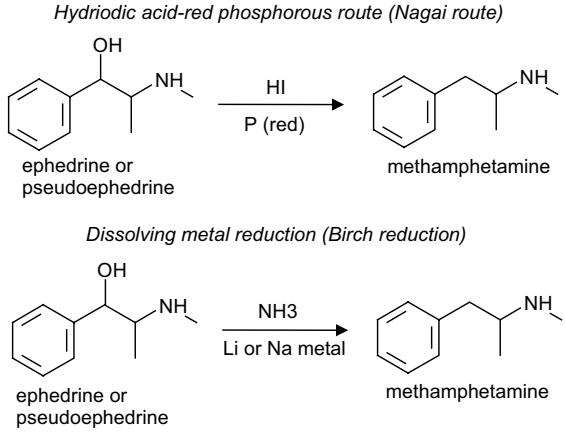
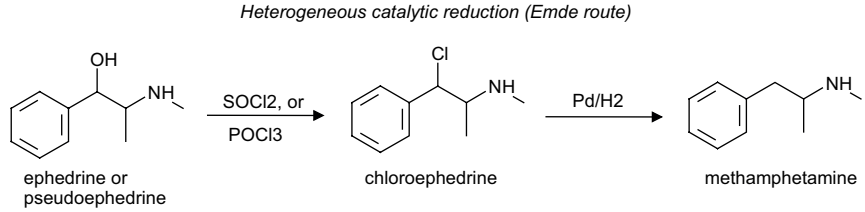


Figure IV. *(continued)*



Other reactions, such as the "nitropropene" or "oxime" route, are rarely encountered.

Ephedrine and pseudoephedrine are widely available in pharmaceutical cough preparations, many of which are available over-the-counter. The Chinese herb Ma-huang, which is encountered in a number of food supplement and lifestyle products is another source of those precursors.

Unlike P-2-P, ephedrine and pseudoephedrine are chiral substances. They are diastereomers, and exist in two enantiomeric forms, each (d- and l-ephedrine, and d- and l-pseudoephedrine), in addition to the two racemates. The chiral analysis of ephedrine or pseudoephedrine isomers can also help in the determination of the manufacturing process of illicit methamphetamine.

All manufacturing methods starting from l-ephedrine or d-pseudoephedrine produce (+)-(S)-methamphetamine as the single optical isomer, which is at least twice as potent as the racemic mixture produced by reactions starting from P-2-P.

Hydriodic acid-red phosphorus route: this is typically carried out by heating ephedrine or pseudoephedrine with red phosphorus and hydriodic acid. The reaction mixture is then filtered, basified and extracted into a solvent. The resulting methamphetamine base is an oily liquid, commonly referred to as "meth oil". The hydrochloride salt is crystallized from this liquid using ether/acetone and hydrochloric acid. Alternatively, hydrogen chloride gas (from a cylinder, an aqueous solution, or generated using sulphuric acid and sodium chloride) is bubbled through the meth oil causing the hydrochloride salt to precipitate out of the solution.

Hl and red phosphorus can be replaced by iodine and hypophosphoric acid (sodium hypophosphate), or by water and iodine. In rare occasions, the reaction mixture, sometimes known as "ox-blood", is used without further purification, mostly by injection. The red coloured mixture, caused by an excess of iodine, contains "meth oil" and different impurities related to the Hl/red P route.

Typical impurities found in samples produced by reductions involving Hl/red P or iodine/hypophosphoric acid are ephedrine or pseudoephedrine, aziridines and dimethylnaphthalenes. Aziridines cannot be considered as route-specific impurities since they can be also produced from chloroephedrine by halogen elimination and ring closure (Emde method), or from an oxime intermediate and N-hydroxymethamphetamine.

Birch reduction: this proceeds via a dissolving metal reduction of ephedrine or pseudoephedrine in the presence of ammonia. The reaction involves mixing the ephedrine or pseudoephedrine with anhydrous ammonia gas and either sodium or lithium metal. The mixture is then allowed to stand until the ammonia has evaporated. Isolation of the meth oil is carried out by direct solvent extraction and filtration. The reaction product is further purified by formation of the hydrochloride salt and re-crystallisation. In illicit practice, Birch reduction is usually completed in a one-step reaction using widely available ammonia, and lithium strips from batteries. Despite this, Birch reduction usually produces a very "clean" end-product. Several route-specific impurities such as N-methyl-1-(1-(1,4-cyclohexadienyl))-2-propanamine are reported in the literature. The reaction involving anhydrous ammonia is hazardous and explosions in clandestine laboratories are not uncommon.

Emde method: ephedrine or pseudoephedrine are typically reacted with thionyl chloride to give the intermediate chloroephedrine, which is then hydrogenated over a platinum or palladium catalyst to yield methamphetamine. In GC-based analytical schemes, the chloroephedrine intermediate is rarely found as an impurity, because it decomposes during analysis to form aziridines. Chloroephedrine also decomposes rapidly during basic extraction of methamphetamine.

C. SYNTHESIS OF RING-SUBSTITUTED ATS

The most commonly encountered methylenedioxy-type ATS is MDMA, and occasionally MDEA and MDA. In general, the synthesis routes used for MDMA are applicable, with minor modifications, to other methylenedioxy-substituted analogues.

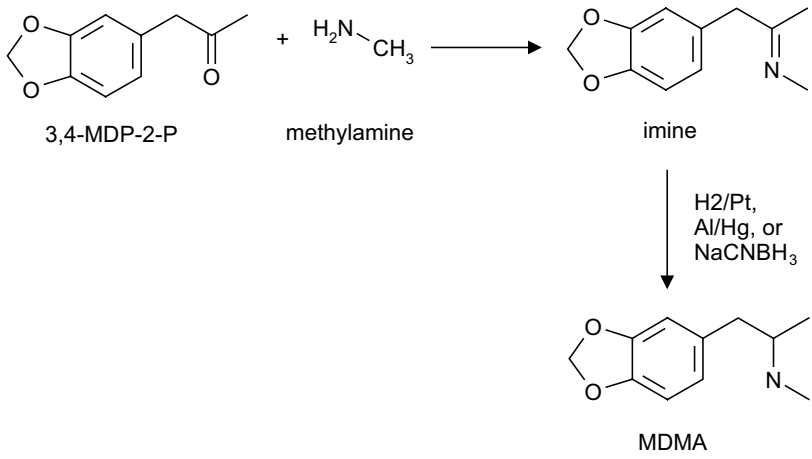
The key precursor used for such syntheses is 3,4-methylenedioxyphenyl-2-propanone (also known as 3,4-MDP-2-P, 3,4-methylenedioxy-phenylacetone, piperonyl methyl ketone, or PMK). 3,4-MDP-2P is a commercially available, internationally controlled precursor.

MDMA can also be synthetised from safrole (3,4-methylenedioxyallylbenzene), either directly, or via isosafrole obtained by isomerization of safrole. Safrole itself is commercially available, or can be extracted from sassafras oil and other safrole-rich essential oils or plant parts. Piperonal (heliotropin, 3,4-methylenedioxybenzaldehyde), a widely used industrial chemical, is another alternative precursor for the synthesis of 3,4-MDP-2-P.

The most direct method for MDMA synthesis is via reductive amination of 3,4-MDP-2-P with methylamine and hydrogen gas over a platinum catalyst (catalytic metal reduction). The reduction can also be achieved by dissolving metal reduction using aluminium amalgam (aluminium foil and mercuric chloride), or by metal hydride reduction using sodium cyanoborohydride (see figure V).

Substituting ethylamine for methylamine produces MDE; ammonia gas produces MDA, while dimethylamine produces MDDMA.

Figure V. Reductive amination used in illicit MDMA manufacture



Analogous to illicit amphetamine manufacture, a method commonly used in illicit manufacture of MDMA is the Leuckart method. 3,4-MDP-2-P and N-methylformamide are reduced using formic acid. The resulting intermediate N-formyl-MDMA is hydrolized by refluxing with strong acid or base to produce MDMA.

The nitropropene method has become popular since the early 1990s. It involves the reaction of piperonal with nitroethane in the presence of basic catalyst, commonly n-butylamine. Various procedures for the production of the intermediate phenyl-nitropropene are reported in the literature, but typically a mixture of piperonal and nitroethane is simply allowed to stand for a few days in the dark. Alternatively, piperonal and nitroethane are refluxed in acetic acid and ammonium acetate.

The intermediates formed in these reactions are very characteristic in appearance and usually precipitate out of the reaction solution as bright yellow crystals. To produce MDA, the intermediate is usually reduced with lithium aluminum hydride. For the synthesis of MDMA, MDEA or other ATS, the nitropropene intermediate is converted into 3,4-MDP-2-P, which is then reduced by one of the reductive amination methods described previously. The nitropropene/nitrostyrene intermediates are bright yellow or bright orange crystalline substances.

MDMA is also commonly manufactured using the so-called “bromosafrole” route. The reaction proceeds via bromination of safrole with hydrobromic acid at low temperature, followed by treatment with methylamine to form the final product. The yield will depend on the water content of the reaction mixture. Substitution of methylamine with other amines produces different MDMA-type products (e.g., MDA, MDEA, or MDDMA).

Methoxyamphetamine-type drugs are typically synthesized using the appropriate ring-substituted aldehyde and nitroethane, while for mescaline, 2C-B, and other ring-substituted phenethylamines, nitromethane is used.

2C-B is manufactured by reacting 2,5-dimethoxybenzaldehyde and nitromethane, followed by $LiAlH_4$ reduction to form the 2,5-dimethoxyphenethylamine. 2,5-dimethoxyphenethylamine is then brominated to form the final product.

VI. QUALITATIVE AND QUANTITATIVE ANALYSIS OF ATS

Generally, in attempting to establish the identity of a controlled drug in suspected material, the analytical approach must entail the determination of at least two uncorrelated parameters. 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.

A. PRESUMPTIVE TESTS

Presumptive tests are fast screening procedures that usually consist of two or three independent tests. They are designed to provide an indication of the presence or absence of drug classes in the test sample and quickly eliminate negative samples. Good presumptive test techniques, as all analytical techniques, maximize the probability of a "true" result, and minimize the probability of a false positive. However, presumptive tests are not considered sufficient for drug identification and results must be confirmed by additional laboratory tests.

In recent times, presumptive tests are more often used as field tests, although they are also carried out in laboratories as a first screening procedure. For ATS screening, colour tests, or spot tests, are typically used, although immunoassay tests and a number of fast and portable instrumental techniques are also available. Instrumental screening methods such as ion mobility spectrophotometer (ion-scan), portable mass spectrometer, FTIR or Raman spectrometer, recently have gained popularity. Many commercial test kits for ATS screening are available, however, they should be evaluated "in house" for specificity and sensitivity.

1. COLOUR TESTS

Colour tests are usually the simplest and quickest chemical test that an analyst can apply to a sample. Most colour tests are quite sensitive; thus, only minute quantities of sample are necessary to complete a successful test, and often the

best results are obtained with the smallest of sample quantities, frequently less than one mg.

Because samples may vary in purity (ATS concentration), and unrelated substances may be present, the colours exhibited by these tests should be interpreted with care. In addition, the subjective aspect of colour evaluation should always be borne in mind.

Colour test techniques

Several different reagents are typically employed for colour testing of amphetamine type substances and their ring substituted analogues. The most important colour tests for these substances are Marquis, Simon's and Chen's test. The *Marquis test* allows the distinction between amphetamine and its ring-substituted analogues. *Simon's test* is generally used as a test for secondary amines, such as methamphetamine and secondary ring-substituted amphetamines, including MDMA and MDE. However, other secondary amines, for example, diethylamine and piperidine, may give similar colours. In general, colours are intense but may fade quickly in the presence of some impurities. For these reasons, it is essential for the analyst to confirm Simon's test results by performing a supplementary test, e.g., the Marquis test. *Chen's test* is used to distinguish ephedrine, pseudoephedrine, norephedrine, phenylpropanolamine and methcathinone from amphetamine and methamphetamine, which do not react with Chen's test reagent.

A fourth test, the *gallic acid test*, provides a simple means for the distinction of MDMA, MDA and MDEA from amphetamine or methamphetamine, because it reacts specifically with methylenedioxy-substituted aromatic compounds. Precursors containing the methylenedioxy-substructure, such as safrole and isosafrole also react.

Methods

Preparation of reagents is described in annex II. Reagents should be prepared freshly.

Marquis test

- Place a small amount (1-2 mg of powder, or 1-2 drops of a liquid) of the suspected material in a depression on a spot plate.
- Add one drop of Marquis Reagent 1, then one drop of Reagent 2, and stir.
- Observe the colour of the mixture.

Simon's test

- Place a small amount (1-2 mg of powder, or 1-2 drops of a liquid) of the suspected material in a depression on a spot plate.

- Add one drop of Simon Reagent 1 and stir.
- Add one drop of Simon Reagent 2, and then one drop of Reagent 3.
- Observe the colour of the mixture.

Chen's test

- Place a small amount (1-2 mg of powder, or 1-2 drops of a liquid) of the suspected material in a depression on a spot plate.
- Add 2 drops of Chen Reagent 1.
- Add 2 drops of Chen Reagent 2, then add 2 drops of Reagent 3 and stir.
- Observe the colour of the mixture.

Gallic acid test

- Place a small amount (1-2 mg of powder, or 1-2 drops of a liquid) of the suspected material in a small test tube.
- Add one drop of Gallic acid Reagent.
- Observe the colour of the mixture.

Results

Table 1 provides results of the three main colour tests for the most commonly encountered amphetamine-type stimulants and their ring-substituted analogues.

Because the gallic acid test is mainly used to identify, generally, the methylene-dioxy ring substituent and not individual ATS with that sub-structure, results are not included separately, for individual substances, in the table. A bright to dark green colour indicates the presence of MDA, MDMA, MDE, N-hydroxy-MDA or MMDA. In some cases, e.g., MDE and N-hydroxy-MDA, the green colour may change to brown during the course of the test.

Table 1. Colour test results

Compound	*Marquis reagent*	*Simon reagent*	*Chen reagent*
Amphetamine	Orange, slowly turning brown	NR*	NR*
Cathinone	NR	NR*	Gradually turns to yellow or orange
Dimethylamphetamine	Orange	NR*	NR*
Ephedrine Pseudoephedrine	NR	NR*	Purple
N-Ethylamphetamine	Yellow, turning brown		
Methamphetamine	Orange, slowly turning brown	Deep blue	NR*
Methcathinone	NR	slightly blue, spot- or ring-like precipitate	Gradually turns to yellow or orange
Norephedrine	NR	NR	Purple
2C-B	Yellow --> green	NR*	NR*
2C-T-2	Light pink, orange	NR*	
2C-T-7	NR	NR*	

Table 1. *(continued)*

Compound	*Marquis reagent*	*Simon reagent*	*Chen reagent*
DMA	green --> dark green	NR*	
DOB	Yellow --> green	NR*	NR*
DOET	yellow brown	NR*	
FLEA	Dark blue/black		
MBDB	Dark blue/black	Deep blue	NR*
MDA	Dark blue/black	NR*	NR*
MDDM	Dark blue/black		
MDEA	Dark blue/black	(Deep) blue --> brown	NR*
MDMA	Dark blue/black	Deep blue	NR*
MDOH	Dark blue/black		
MMDA	Purple	NR*	NR*
4-MTA	NR	NR*	
PMA	NR --> light green	NR*	
STP / DOM	Yellow	NR*	NR*
TMA	Orange	NR*	

Note: NR = no reaction
*The colour of the reagent should be considered as negative.

Analytical notes

(a) ATS specific

Because ATS, especially ring-substituted analogues and methamphetamine in Southeast Asia, are frequently encountered in the form of brightly coloured tablets, the result of colour tests may be masked, or a change of the colour test result may occur.

Although a wide variety of colours are used for the production of ATS tablets, most colours are soluble in water, and their solubility may be manipulated by changing the pH of the solution. In such cases, therefore, the analyst should adjust the extraction procedure and eliminate the colour altogether before proceeding to the colour test itself.

In cases, when a colour test cannot be clearly interpreted due to presence of a tablet dye, the following procedure will often produce acceptable results:

Place a small amount (approximately 10 mg) of the sample into a small test tube. Add approximately 1 ml of methanol (or 1 ml of a 4:1 mixture of methanol:methylene chloride). After filtering through glass wool, evaporate to dryness. Reconstitute the
sample in a minimum amount of water, and then proceed with the colour test by carefully depositing the aqueous sample solution on a spot plate and adding colour reagent. Since the sample is already diluted, 3 reagent drops per one drop of sample solution will be sufficient.

The presence of diluents and adulterants may also disturb colour reactions and result in false negative test results. While Simon's test can lead to false negative results when adulterants or diluents are present in ATS samples, the Marquis test is less sensitive to adulterated samples and is, therefore, preferable when the concentration of ATS in the sample is very low.

(b) General

The colours described are subjective judgements due to individual perception of colours. Because of this, subjective aspect of colour evaluation, it is necessary for each analyst to test appropriate reference standards in order to ensure that he or she can recognize each colour test result. Similarly, it is advisable to carry out a blank test without the target substance to ensure familiarity with the colour of the reagent.

When preformed properly, a negative colour test is generally quite reliable in establishing the absence of a target compound; however, positive results are only presumptive indications of the possible presence of a compound. Many other compounds, often harmless and uncontrolled by national legislation or international treaties, may give similar colours with a given colour test reagent.

Therefore, it is mandatory for the analyst to confirm a positive colour test for any legally controlled compound by the use of additional laboratory tests.

To eliminate the possibility of a false positive result due to a contaminated spot plate, it may be advantageous to place the reagent onto the spot plate, and then add a small quantity of the sample to the reagent.

Further reading

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2. ANION TESTS

Anion testing for forensic purposes typically makes use of solubilities combined with selected reactions where results are determined by the presence or absence, and solubility, of a precipitate.

The solubility of different ATS and their salts in water and several common solvent systems are described below.

Amphetamine and its salts

	Base	*Hydrochloride*	*Phosphate*	*Sulphate*
Water	Slightly soluble	Soluble	Soluble	Soluble
Methanol or ethanol	Soluble	Soluble	Slightly soluble	Slightly soluble
Diethyl ether	Soluble	Insoluble	Insoluble	Insoluble
Chloroform	Soluble	Soluble	Insoluble	Insoluble

Methamphetamine and its salts

	Base	*Hydrochloride*
Water	Slightly soluble	Soluble
Methanol or ethanol	Soluble	Soluble
Diethyl ether	Soluble	Insoluble
Chloroform	Soluble	Soluble

Ring-substituted ATS and their salts

The free bases of ring-substituted ATS are generally insoluble in water and soluble in ethanol, diethyl ether, chloroform and other organic solvents. Their hydrochloride salts are soluble in water and ethanol, slightly soluble in chloroform, and insoluble in diethyl ether. Solubilities of individual substances of this group depend on the specific ring-substituted ATS in question.

Methods

All reagents should be prepared according to an established procedure. Details of the preparation of reagents are described in annex II.

Silver nitrate test

The unknown ATS sample is dissolved in several drops of deionized water and treated with 1-2 drops of $AgNO_3$ solution. The results for common anions are listed below. Because other anions can also give similar results, additional tests or familiarization of the test limitations should be performed.

Chloride: Forms a white curdy precipitate that is insoluble in concentrated nitric acid. After washing with water, the precipitate is soluble in dilute ammonia solution, from which it can be re-precipitated by the addition of nitric acid.

Bromide: Forms a pale yellow to cream coloured precipitate that is insoluble in nitric acid. After washing with water, the precipitate very slowly soluble in dilute ammonia solutions and soluble in concentrated ammonia solutions. It can be re-precipitated from both solutions by the addition of nitric acid.

Iodide: Forms a bright yellow precipitate that is slightly soluble in a concentrated ammonia solution but soluble in a thiosulfate solution.

Sulphate: Forms a light coloured crystalline precipitate that can easily be identified by placing the testing solution on a microscope slide and looking for the characteristic "diamond" shaped silver sulphate crystals.

Phosphate: Forms a light yellow precipitate, which is soluble in a dilute ammonia solution, or cold nitric acid.

For sulphate and phosphate salts, additional specific tests may be performed:

Sulphate salt test

The unknown ATS sample (about 100 mg) is dissolved in water and treated with a solution of barium chloride. A white precipitate, which is insoluble in hydrochloric acid, indicates the presence of a sulphate salt.

Phosphate salt test

The unknown ATS sample (about 100 mg) is dissolved in a solution made of equal volumes (for example, 1ml each) of nitric acid solution (10% v/v) and ammonium molybdate solution (10% w/v). With gentle heating, formation of a bright canary-yellow precipitate, which is soluble in ammonia solution, indicates the presence of a phosphate salt.

Analytical notes

Since all anion tests are carried out in aqueous solutions, water-solubility of the ATS salts is a pre-condition to meaningful results.

Washing the precipitate with water before performing the test for dissolution of the precipitate is critical to remove any soluble (non-precipitated) anion.

Further reading

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3. MICROCRYSTAL TESTS

Microcrystal tests are quick, simple, and extremely sensitive tests for the identification of substances and their optical isomers. They involve formation of crystals from the reaction of the target compound with a chemical reagent, followed

by the analysis of the resulting crystals by means of a polarizing microscope and comparison with reference material, usually photographs of known crystals.

Methods

The simplest form of the test consists of adding a drop of suitable reagent to the test substance, followed by observing and analyzing the crystals formed under the polarizing microscope.

In order to maintain an accurate record, the characteristic features of the crystals should be described. The most accurate record of the test results is by photographs. If a photograph is not available a sketch of crystal forms is helpful.

Methods and procedural details for microcystal tests of ATS are described in chapter VI.G.2. Descriptive terms for crystal forms and photographs of crystals of major ATS as well as basic instrument and equipment requirements are given in annex III.

Further reading

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Fulton, C. C. (1969). *Modern Microcrystal Tests for Drugs*. Wiley-Interscience, A Division of John Wiley & Sons, New York-London-Sydney-Toronto.

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B. THIN LAYER CHROMATOGRAPHY (TLC)*

TLC has become one of the most commonly used techniques for the separation and identification of illicitly manufactured drugs. It is rapid (an analysis is rarely longer than thirty minutes), sensitive (sub-milligram quantities of analyte required), enormously flexible in both the stationary and the mobile phase, and thus amenable to a wide variety of substances, in base and salt form, ranging from the most polar to the most non-polar materials. It is also amenable to a variety of visualization techniques, and it is inexpensive.

Despite the obvious advantages of TLC, in many countries, it is not accepted as a single technique for drug identification.

*For more standardized TLC systems, see: *Thin-layer chromatographic R_f values of toxicologically relevant substances on standardized systems,* second, revised and enlarged edition, Report XVII of the DFG Commission for clinical-toxicological analysis, Special issue of the TIAFT Bulletin, VCH Verlagsgesellschaft mbH, 1992.

TLC plates (stationary phases)

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

Note: Plates prepared by the analyst must be activated before use by placing them into an oven for at least 10 to 30 minutes at 120°C. Plates are then stored in a grease-free desiccator over blue silica gel. Heat activation is normally not required for chemically bonded layers (commercial plates).

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

Solvent systems (mobile phases)

System A:	Methanol	100
	Concentrated ammonia	1.5
System B:	Ethyl acetate	85
	Methanol	10
	Concentrated ammonia	5
System C:	Cyclohexane	75
	Toluene	15
	Diethylamine	10

Methods

Recommended methods and selected results are provided below, but it remains the responsibility of the analyst to familiarize him/herself with the specific results of individual ATS.

Solvent systems
Prepare developing solvent systems as accurately as possible by use of pipettes and measuring cylinders. Leave the solvent system in the TLC tank for a time sufficient to allow vapour phase saturation to be achieved prior to the analysis (with adsorbent paper-lined tanks, this takes approximately 5 minutes).

Preparation of ATS standard and sample solutions
The form of standards and samples, salt or base, is unimportant. Either form will be satisfactory. Because of the basic nature of the developing solvents, the compounds migrate as free bases.

ATS standard solutions: Standard solutions of ATS should be prepared at a concentration of approximately 2 mg/ml in methanol. They must be stored in a dark and cold place.

ATS sample solutions (unknown ATS sample): Sample solutions should be prepared at a concentration of approximately 5mg/ml in methanol. In cases, where the ATS purity is suspected to be very low due to adulteration, it may be necessary to prepare more concentrated sample solutions (ten-times more concentrated solutions are recommended as a starting point).

For ATS samples in other forms than powder, the sample solutions should be prepared as follows:

Tablets: Grind a representative number of tablets (following general sampling plans) to a fine powder and prepare a solution as for powders.
Capsules: Remove the contents of a representative sample of capsules (following general sampling plans) and prepare a solution as for powders.
Aqueous solutions: Spot directly, or the equivalent of 5mg/ml, if the concentration of the ATS is known.

Spotting and developing

Place both a 1 μl and a 5 μl spot of sample solution, together with 2 μl of the standard solution(s) onto the TLC plate (preferably, also a solvent spot as a negative control should be applied to the plate). Spotting must be done carefully, without damaging the plate's surface.

The starting point of the run, i.e., the "spotting line," should be 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 ($\leq$ 2 mm).

Allow spots to dry, and place plate into solvent-saturated TLC 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 the solvent reaches the development line marked beforehand; otherwise, diffuse spots will occur.

Visualization/detection

Plates must be dried prior to visualization: The solvent can be allowed to evaporate at room temperature, or removed with a hot air blower. If hot air is used, care must be taken because of the volatility of the ATS free bases. It is important for proper colour development that all traces of ammonia be removed from the plate.

The following is a selection of visualization methods. Based on the results of the presumptive tests, anticipated ATS should be targeted using one or a combination of the following methods and reagents:

Method/Visualization reagent	*Target analytes and results*
A. UV light at 254 nm	Universal method. Many substances, including ATS, give purple spots on an otherwise green-fluorescent plate
B. Ninhydrin reagent	Many primary and secondary amines attached to an aliphatic carbon atom, such as amphetamine and methamphetamine, result in violet or pink spots.
C. Acidified potassium iodoplatinate reagent	Sensitive general reagent. Most primary and secondary amines give light blue spots.
D. Fast Black K	Primary and secondary amines give spots varying in colour from violet (primary amines) to orange or orange-red (secondary amines).
E. Marquis reagent	Distinction between unsubstituted and ring-substituted ATS
F. Fluorescamine reagent (Fluram)	Sensitive reagent for primary amines. Recommended for the detection of low concentrations of primary amines.
G. Simon reagent	General reagent for secondary amines (ephedrine and pseudoephedrine do not react)
H. Dragendorff ragent	General reagent for alkaloids and nitrogenous bases

Key:

Method/Visualization reagent

A. UV light

Observe the dried plate under UV light at 254nm and 366nm.

B. Ninhydrin reagent

Prepare a 10% solution in ethanol.

Spray the plate with the ninhydrin reagent and heat in an oven at 120°C for at least 15 minutes. Violet or pink spots are given by primary amines such as amphetamine and secondary amines such as methamphetamine. Ephedrine also produces a violet spot.

C. Acidified potassium iodoplatinate reagent

Dissolve 0.25g platinic chloride and 5g potassium iodide in water and make up to 100 ml. Add 2 ml of concentrated hydrochloric acid.

Spray the plate with acidified potassium iodoplatinate solution and observe any coloured spots Amphetamine and methamphetamine give dirty grey-violet-brown spots on a pink background.

The solution may also be used to overspray plates that have previously been sprayed with ninhydrin.

D. Fast Black K

Solution A: Prepare 1% solution of Fast Black K salt in water [2,5-Dimethoxy-4-((4-nitrophenyl)azo)benzene diazonium tetrachlorozincate (2:1)]

Solution B: 1N NaOH

Spray the plate with solution A and observe any coloured spots. Secondary amines such as methamphetamine and MDMA produce spots immediately. Overspraying with solution B produces coloured spots for amphetamine and other ring-substituted ATS. Air dry the plates and spray once more with solution A. This produces more intensely coloured spots. The colours vary from violet for primary amines to orange or orange-red for secondary amines such as methamphetamine and MDMA.

E. Marquis reagent

Add 8-10 drops of 40% formaldehyde solution to 10 ml of concentrated sulphuric acid. Prepare fresh prior to each use.

Spraying with Marquis reagent is not recommended because of the concentrated sulphuric acid involved. However, it provides additional information useful for differentiating between ATS, for example, after detection with ninhydrin. To that end, drop the Marquis reagent with a Pasteur pipette on the spots already detected.

F. Fluorescamine reagent (Fluram)

Dissolve 10 mg fluorescamine in 50 ml acetone. Prepare daily

Spray the plate with the fluorescamine reagent. Air dry it with a hot air blower. Observe the plate under a UV light at 366 nm. Amphetamine gives a bright yellow fluorescent spot. Methamphetamine is not detected. (For stabilization on 366 nm, spray with 10% v/v solution of trietylamine in dichloromethane).

G. Simon reagent

Dissolve 100 mg of sodium nitroprusside and 2 g of sodium carbonate in 10 ml of water (i.e., an aqueous solution containing sodium nitroprusside at a concentration of 1%, and sodium carbonate at 20%). Prepare reagent freshly before use.

Spray the plate with the Simon reagent. Place the plate in an empty developing tank along with a beaker containing acetaldehyde. Cover the tank. The acetaldehyde vapour will cause a methamphetamine spot to become an intense blue colour. (This modified method improves the sensitivity for methamphetamine to 0.1 μg (LOD). Primary amines cannot be detected, because of the system's low sensitivity for this group of substances, and the interference with the background colour when ammonia is used in the developing solvent.)

H. Dragendorff reagent

Stock solution: Dissolve 0.85 g bismuth subnitrate (basic) in 10 ml acetic acid. Dilute to 50 ml with water and add 8 g of potassium iodide in 20 ml water

Spray the plate with a solution prepared from 1 ml Dragendorff stock solution, 2 ml acetic acid and 10 ml water. Colours vary from orange to violet.

Interpretation

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

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

It is very common to express retention factors as R_f x 100, referred to as hR_f.

Results

Compare colours and R_f values of the unknown ATS sample with those of the authentic ATS reference standards that were run simultaneously on the same plate. R_f values for some of the most common ATS are given in Table 2.

Table 2. R_f values of commonly encountered ATS and adulterants

	*TLC system**		
ATS name	*A*	*B*	*C*
Amphetamine	0.48 (0.43)**	0.37 (0.43)	(0.20)
Cathinone	0.66	0.56	
DOB	0.37	0.32	(0.13)
DOET	0.36	0.32	(0.24)
DMA	0.37	0.33	(0.19)
N-Ethylamphetamine	0.47	0.37	(0.47)
Methamphetamine	0.35 (0.31)	0.22 (0.42)	(0.28)
MDA	0.36 (0.39)	0.33 (0.42)	(0.18)

	*TLC system**		
ATS name	*A*	*B*	*C*
MDMA	0.31 (0.33)	0.21 (0.39)	(0.24)
MMDA	0.40	0.31	
PMA	0.41 (0.73)	0.33 (0.43)	(0.23)
STP/DOM	0.35 (0.51)	0.31 (0.41)	(0.15)
TMA	0.35	0.20	
Ephedrine	(0.30)	(0.25)	(0.05)
Caffeine	(0.52)	(0.52)	(0.03)

*Solvent system: A, B or C; TLC plate: Silica gel G with layer thickness of 0.25 mm
System A: Methanol: concentrated ammonia (100:1.5)
System B: Ethyl acetate: Methanol: concentrated ammonia (85:10:5)
System C: Cyclohexane: Toluene: Diethylamine (75:15:10)

**R_f values in brackets were obtained using Silica plates impregnated with methanolic KOH (0.1 mol/l).

Analytical note

Because small changes in TLC plate composition and activation, in solvent systems, tank saturation or development distance can result in significant changes in the R_f values, the values provided should only be considered as an indication of the chromatographic behaviour of the ATS and adulterants listed. It is essential that ATS reference standards be run simultaneously on the same plate. Alternatively, reproducibility can be significantly enhanced by the use of reference compounds and corrected R_f values (R_f^c).

For identification purposes, both the R_f values and the colour of the spots after spraying with different visualization reagents should always be considered.

Further reading

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C. GAS CHROMATOGRAPHY (GC)—FLAME IONIZATION DETECTOR (FID)

For the GC analysis of ATS, the general principles of the techniques apply. Today, the GC instrument of choice for routine analytical work is the narrow bore capillary gas chromatograph, using capillary columns with internal diameters between 0.2 and 0.32 mm.

It is recognized that there are laboratories, which for a variety of reasons, may wish to maintain a packed column system. For those laboratories, a method using packed columns was described in an earlier edition of this manual. GC procedures utilizing a megabore capillary column (0.53 mm internal diameter) represent a means to improve on resolving power compared to packed columns, and are more robust than narrow bore capillary column systems. Older GC systems that are designed for packed columns can be converted for use with megabore columns.

Methods

1. QUALITATIVE ANALYSIS

Preparation of ATS standard and sample solutions

ATS standard solutions: weigh approximately 25 mg of ATS standard salt(s)* of interest into a 25 ml volumetric flask and make up to the mark with water. Pipette an aliquot of 1 to 5 ml of this solution into a 10 ml glass stoppered test tube. Add drop-wise a 5% solution of sodium hydroxide until pH 10. Then add 5 ml of extracting solvent**.

Stopper and invert the test tube at least 10 times or vortex for 1 min and let stand until layers separate.*** Using a Pasteur pipette, transfer the solvent layer (e.g., chloroform) through anhydrous sodium sulphate layer into a GC vial.

Inject 1-2 μl of the solvent layer into the GC.

ATS sample solutions (unknown ATS sample): weigh 25 to 150 mg of sample, depending on the anticipated purity, to obtain a final concentration of about

*Occasionally, ATS standards can be obtained in the base form. In those cases, extraction is not required. In general, however, it is important that the form of standards and samples be always the same.

**All analytes must be completely soluble in the extraction solvent. The extraction solvent must be immiscible with aqueous layer. Suitable solvents include n-hexane, chloroform, methylene chloride or butyl acetate.

***When chloroform is used as extracting solvent, emulsions may form. In such cases, addition of NaCl improves the extraction rate by breaking the emulsion. If modern shakers are used and the mixture is then centrifuged for separation of the two layers, the formation of emulsions normally does not occur.

1 mg/ml of analyte salt, into a 25 ml volumetric flask and make up to the mark with water. The anticipated sample purity is determined empirically.*

Pipette an aliquot of 1 to 5 ml of this solution into a 10 ml glass stoppered test tube. Add drop-wise a 5% solution of sodium hydroxide until pH 10. Then add 5 ml of the extracting solvent (e.g., chloroform).

Stopper and invert the test tube at least 10 times or vortex for 1 min and let stand until the layers separate. Using a Pasteur pipette, transfer the solvent layer through anhydrous sodium sulphate layer into a GC vial.

Inject 1-2 μl of the solvent layer into the GC.

If quick sample throughput is required, samples may be taken up directly in ethanol/aqueous ammonia (99:1), and injected onto the gas chromatograph. Using this method, the condition of the injector and column may deteriorate more quickly than with extracted samples (Note: This method is not suitable for quantitative analysis since it is hard to produce good chromatography in the presence of ammonia).

GC operating conditions

Detector:	FID (or NPD, if available/desired)
Column:	DB-5 (5% phenyl 95% dimethylpolysiloxane), DB-1 (100% dimethyl-polysiloxane), or equivalent
Length:	10-30 m, ID 0.20-0.53 mm
Film thickness:	0.10-0.50 μm
Carrier gas:**	Nitrogen, helium or hydrogen, at approx. 0.8ml/min. (N_2) or 1-1.2 ml/min. (He or H_2)
Split ratio:	20:1 to 50:1
Column temperature:	initial temperature has to be low enough (e.g., 60-90°C) to account for high volatility of ATS bases, e.g.: 60°C, hold for 0.5 min., to 280°C, at a rate of 12°C/min., hold final temperature for 30 min.
Injector temperature:	210-250°C
Detector temperature:	310°C

Results

Identification is accomplished by comparing the retention time of the analyte with that of a reference standard. The elution order is as follows:

*Determination of sample purity may be done based on chemist's experience, or using the following rough method: Take 100 mg of sample and dissolve in 2-3 ml of chloroform. Filter the solution, collect the insolubles, dry and weigh. The soluble amount (i.e., original weight minus dry insolubles) is the anticipated sample purity. Note, however, that this method may result in a lower than actual anticipated sample purity for salts that are not soluble in chloroform (see chapter VI.A.2, on "anion tests", above).

**The gas flow depends on column ID; pressure gradient programming can be used if available.

amphetamine < methamphetamine < pseudoephedrine < ephedrine < PMA < PMMA < MDA < MDMA < 4-MTA < MDEA < MBDB < 2C-B.

Under the conditions described, caffeine and ketamine, which are frequently found in ATS samples in some regions, elute after 2C-B.

2. QUANTITATIVE ANALYSIS

Three methods for the quantitative GC-FID analysis of ATS are provided below: A single standard method (A) and two multiple standard methods (B and C). Methods A and B do not require derivatization, while method C requires silylation. All three methods are described here for general use. Annex IV provides examples of validated GC methods for quantitation of selected ATS.

Method A: Single standard method

Method A is suitable for quantitation of most ATS. It involves the preparation of only one ATS standard solution in a concentration similar to the anticipated concentration of the analyte.

Preparation of internal standard solution (IS): weigh accurately approximately 25 mg of the selected internal standard (for example, n-tetradecane or other n-alkanes with an even number of carbon atoms, or diphenylamine) and dissolve it in 25 ml of extracting solvent (e.g., chloroform). If the internal standard solution is used for extraction, the target concentration should be prepared within the instrument linear range (no more than 0.5 mg/ml).

Preparation of ATS standard and sample solutions:* the preparation of ATS standard and sample solutions should follow the procedures outlined above for qualitative analysis, using the internal standard solution for the extraction of both ATS standards and samples, as follows:

(a) ATS standard solutions

Weigh accurately approximately 25 mg of ATS standard salt(s)** of interest into a 25 ml volumetric flask and make up to the mark with water. Accurately pipette an aliquot of 1 to 5 ml of this solution into a 10 ml glass stoppered test tube. Add drop-wise a 5% solution of sodium hydroxide until pH 10. Then add 5 ml of internal standard solution, accurately dispensed.

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*ATS standard and samples solutions and their concentrations are designed for use with capillary columns and the procedures described below. The use of alternative columns and GC systems may necessitate changes in terms of both relative composition and concentrations of individual components.

**If ATS standards in the base form are used, extraction is not required. In those cases, weigh no less than approximately 10 mg of ATS standard and dissolve directly in 10 ml of accurately dispensed internal standard solution. For accurate quantitation, it is important that the form of the analyte (salt or base) be the same as that of the ATS standard.

Stopper and invert the test tube at least 10 times or vortex for 1 min and let stand until layers separate. Using a Pasteur pipette, transfer the solvent layer through anhydrous sodium sulphate layer into a GC vial.

Inject 1-2 μl of the solvent layer into the GC. Analyze standard solution in triplicate or more.

(b) ATS sample solutions (unknown ATS sample)

Weigh accurately 25 to 150 mg of sample, depending on the anticipated purity, to obtain a final concentration of about 1 mg/ml of analyte salt, into a 25 ml volumetric flask and make up to the mark with water. The anticipated sample purity is determined empirically.

Accurately pipette an aliquot of 1 to 5 ml of this solution into a 10 ml glass stoppered test tube. Add drop-wise a 5% solution of sodium hydroxide until pH 10. Then add 5 ml of internal standard solution, accurately dispensed.

Stopper and invert the test tube at least 10 times or vortex for 1 min and let stand until the layers separate. Using a Pasteur pipette, transfer the solvent layer through anhydrous sodium sulphate layer into a GC vial.

Inject 1-2 μl of the solvent layer into the GC. Analyze unknown ATS sample solution preferably two times or more.

GC operating conditions

Same as for qualitative GC analysis (see p. 30).

Calculations

From the multiple injections, calculate average peak area ratios of: *(a)* the relevant ATS standard to the internal standard ($A_{St/IS}$), and *(b)* the unknown ATS to the internal standard ($A_{ATS/IS}$).

The percentage drug content of the sample can be calculated using the general formula:

$$\text{ATS (\%)} = \frac{C_{St}}{C_{ATS}} * \frac{A_{ATS/IS}}{A_{St/IS}} * 100$$

where

ATS (%) = Content of the unknown ATS (as base or salt; see footnote ** on p. 32) in the original sample material (= sample purity)

C_{St} = Concentration of ATS standard solution (mg/ml), as prepared under *(a)*, above (= weight of pure ATS standard per millilitre solvent)

C_{ATS} = Concentration of the unknown ATS sample solution (mg/ml), as prepared under *(b)*, above (= weight of unknown ATS sample per millilitre solvent)

$A_{ATS/IS}$ = Peak area counts of the unknown ATS divided by peak area counts of the internal standard (preferably, average of duplicate determinations)

$A_{St/IS}$ = Peak area counts of ATS standard divided by peak area counts of the internal standard (average of triplicate determinations)

Method A can be modified from a single to a multiple standard method by sequentially diluting aliquots of the ATS standard solution with the internal standard solution using 10 ml volumetric flasks. That way, ATS standard concentrations can be prepared, targeting the appropriate concentration within the linear range of the instrument, and achieving a multiple-point calibration.

Method B: Multiple-standard method without derivatization

The method below is a recommended multiple-point calibration method, validated GC methods for the quantitative analysis of ATS, with and without derivatization, are given in annex IV.

Preparation of internal standard solution (IS)

Weigh accurately 0.3 to 0.4 g of the selected internal standard (n-tetradecane, other n-alkanes, diphenylamine, or a structurally related ATS in base form)* into a 500 ml volumetric flask and dilute to volume with chloroform to give an internal standard solution of 0.6 to 0.8 mg/ml.

Preparation of ATS standard solutions (GC calibration solutions)

Standard stock solutions should contain all compounds of interest in concentrations of approximately 1000 mg/l. They may be kept in a closed flask in a refrigerator for up to one year. For the preparation of stock solutions:

(a) Accurately weigh approximately 1000 mg of the ATS salt(s) of interest into a 1000 ml volumetric flask and make to the mark with water.

(b) Accurately pipette 5 ml of this solution into a 20 ml glass stoppered test tube. Basify to litmus by adding a few drops of concentrated ammonia solution. Accurately add 5 ml of chloroform.

(c) Stopper and shake well, then let stand until the layers separate. Using a Pasteur pipette, transfer approximately 1 ml of the chloroform layer through anhydrous sodium sulphate into a small beaker. This standard solution must not be left to stand more than half an hour before used for calibration.

(d) For the preparation of calibration standards for a 5-point calibration, prepare the different levels as shown in the following table:

*If an internal standard in salt form (e.g., the salt of a structurally related ATS) is used, an extraction is required. In those cases, weigh no less than approximately 10 mg of ATS standard and dissolve directly in 10 ml of accurately dispensed internal standard solution.

Preparation of multiple point calibration standards

Calibration level	*ATS standard solution (μl)*	*IS solution (μl)*	*$CHCl_3$ (μl)*	*Approx. concentration of ATS-salt (mg/l)*
Level 1	20	100	880	20
Level 2	40	100	860	40
Level 3	60	100	840	60
Level 4	80	100	820	80
Level 5	100	100	800	100

Inject, at least in triplicate, 1-2 μl of each level into the GC and use average values for establishment of the calibration curve.

Preparation of ATS sample solutions (unknown ATS sample)

In general, but specifically for quantitative analyses, homogenize samples before starting any tests or sub-sampling.

(a) Accurately weigh a sufficient sample quantity into a 25 ml volumetric flask to obtain a final concentration of approximately 0.2-1 mg/ml of analyte. Make to the mark with water. (The amount of the sample to be weighed will depend on the anticipated purity, as indicated by the preliminary screening method. As an example, if the anticipated purity is about 40%, the sample amount used should be approx. 60 mg.)

(b) Accurately pipette 5 ml of this solution into a 20 ml glass stoppered test tube. Basify to litmus by adding a few drops of concentrated ammonia solution. Accurately add 5 ml of chloroform.

(c) Stopper and shake well, then let stand until the layers separate. Using a Pasteur pipette, transfer approximately 1 ml of this sample solution through anhydrous sodium sulphate into a small beaker. Measure 100 μl of sample solution, 100 μl of internal standard solution and 800 μl of chloroform into GC sample vial.

(d) Inject 1-2 μl into the gas chromatograph. Analyze unknown ATS sample solution preferably two times or more.

GC operating conditions

For quantitative analyses, a GC equipped with an autosampler is preferable. Operating conditions may be the same as for qualitative analysis (see p. 30).

Calculations

The percentage drug content of the sample is calculated from the concentration of the unknown ATS sample solution and the corresponding values from the calibration curve. With modern GC instrumentation and software, and after input by the operator of the concentrations of the different calibration standards and the unknown sample solution, the calibration curve will be established and calculations will be performed automatically for any single point along the curve upon

completion of the analytical run. Typically, the result will then be expressed as the percentage content of the unknown drug in the original sample material, i.e, as the sample purity (weight of the analyte relative to the sample weight).

Method C: Multiple-standard method with derivatization

Prepare derivatized ATS standard and sample solutions by measuring an aliquot (e.g., 100 μl) of the liberated dried base (i.e., after having transferred sample or standard solutions through anhydrous sodium sulphate into a small beaker; step *(c)* above) together with an aliquot of 100 μl of internal standard solution, 750 μl of chloroform and 50 μl of BSTFA (or BSTFA + 1% TMCS) into a GC sample vial.

For multiple point calibration, follow method B (see table above entitled "Preparation of multiple point calibration standards"), using 50 μl BSTFA in each step for the preparation of standard and sample solutions, and adding chloroform to make up to 1 ml.

D. GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

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

Preparation of ATS standard and sample solutions

Samples are prepared as described in the previously provided GC methods.

The sensitivity of the analysis and the specificity of the mass spectra of ATS are improved by derivatization (see annex VII). Preparation of derivatives is particularly desirable when the mass spectrum of the underivatized molecule is of low diagnostic value. Most underivatized ATS have fragment ions of low m/z ratio, low intensity, and only one fragment ion of higher abundance (base peak). Derivatization of ATS usually produces fragment ions of higher m/z ratio and higher abundance. High mass ions are more specific and they have greater diagnostic value, since they are not affected by interfering background ions such as column bleed or other contaminants.

Similar to the previously described GC analysis, if quick sample throughput is required, samples may be taken up directly in ethanol/aqueous ammonia (99:1) and injected into the GC-MS. However, in this case, the condition of the injector

and column may deteriorate more quickly than with extracted samples. For use in GC-MS, samples of primary amines may also be taken up directly in carbon disulfide (CS_2), which results in the formation of an isothiocyanate, a derivative that gives a more characteristic mass spectrum than the parent compounds.

GC-MS operating conditions

GC oven conditions:	same as for GC analysis (Conditions from method A, B or C can be used)
Column:	same as for GC analysis, for example: DB-5 (5% phenyl 95% dimethylpolysiloxane), DB-1 (100% dimethylpolysiloxane), 0.25 mm x 30 m x 0.25 μm, or equivalent

Inlet

Mode:	Split/Splitless
Temp:	250°C
Carrier gas:	Helium, 1 ml/min
Mode:	Constant flow (or constant pressure)

Detector

Ionisation mode:	EI mode, 70 eV (CI mode if desired)
Transfer line temp:	280°C
Ion source temp:	230°C
Scan parameters:	TIC (SIM if required), scan range: 35-450 (for substances such as 2C-B one may start at m/z 29)

Results

Identification is accomplished by comparing the retention time and mass spectrum of the analyte with that of a reference standard.

All compounds identified by GC-MS and reported by the analyst must be compared to a current mass spectrum of the appropriate reference standard, preferably obtained from the same instrument, operated under the same conditions. There are several commercial mass spectra reference libraries available. With most GC-MS instruments, many of those libraries are also available as an on-line option. In general, it is, however, important to use mass spectral libraries, whether from a commercial source or user generated, for reference purposes only.

Further reading

A single literature source for mass spectral interpretation of ATS is not available but there are several good general literature sources, which together present reasonably comprehensive data, e.g.:

Karl Pfleger, Hans H. Maurer, Armin Weber (2000), *Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and Their Metabolites: Parts I-IV,* Wiley.

UNODC also has a limited collection of mass spectra related to major ATS. The collection can be accessed from the Internet or, upon request, it can also be made available on CD-ROM.

E. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

In addition to GC, HPLC is another major separation technique commonly used in forensic drug analysis.

For ease of sample preparation, best reproducibility and detectability, reversed phase chromatography is recommended for the analysis of ATS and their ring substituted analogues. 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.

Since there are a large variety of stationary and mobile phases available to the analyst, only guidelines are presented below.

Method

Preparation of ATS standard and sample solutions

Dissolve an appropriate amount of standard or sample in the mobile phase, targeting a concentration of the active component between 0.05-0.50 mg/ml. Sample solutions should be filtered prior to analysis.

Stock and standard solutions must be prepared from reference standards. Working standards should be within the linear range of the detector and approximately 80-120% of the target concentration. Multiple point calibration is desirable but a single standard method is also acceptable.

Operating conditions

Detector:	Diode array detector, rapid scanning or variable wavelength detector, UV 200-210 nm (also use 280-290 nm for methylenedioxy-substituted phenethylamines)
Stationary phase:	C8 or C18 with 5 μm particle size or smaller
Column length:	$\leq$ 30 cm
Column diameter:	$\leq$ 5.0 mm

Pre-column:	Diameter: 2-4 mm, length: 25 mm, reversed phase C8 or C18
Column temperature:	15-35°C (temperature control is strongly recommended in non-thermostated environments)
Mobile phase buffer:	phosphate buffer pH 2.0-3.2 (e.g. 50 mM monobasic sodium phosphate adjusted with phosphoric acid). For tailing peaks, mobile phase additives such as alkyl sulphates or alkyl sulphonates or amines may be required.
Mobile phase organic modifier:	methanol or acetonitrile between 2% and 20% (with higher alkyl groups, on alkyl sulphates or alkyl sulphonates, higher organic concentrations may be required).
Flow rate:	0.1-2.0 ml/min.
Injection volume:	1-100 μl
Separation:	Isocratic or gradient analysis should be performed, targeting retention times of less than 30 minutes.

Results

Identification is accomplished by comparing the retention time of the analyte with that of a reference standard and, if available, by using multiple UV wavelengths or diode array or rapid scanning UV detection. For ring substituted analogues such as MDA, MDMA, and MDEA, fluorescence scans also could be used. Specificity of the method is important, since there is always the possibility that similar compounds elute at the same retention time.

A typical elution order is as follows: norephedrine, ephedrine, amphetamine, methamphetamine, MDA, MDMA and MDEA. The separation of the ephedrine/ pseudoephedrine and norephedrine/ norpseudoephedrine pairs can be difficult, and may require slight adjustment of the HPLC conditions.

Quantitation:

External or internal standard calibration may be used (external standard calibration is recommended especially if a valve-based injector is used in the overfill mode). The use of peak area for HPLC quantitation is recommended, because peak broadening (decreases in peak height) may occur as a result of deterioration of the stationary phase, rendering peak height unsuitable for quantitation.

The specificity of detection can be enhanced by using multiple UV wavelengths or fluorescence detection. A validated HPLC method for quantitation of ATS is described in annex V.

Further reading

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Clark, C. R., DeRuiter, J., Valer, A. and Noggle, F. T. (1995). Gas Chromatographic-Mass Spectrometric and Liquid Chromatographic Analysis of Designer Butanamines Related to MDMA, *J. Chromatogr. Sci.,* vol. 33 pp. 328-337.

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F. FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

Infrared spectroscopy is widely employed as a qualitative and, in recent times, also quantitative method for the determination and structural analysis of ATS. FTIR analysis can be a particularly useful tool for the rapid screening of ATS tablets and powders, providing indications as to the homogeneity of tablets or the presence of mixed batches.

Sample preparation

The preferred methods for sample preparation are dissolution of the sample in an appropriate solvent, or its suspension in an oil mull. A less desirable technique is

the halide disc method, where the sample is dispersed into finely ground potassium halide (KBr or KCl) and pressed into a thin disc.

However, most forensic laboratories favour the halide disc method for two reasons: *(a)* potassium halides are IR transparent in the so-called finger print region (2000-400 cm^{-1}); and *(b)* the potassium halide disc can generally be re-analysed many times, if stored over a desiccant.

The *halide disk method** consists of grinding a dry sample to a very fine powder, then mixing about 1 mg of homogenized sample powder with 200 mg of carefully dried and ground alkali halide. After grinding, the mixture is pressed into a thin, transparent disk.

Both KCl and KBr work equally well. However, KCl is slightly less hygroscopic, and is generally recommended over KBr, especially when the analyte is a hydrochloride salt (to eliminate the problem of halide exchange). Whether KBr or KCl is used, it should be "IR grade" and dried at 110°C for a minimum of one hour. It can be stored above a strong desiccant (e.g., phosphorus pentoxide) in a desiccator, or left in the oven and removed on an "as needed" basis. This may be important in any subsequent legal proceeding. Also, the material under investigation can be recovered from the halide disc for further testing.

The *Nujol mull method* requires the mixing of a finely powdered sample (2-3 mg) with one drop of nujol (liquid paraffin or perfluorinated long-chain alkane), and then grinding the mixture in an agate mortar. The quantity of nujol added is adjusted so that the final mull is the consistency of a thin cream. The resulting mull is spread on an alkali halide disc (usually KBr) and a similar disc placed on top. The film between the halide discs should contain no air bubbles. The obvious disadvantage of this method is interference from the Nujol in the spectrum. A similar approach, the *thin film technique*, is also used for the direct analysis of ATS free bases, which are usually oily liquids.

Single reflection diamond ATR method (e.g. the "Golden Gate" device) is a relatively new method for both liquids and solids, which needs no sample preparation. However, spectra obtained with this method cannot be directly compared with the ones obtained from any of the methods described above. Finally, the gas cell method is a practical tool for quick analysis of solvents and some precursors from clandestine laboratories.

Isolation of pure drug from sample

Isolation of the ATS free base

Dissolve 25-50 mg of the sample in 1 ml of 0.1N tartaric acid. Add 4-5 drops of ammonium hydroxide and extract with chloroform. Pass the chloroform layer through a small column containing a cotton plug to remove suspended particles.

*For solids it is important *(a)* to reduce the size of the particles by grinding to a dimension of less than the shortest analytical wavelength (to avoid scattering), and *(b)* to dilute the sample in order to minimize absorption in the prepared disk.

Allow a portion of the chloroform solution to evaporate directly onto a KBr disc and record the infrared spectrum of the free base, for example, by the thin film technique on KBr discs.

Isolation of the ATS salt

Triturate a 20-50 mg portion of the sample with 1-2 ml of chloroform. Filter, collect the extract and concentrate by using a gentle stream of nitrogen. Induce crystallization, filter, dry crystals, and run the infrared spectrum of the resulting ATS salt by one of the methods described below.

Methods

Spectra of ATS *salts* are recorded using samples prepared by the following methods:

- KBr halide disc (1-1.5%)
- Nujol mull methods
- Direct method, e.g. diffuse reflectance ATR
- Diffuse reflectance method

Spectra of ATS *bases* are recorded using samples prepared by the following methods:

- Thin film method
- IR cards
- Direct method, e.g. diffuse reflectance ATR

Alternative IR sample preparation methods for methamphetamine

Serial dry extraction method

Place 200 mg of powdered methamphetamine sample into a disposable Pasteur pipette with a glass wool plug at the end. Wash the sample with two 1 ml-portions of acetone and collect the acetone fraction. After air-drying wash the column with two 1 ml-portions of chloroform and collect the chloroform fraction. Allow the column to dry again and then rinse with two 1 ml-aliquots of methanol and collect the methanol fraction. The insoluble material can then be removed from the pipette. All fractions are examined by infrared spectroscopy for identification.

Physical separation/selective re-crystallisation

The procedures below work well for the type of mixtures indicated; they do not work as well with amphetamine hydrochloride.

A mixture known to contain methamphetamine: Add in a beaker 25 mg of sample to 20 ml of a 2:1 mixture of chloroform and hexane, and concentrate the solution to approximately half the original volume. Add diethyl ether to precipitate the methamphetamine. Filter, dry and obtain IR spectrum (methamphetamine hydrochloride).

A mixture known to contain methamphetamine, pseudoephedrine, and ephedrine: Place 100 mg of sample in a piece of filter paper and wash with 40 ml of a 3:1 mixture of chloroform and hexane. Wash the insoluble portion with chloroform, and dry and recover for examination (ephedrine hydrochloride). Evaporate the original solute to dryness and divide into two equal portions. Dissolve one half of this sample in 20 ml of a 2:1 mixture of chloroform and hexane, concentrate to approximately half the original volume, and add diethyl ether to precipitate the methamphetamine. Filter, dry and obtain IR spectrum (methamphetamine hydrochloride). Dissolve the other half of the sample in 2 ml of chloroform and add 1.6 ml of hexane to precipitate the pseudoephedrine. Filter, dry and obtain IR spectrum (pseudoephedrine hydrochloride).

Results

Identification is accomplished by comparing the spectrum of the analyte with that of a reference standard, or from a spectral library.

Further reading

Alternative IR sample preparation methods for methamphetamine

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G. ANALYSIS OF OPTICAL ISOMERS

Most ATS have one asymmetric carbon atom resulting in a pair of enantiomers for each ATS. Depending on the source, therefore, different enantiomeric forms

of amphetamine, methamphetamine or other ATS may be encountered in seized samples submitted for analysis.

Under the 1971 UN Convention on Psychotropic Substances, each optical isomer (d- and l-) as well as the racemic mixture (dl) of amphetamine and methamphetamine are scheduled. With most ring-substituted ATS, only the racemates are listed, and individual enantiomers are included by means of a generic phrase.

Optical isomers differ to some extent in pharmacological activity and are subject to different regulatory measures in certain countries. In countries where national legislation requires that the specific optical isomer present be identified, the following analytical procedures may be used.

1. MELTING POINT

Comparison of the melting point of the sample mixed with that of an enatiomerically pure reference standard is a rapid and simple test to distinguish optical isomers.

For example, d- and l-methamphetamine hydrochloride have the same melting point (170-175°C), but a mixture of equal amounts of both optical isomers (racemic mixture) has a lower melting point (130-135°C).

This method requires fairly pure samples. Generally, melting points should be determined using dried samples.

2. MICROCRYSTAL TESTS

(See also section on microcrystal test as a presumptive test, above)

For ATS, the "hanging drop" technique, or volatility test, is typically employed. This technique requires a cavity slide, a cover glass, the testing reagent and a volatilizing reagent. Preparation of testing and volatilization reagents is described in annex III.

Microcrystal tests for optical isomers of amphetamine:

Gold chloride test [5% $HAuCl_4$ in H_3PO_4]

Transfer a small quantity of the sample powder into the depression of the cavity slide, and add one or two drops of the volatilizing reagent (5% NaOH solution). This liberates the free amine in the form of a volatile free base, which rises from the solution as a vapour. Immediately transfer a drop of the testing reagent (5% $HAuCl_4$ in H_3PO_4) onto a glass slide and invert the slide crosswise over the sample cavity. The reagent then reacts with the amine vapour present in the cavity. After an appropriate interval, reinvert the reagent slide and examine for crystals in the reagent or at the edge of the reagent drop.

Results

Both d- and l- amphetamine give identical microcrystals, resembling long yellow rods or coarse needles and long narrow blades. The way to distinguish them is to form the racemate, which does give different crystals. The dl-amphetamine racemate gives at first "oily" drops then coloured platy crystals. These crystals largely form after inversion.

Distinction of d- and l-amphetamine

If the above test indicates that the sample is d- or l-amphetamine, distinction should be undertaken as to which is present. To this end, add some of the unknown sample powder to a small amount of known d-amphetamine salt in one cavity and to a small amount of l-amphetamine salt in another. Repeat the test above. The mixture that is (d+d) or (l+l) will give the long yellow rods etc. The mixture that proves to be (d+l) will give the platy crystals of the racemate as previously described.

Methamphetamine

d-Methamphetamine tested with 5% $HAuCl_4$ in H_3PO_4 gives "V" blades with one side shorter than the other side. These develop very rapidly if the test is done directly on the dry sample, or more slowly if the sample is diluted or performed using the hanging drop technique.

l-Methamphetamine gives single, crossed blades, and "v" blades, which form characteristic cigar-shaped ends (they taper on both sides of the blade tip).

d,l-Methamphetamine forms single and "X" blades which are sometimes "knife"-shaped. The blade ends only taper on one side similar to a knife.

MDMA

MDMA tested with 5% $HAuCl_4$ in H_3PO_4 gives high birefringed white X-shaped crystals with star-like clusters under polarized light. These crystals are similar to those of d,l-methamphetamine with gold chloride, but can be differentiated with practice.
Note: Because MDMA is very sensitive in reacting with the gold chloride reagent, only a very small portion is sufficient for good results.

The gold chloride test is also useful for the precursors ephedrine and pseudoephedrine, and can be useful for nicotinamide and caffeine. Reference samples of these substances should be used to obtain reference crystals.

Microcrystal tests for optical isomers of methamphetamine: [H_3BiI_6 in H_2SO_4]

Use hanging drop procedure as described above for amphetamine but using H_3BiI_6 in H_2SO_4 testing reagent (see annex III for preparation of the reagent).

Results

d-Methamphetamine gives long orange needles. dl-Methamphetamine gives characteristic orange-red rods with slanting ends.

Further reading

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3. INSTRUMENTAL TECHNIQUES

There are a number of direct instrumental methods (chiral GC, HPLC or CE) and indirect derivatization methods available for the analysis of optical isomers of ATS. The choice of the method depends on the scope of the analysis, the availability of equipment and other laboratory requirements. Both direct and indirect methods should be considered as complementary since neither offers a universal solution for chiral separation. The strengths and weaknesses of both approaches should be considered with care.

Direct instrumental methods allow the analysis of optical isomers without derivatization, using chiral stationary phases and/or chiral additives to the run buffer (CE).

Indirect methods are based on the derivatization of the analyte with a chiral reagent to produce two different diastereoisomers with different physico-chemical properties that can be separated on an achiral stationary phase. The choice of the chiral reagent depends on number of factors such as separation power, sensitivity, efficiency of derivatization, and its compatibility with the instrumental technique. Methods using chiral derivatization are essentially less expensive and do not require specialized equipment or columns. The use of normal, achiral columns allows easy integration of chiral separations into routine analysis schemes.

GC techniques

Gas chromatography (GC) is a well established technique for chiral separation. It can be performed using direct methods, employing chiral capillary columns, or indirect methods, where separation is achieved by using chiral reagents and achiral stationary phases.

Chiral gas chromatography (direct GC method)

Commercially available stationary phases for the GC separation of optical isomers are usually produced by addition of derivatized cyclodextrin macromolecules to a common stationary phase. The most commonly used chiral stationary phase for ATS is beta-cyclodextrin based.

Method

Sample preparation (extraction) for chiral GC analysis is the same as for normal GC (see above).

GC operating conditions

Column:	Dexcst, 0.25 mm x 30 m x 0.25 micron film, or equivalent
Carrier gas:	Helium at approx. 1.2 ml/min
Oven temperature:	120°C for 1 min., then 1.5°C/min to 175°C, hold for 1.5 min.
Injection volume:	1 μl
Injector temperature:	190°C
Detector temperature:	280°C

Note: Other GC operating conditions can be used, but need to be tested for optimal separation of chiral compounds. Use of nitrogen as carrier gas requires lower flow rates (approx. 0.8 ml/min.) for optimal velocity, resulting in broader peaks.

Chiral derivatization (indirect GC method)

Diastereoisomers of ATS can be prepared using different reagents such as acylchlorides, alkylsulphonates, isothiocyanates, chloroformates. Mosher's acid [R(+)- or S(-)-α-methoxy-α-(trifluoromethyl)-phenylacetic acid], Mosher's acid chloride, and N-trifluoroacetyl-1-prolyl chloride (TPC, also known as TFAP-Cl) are the most popular.

Mosher's acid and acid chloride results in quantitative derivatization of most amines, with the exception of ephedrine and pseudoephedrine. It can be used as reagents for both GC and HPLC analysis.

TPC is known to produce stable derivatives of almost all ATS including ephedrines. It is more amenable for GC analysis.

Details of ATS sample preparation using Mosher's acid and TPC for chiral derivatizations are described in annex VII.

The GC operating conditions for qualitative GC analysis (see above) can also be used for the analysis of chiral derivatives.

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HPLC techniques

For HPLC, various approaches have been used, including derivatization with chiral reagents, incorporation of chiral additives in the mobile phase, and the use of chiral stationary phases. A variety of chiral HPLC columns are commercially available. The performance of chiral stationary phases in HPLC has been dramatically improved in recent years, although such analyses are still expensive.

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CE techniques

Capillary zone electrophoresis is highly advantageous for chiral analysis since it allows for the highly efficient separation of enantiomers without derivatization and specialty columns (capillaries). For the separation of ATS, chiral additives to the run buffer such as hydroxyl-propyl beta-cyclodextrin are commonly used.

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Infrared (IR) techniques

Although enantiomers have the same infrared spectrum, infrared (IR) spectroscopy can be used to distinguish between enantiomers of a given compound after converting them into the corresponding diastereomers.

ATS, as all organic bases can easily be reacted with chiral organic acids to form diastereomers. D- and l-amphetamine, for instance, can be coupled with d-mandelic acid to form two diastereomers, d-amphetamine-d-mandelate and l-amphetamine-d-mandelate, which have different IR spectra.

Method

An aqueous solution of ATS salt (10-50 mg) is made alkaline, and the ATS extracted into methylene chloride. The methylene chloride is dried over anhydrous sodium sulphate and concentrated to approximately 2 ml. A saturated solution of d-mandelic acid in methylene chloride is added, several drops at a time, until the ATS solution is neutralized (pH paper). The d-mandelate-ATS salt is allowed to crystallize, the solution filtered through suction, and the crystals are washed with a small portion of methylene chloride. After drying, a KBr disc of the crystals is prepared, and the infrared spectrum acquired. The procedure is repeated using known optically pure isomers of corresponding ATS.

Results

Identification of optical isomers is accomplished by comparing the resulting spectra with those obtained from appropriate pure reference standards. IR bands in the 800-1600cm^{-1} region provide the most analytically distinctive information.

IR spectra of the salts of optical isomers of amphetamine with mandelic acid are given in the earlier edition of the UN manual "Recommended methods for testing amphetamine and methamphetamine" (ST/NAR/9). Data can also be accessed at the Laboratory and Scientific Section's web page.

Further reading

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VII. ADDITIONAL ANALYTICAL TECHNIQUES FOR THE ANALYSIS OF ATS

There are a number of additional analytical techniques suitable for the forensic identification and/or quantitation of ATS, such as:

- Capillary electrophoresis (CE)
- Gas chromatography-Fourier transformed infrared spectroscopy (GC-FTIR)
- LC-MS and CE-MS
- Near Infrared (NIR) Spectroscopy
- Nuclear magnetic resonance (NMR) spectroscopy (qualitative and quantitative)
- Quantitative FTIR
- Quantitative TLC
- Raman FTIR spectroscopy
- Solid phase-micro extraction-gas chromatography (SPME-GC)

A description of most of these techniques is beyond the scope of this "Manual on recommended analytical methods for ATS", and the analyst is referred to a complementary "Manual on analytical techniques generally, their characteristics and practical use for drug analysis". Four techniques, qualitative NMR, CE, SPME-GC, and GC-FTIR are briefly described below, because they offer specific, attractive options for the analysis of ATS.

A. ^{1}H-NUCLEAR MAGNETIC RESONANCE (NMR) TECHNIQUES

The availability of a large number of positional isomers of structurally related ATS, especially ring-substituted ATS, requires effective tools that provide the necessary structural information for their differentiation. NMR enables the analyst to unequivocally distinguish between different ring-substituted amphetamine derivatives, even in the presence of diluents and other adulterants. Although certain substitution patterns resemble one another in the area corresponding to the protons of the alkyl side chain, the integrated spectrum and the pattern of the aromatic proton signals allow their distinction from one another. While being a powerful tool for the identification of analogues, the cost of NMR spectroscopy and the technical expertise required prevent its widespread application in routine analysis.

Method

Dissolve about 20 mg of the drug sample in 1 ml of D_2O. If insoluble materials are present, centrifuge, otherwise transfer directly the supernatant into an NMR tube. Record the spectrum of this solution containing the salt form of the ATS.

Liberate the free base of the ATS in situ by the addition of 20-30 mg of solid K_2CO_3 and 0.5 ml of $CDCl_3$ and record the spectrum of the free base. Compare the spectrum of the unknown with the reference spectra, which were recorded on a Fourier transform instrument at 90 MHz using a flip angle of 18° (1 μsec) with no delay following data acquisition.

Reference NMR spectra of selected ATS are given in the earlier edition of the two UN manuals related to the analysis of ATS, namely, "Recommended methods for testing amphetamine and methamphetamine" (ST/NAR/9) and "Recommended methods for testing illicit ring-substituted amphetamine derivatives" (ST/NAR/12). Data can also be accessed at the Laboratory and Scientific Section's web page.

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B. CAPILLARY ELECTROPHORESIS (CE)

CE, similar to HPLC, requires no derivatization or extraction steps, and therefore is advantageous over GC for the analysis of ATS compounds. In contrast to HPLC, CE offers higher resolving power for the analysis of these solutes, which translates into faster analysis. The use of dynamically coated capillaries provides for significant improvement in precision, peak efficiency and/or separation times for ATS compounds over comparable methods using uncoated capillaries. In addition the dynamically coated capillary approach can allow for the rapid chiral analysis

of a sample using the same capillary and sample vial but a different run buffer. See annex VI for validated CE method for quantitation of selected ATS and for methodology to differentiate optical isomers.

Further reading

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C. SOLID PHASE-MICRO EXTRACTION-GAS CHROMATOGRAPHY (SPME-GC)

SPME (solid phase micro extraction) is a solvent free sample preparation technique, which can be used for headspace analysis over solutions, or directly over ATS powders, or it can be used for (trace) analysis of aqueous solutions containing ATS. SPME is typically performed with a special syringe fitted with a silica fibre placed on top of the syringe piston. The fibre is coated with a polymeric phase like the ones used in capillary columns. During sampling, the coating fibre absorbs the compounds from the gas phase in the headspace, or directly from the aqueous phase. Many different fibre coatings are available for analysis of ATS and other substances. One of the most used is, for example, a polydimethylsiloxane (PDMS) coated fibre.

Headspace method

An aqueous ATS sample is made alkaline to convert the amines into free bases, thus increasing their volatility. The sample can additionally be heated in order to increase the amount of amines in the gas phase above the sample solution. The syringe with the exposed fibre is placed in the headspace above the solution and the ATS free bases are absorbed on the fibre. At the equilibrium, the extracted amount of every compound absorbed on the fibre is proportional to their concentration in the solution, though with different distribution coefficients. After the extraction is completed, the syringe is transferred to the chosen analytical instrument.

When the fibre is inserted into the heated GC injector port, the extracted amines are thermally desorbed. In HPLC, CEC and CE, the solvent mixture elutes the amines form the fibre.

(Trace) analysis from aqueous samples

The fibre is dipped directly into the aqueous sample solution, which was made alkaline in order to release the ATS free bases. The sample solution is stirred to increase the exchange of the compounds between the solution and the fibre for a speedier extraction. Sample analysis is carried out in the same way as described in the headspace method.

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D. GAS CHROMATOGRAPHY-FOURIER TRANSFORM INFRARED SPECTROSCOPY (GC-FTIR)

GC-FTIR, as another hyphenated technique, unifies the advantages of a the GC separation technique with the high analyte specificity of IR. GC-FTIR with a light pipe flow cell has no limitations as to the carrier gas flow, which makes it ideal for use with short wide bore columns, thus resulting in efficient and fast analysis. For instance, most common ATS can be analyzed within less than five minutes. However, since the technique is rather insensitive, large amounts of substance have to be injected, and the film thickness of the stationary phase is critical in order not to overload the column.

Method

ATS sample preparation

Sample preparation is the same as for qualitative GC analysis above, but the target analyte concentrations should be 1-10 mg/ml.

GC operating conditions

General GC conditions shown above (e.g., GC-FID or GC-MS method) can be used, with the following modifications:

GC column:	Wide bore column 0.32-0.53 mm ID
Column length:	3-10 m
Film thickness:	$\geq$ 1.0 μm
Temperature of transfer line:	300°C
Spectral resolution:	8 cm^{-1}

Results

Identification is accomplished by comparing the retention time and FTIR spectrum of the analyte with that of a reference standard.

Further reading

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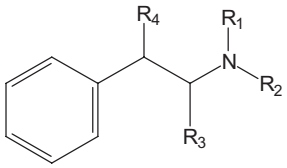
Annex I. Chemical structures of selected ATS

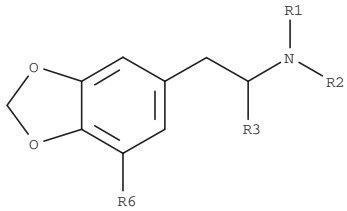
Table A1. Non-ring substituted amphetamines



Note: Unless indicated specifically, names do not refer to individual enantiomers

Common name	*IUPAC name*	*R1*	*R2*	*R3*	*R4*
Amphetamine	1-methyl-2-phenylethylamine	H	H	CH_3	H
Methamphetamine	*N*-methyl-*N*-(1-methyl-2-phenylethyl)amine	CH_3	H	CH_3	H
N-Ethylamphetamine	*N*-ethyl-*N*-(1-methyl-2-phenylethyl)amine	C_2H_5	H	CH_3	H
Dimethylamphetamine	*N,N*-dimethyl-*N*-(1-methyl-2-phenylethyl)amine	CH_3	CH_3	CH_3	H
N-Hydroxyamphetamine	*N*-(1-methyl-2-phenylethyl) hydroxylamine	H	OH	CH_3	H
N-Hydroxymethamphetamine	*N*-methyl-*N*-(1-methyl-2-phenylethyl) hydroxylamine	CH_3	OH	CH_3	H
Cathine	(1S,2S)-2-amino-1-phenylpropan-1-ol	H	H	CH_3	OH
Cathinone	2-amino-1-phenylpropan-1-one	H	H	CH_3	=O
Methcathinone	2-(methylamino)-1-phenylpropan-1-one	CH_3	H	CH_3	=O
Fenetylline	1,3-dimethyl-7-{2-[(1-methyl-2-phenylethyl)amino] ethyl}-3,7-dihydro-1*H*-purine-2,6-dione	H	theo-phylline	CH_3	H
Phenylpropylmethylamine (PPMA)	*N*-methyl-*N*-(2-phenylpropyl)amine	CH3	H	CH_3	CH_3

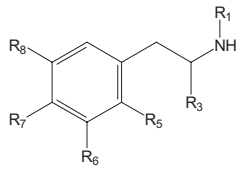
Table A2. Methylenedioxy substituted amphetamines



Note: Unless indicated specifically, names do not refer to individual enantiomers

Common name	*IUPAC name*	*R1*	*R2*	*R3*	*R4*
3,4-methylenedioxy-amphetamine (MDA, tenamfetamine)	1-(1,3-benzodioxol-5-yl) propan-2-amine	H	H	CH_3	H
3,4-methylenedioxy-methamphetamine (MDMA)	*N*-[2-(1,3-benzodioxol-5-yl)-1-methylethyl]-*N*-methylamine	CH_3	H	CH_3	H
3,4-methylenedioxy-ethylamphetamine (MDE, MDEA)	*N*-[2-(1,3-benzodioxol-5-yl)-1-methylethyl]-*N*-ethylamine	C_2H_5	H	CH_3	H
3,4-methylenedioxy-N,N-dimethylamphetamine (MDDM)	*N*-[2-(1,3-benzodioxol-5-yl)-1-methylethyl]-*N,N*-dimethylamine	CH_3	CH_3	CH_3	H
N-hydroxy-3,4-methylene-dioxyamphetamine (N-hydroxy-MDA, N-hydroxytenamfetamine)	*N*-[2-(1,3-benzodioxol-5-yl)-1-methylethyl]hydroxylamine	H	OH	CH_3	H
N-hydroxy-N-methyl-3,4-methylenedioxyamphetamine (N-hydroxy-MDMA, FLEA)	*N*-[2-(1,3-benzodioxol-5-yl)-1-methylethyl]-*N*-methylhydroxylamine	CH_3	OH	CH_3	H
N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB)	*N*-[1-(1,3-benzodioxol-5-ylmethyl) propyl]-N-methylamine	CH_3	H	C_2H_5	H
1-(3,4-methylenedioxyphenyl)-2-butanamine (BDB)	1-(1,3-benzodioxol-5-yl) butan-2-amine	H	H	C_2H_5	H
5-methoxy-3,4-methylenedioxyamphetamine (MMDA)	1-(7-methoxy-1,3-benzodioxol-5-yl) propan-2-amine	H	H	CH_3	OCH_3

Table A3. Other ring substituted amphetamines



Note: Unless indicated specifically, names do not refer to individual enantiomers

Common name	*IUPAC name*	*R1*	*R3*	*R5*	*R6*	*R7*	*R8*
2,4,5-Ring substituted phenethylamines							
4-Bromo-2,5-dimethoxy-phenetylamine (2C-B, Nexus)	2-(4-bromo-2,5-dimethoxy-phenyl)ethanamine	H	H	OCH_3	H	Br	OCH_3
4-Methylthio-2,5-dimethoxy-phenethylamine (2C-T)	2-[2,5-dimethoxy-4-(methyl-thio)phenyl]ethanamine	H	H	OCH_3	H	SCH_3	OCH_3
4-Ethylthio-2,5-dimethoxy-phenethylamine (2C-T-2)	2-[4-(ethylthio)-2,5-di-methoxyphenyl]ethanamine	H	H	OCH_3	H	SC_2H_5	OCH_3
4-Propylthio-2,5-dimethoxy-phenethylamine (2C-T-7)	2-[2,5-dimethoxy-4-(propyl-thio)phenyl]ethanamine	H	H	OCH_3	H	SC_3H_7	OCH_3
4-Chloro-2,5-dimethoxy-phenethylamine (2C-C)	2-(4-chloro-2,5-dimethoxy-phenyl)ethanamine	H	H	OCH_3	H	Cl	OCH_3
4-Iodo-2,5-dimethoxy-phenethylamine (2C-I)	2-(4-iodo-2,5-dimethoxy-phenyl)ethanamine	H	H	OCH_3	H	I	OCH_3
2,4,5-Ring substituted amphetamines							
2,4,5-Trimethoxyamphetamine (TMA-2)	1-(2,4,5-trimethoxyphenyl) propan-2-amine	H	CH_3	OCH_3	H	OCH_3	OCH_3
4-Methyl-2,5-dimethoxy-amphetamine (DOM, STP)	1-(2,5-dimethoxy-4-methylphenyl)propan-2-amine	H	CH_3	OCH_3	H	CH_3	OCH_3
4-Bromo-2,5-dimethoxy-amphetamine (DOB, Bromo-STP, BDMA)	1-(4-bromo-2,5-dimethoxy-phenyl)propan-2-amine	H	CH_3	OCH_3	H	Br	OCH_3
4-Chloro-2,5-dimethoxy-amphetamine (DOC)	1-(4-chloro-2,5-dimethoxy-phenyl)propan-2-amine	H	CH_3	OCH_3	H	Cl	OCH_3
4-Iodo-2,5-dimethoxy-amphetamine (DOI)	1-(4-iodo-2,5-dimethoxy-phenyl)propan-2-amine	H	CH_3	OCH_3	H	I	OCH_3
4-Ethyl-2,5-dimethoxy-amphetamine (DOET)	1-(4-ethyl-2,5-dimethoxy-phenyl)propan-2-amine	H	CH_3	OCH_3	H	C_2H_5	OCH_3
Other ring substitution patterns (phenethylamines and amphetamines)							
3,4,5-Trimethoxyphenethyl-amine (mescaline)	2-(3,4,5-trimethoxyphenyl) ethanamine	H	H	H	OCH_3	OCH_3	OCH_3
para-Methoxyamphetamine (PMA)	3-(4-methoxyphenyl)-1-methylpropylamine	H	CH_3	H	H	OCH_3	H
para-Methoxy-methamphetamine (PMMA)	*N*-[2-(4-methoxyphenyl)-1-methylethyl]-*N*-methylamine	CH_3	CH_3	H	H	OCH_3	H
2,5-Dimethoxyamphetamine (DMA)	1-(2,5-dimethoxyphenyl) propan-2-amine	H	CH_3	OCH_3	H	H	OCH_3
3,4,5-Trimethoxyamphetamine (TMA)	1-(3,4,5-trimethoxyphenyl) propan-2-amine	H	CH_3	H	OCH_3	OCH_3	OCH_3
4-Methylthioamphetamine (4-MTA)	1-[4-(methylthio)phenyl] propan-2-amine	H	CH_3	H	H	SCH_3	H

Annex II. Preparation of colour and anion test reagents

All reagents should be prepared according to an established procedure.

Chen's test

Reagent 1: Add 1 ml of glacial acetic acid to 100 ml of water (=1% (v/v) aqueous acetic acid solution)

Reagent 2: Dissolve 1 g of copper(II) sulphate in 100 ml of water (=1% (w/v) aqueous $CuSO_4$ solution)

Reagent 3: Dissolve 8 g of sodium hydroxide in 100 ml of water (=2N aqueous sodium hydroxide solution).

Gallic acid test

Reagent: Dissolve 0.1 g of gallic acid in 20 ml of concentrated sulphuric acid (=0.5% (w/v) solution)

Marquis test

Reagent 1: Add 8-10 drops (approx. 0.25 ml) of 37% formaldehyde solution to 10 ml of glacial acetic acid

Reagent 2: Concentrated sulphuric acid

Phosphate test

Ammonium molybdate: Dissolve 10 g of ammonium molybdate [$(NH_4)_6Mo_7O_{24}$ x $4H_2O$] in 100 ml of water (=10% (w/v) aqueous ammonium molybdate solution)

Nitric acid: Carefully add 10 ml of nitric acid to 90 ml water (= 10% (v/v) nitric acid solution)

Silver nitrate test (also known as Chloride test)

Dissolve 1.7 g of silver nitrate in 100ml of water (=1.7% aqueous silver nitrate solution).

Simon's test

Reagent 1: Dissolve 2 g of sodium carbonate in 100 ml of water (=2% aqueous sodium carbonate solution)

Reagent 2: Dissolve 0.9 g of sodium nitroprusside in 90 ml of water (=1% aqueous sodium nitroprusside solution)

Reagent 3: Mix 10 ml of acetaldehyde solution and 10 ml of ethanol (=50% (v/v) ethanolic acetaldehyde solution)

Sulphate test

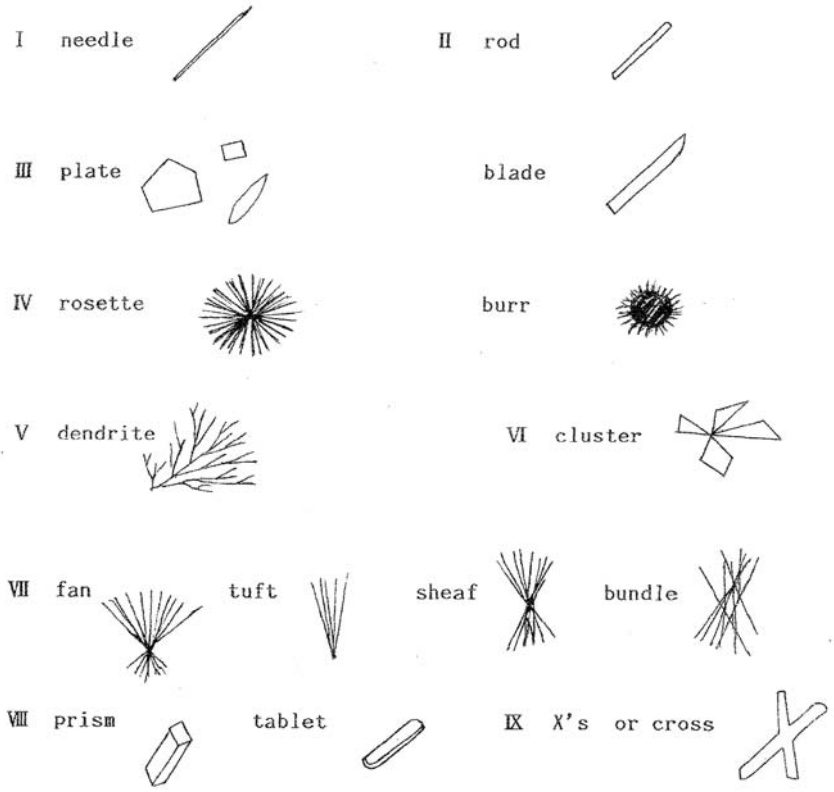
Dissolve 5 g of barium chloride dihydrate in 100 ml of water (= approx. 5% aqueous barium chloride solution).

There are other established procedures for the preparation of colour test reagents, for example, Clarke's, which show slight variation in recipes.

Annex III. Microcrystal tests

Classified typical microcrystals

Typical forms of microcrystal can be classified into nine groups, using the descriptive terms below. In order to allow description of all types of microcrystals, adjectives such as irregular, fine, or square-cut should be added to the basic terms.



Source: Õno, M., *Microcrystal Test,* Japan, 1996

Reagents

Testing reagent—5% $HAuCl_4$ in H_3PO_4

Dissolve 1 g of commercial gold trichloride acid ($HAuCl_4 \times 4H_2O$) in 20 ml of a solution containing one volume of concentrated H_3PO_4 and two volumes of water.

Testing reagent—H_3BiI_6 in H_2SO_4

Dissolve 1.25 g potassium iodide in 2.0 ml of water. Add 2.5 ml of a solution of H_2SO_4 diluted 1:7 with water, 0.5 ml of concentrated $Bi(NO_3)_3$ solution and 0.1 g of sodium hypophosphite. The concentrated $Bi(NO_3)_3$ stock solution is prepared by dissolving 50 g of bismuth subnitrate in 70 ml of a solution of HNO_3 (diluted 1:1 with water) and made up to 100 ml with water. The testing reagent can be kept for several months.

Volatilising reagent

Prepare a 5% aqueous NaOH solution.

Annex IV. Validated GC methods for quantitation of selected ATS

Examples of validated GC methods for the quantitative analysis of selected ATS are provided below. Method B does not require derivatization, while method C requires silylation.

The described ATS standard and samples solutions and their concentrations are designed for use with capillary columns and the procedures described below. The use of alternative columns and GC systems may necessitate changes in terms of both relative composition and concentrations of individual components.

Equipment and reagents

- Grade B volumetric glassware or better.
- Gas Chromatograph fitted with flame ionisation detector
- Analytical balance capable of weighing to an accuracy of ± 0.0001 g.
- All reagents must be of analytical reagent grade.

Method B: Multiple-point calibration method without derivatization

Method B is a validated method for the quantitative GC analysis of underivatized ATS, specifically the following: amphetamine, methamphetamine, MDA, MDMA, MDEA and MBDB.

Preparation of internal standard solution (IS): Phenylbenzylamine (PBA)

Accurately weigh 0.3 to 0.4 g of PBA into a 500 ml volumetric flask and dilute to volume with chloroform to give an internal standard solution of 0.6 to 0.8 mg/ml.

Preparation of ATS standard solutions (GC calibration solutions)

Standard stock solutions should contain all compounds of interest in concentrations of approximately 1000 mg/l. They may be kept in a closed flask in a refrigerator for up to one year. For the preparation of stock solutions:

(a) Accurately weigh approximately 1000 mg of the compound(s) of interest into a 1000 ml volumetric flask and make to the mark with water.

(b) Accurately pipette 5 ml of this solution into a 20 ml glass stoppered test tube. Basify to litmus by adding a few drops of concentrated ammonia solution. Accurately add 5 ml of chloroform.

(c) Stopper and shake well, then let stand until the layers separate. Using a Pasteur pipette, transfer approximately 1 ml of the chloroform layer through anhydrous sodium sulphate

into a small beaker. This standard solution must not be left to stand more than half an hour before used for calibration.

(d) For the preparation of calibration standards for a 5-point calibration, prepare the different levels as follows:

Preparation of 5-point calibration standards

Calibration level	*ATS standard solution (µl)*	*IS solution (µl)*	*$CHCl_3$ (µl)*	*Approx. concentration of ATS-salt (mg/l)*
Level 1	20	100	880	20
Level 2	40	100	860	40
Level 3	60	100	840	60
Level 4	80	100	820	80
Level 5	100	100	800	100

Preparation of ATS sample solutions (unknown ATS sample)

In general, but specifically for quantitative analyses, homogenize samples before starting any tests or sub-sampling.

(a) Accurately weigh a sufficient sample quantity into a 25 ml volumetric flask to obtain a final concentration of approximately 0.2-1 mg/ml of analyte. Make to the mark with water. (Note: The amount of the sample to be weighed will depend on the anticipated purity as indicated by the preliminary screening method. As an example, if the anticipated purity is about 40%, the sample amount used should be approx. 60 mg.)

(b) Accurately pipette 5 ml of this solution into a 20 ml glass stoppered test tube. Basify to litmus by adding a few drops of concentrated ammonia solution. Accurately add 5 ml of chloroform.

(c) Stopper and shake well, then let stand until the layers separate. Using a Pasteur pipette, transfer approximately 1 ml of this sample solution through anhydrous sodium sulphate into a small beaker. Measure 100 µl of sample solution, 100 µl of internal standard solution and 800 µl of chloroform into GC sample vial.

(d) Inject into the gas chromatograph.

GC operating conditions

For quantitative analyses, a GC equipped with an autosampler is preferable. It is acknowledged that use of different instruments may require adjustments in the operating conditions.

Column:	HP-5, 0.32 mm x 30 m x 0.5 µm
Carrier gas:	Helium at approx. 1.2 ml/min (head pressure 12 psi)
Oven temperature:	100°C for 4 min, then 10°C/min to 270°C, and hold for 1 minute
Injection volume:	1 µl
Injector temperature:	190°C
Detector:	Flame ionisation detector at 270°C

Approximate retention times

Amphetamine	7.18 min.
Methamphetamine	8.25 min.
MDA	13.16 min.
MDMA	13.93 min.
MDEA	14.58 min.
MBDB	15.17 min.
Phenylbenzylamine (IS)	16.33 min.
Caffeine	17.92 min.
Ketamine	18.33 min.

Calculations

For routine analysis, the computer software will perform the calculations upon completion of the analytical run. The result will be automatically printed on a report and expressed as %w/w of an analyte as a base (i.e., weight of analyte, relative to sample weight).

Method C: Calibration method using BSTFA as a derivatization reagent (single- or multiple-point calibration)

Method C is a validated method for the quantitative GC analysis of derivatized ATS, specifically the following: ephedrine, pseudoephedrine, BDMA (4-bromo-2,5-dimethoxyamphetamine) and 2C-B

Use of method C is specifically recommended for ATS samples that contain ephedrine and/or pseudoephedrine, which are frequently not resolved from other analytes and produce broad peaks. For further details on derivatization see annex VII.

Preparation of internal standard solution (IS): Phenylbenzylamine (PBA)

Same as for method B, above.

Preparation of ATS standard solutions (GC calibration solutions)

For multiple point calibration using BSTFA, prepare the different levels analogous to method B, above, as follows: take 20, 40, 60, 80 and 100 μL of ATS standard stock solution, to each calibration level, add 100 μL of internal standard solution, and 50 μl of BSTFA. Add chloroform to make up to 1ml.

Preparation of sample solutions (unknown ATS sample)

(a) Accurately weigh sample into a 25 ml volumetric flask to obtain a final concentration of approximately 0.2-1 mg/ml of analyte. Make to the mark with water. (Note: Amount of the sample to be weighed will depend on anticipated purity as indicated by the preliminary screening method. As an example, if the anticipated purity is about 40%, the sample amount used should be approx. 60 mg.)

(b) Accurately pipette 5 ml of this solution into a 20 ml glass stoppered test tube. Basify to litmus by adding a few drops of concentrated ammonia solution. Accurately add 5 ml of chloroform.

(c) Stopper and shake well, then let stand until the layers separate. Using a Pasteur pipette, transfer approximately 1 ml of this sample solution through anhydrous sodium sulphate into a small beaker. Measure 100 μl of sample solution, 100 μl of internal standard solution, 750 μl of chloroform and 50 μl of BSTFA into a GC sample vial.
(d) Inject into the gas chromatograph.

GC operating conditions

Column:	HP-5, 0.32 mm x 30m x 0.5 μm
Carrier gas:	Helium at approx. 1.2 ml/min (head pressure 12 psi)
Oven temperature:	100°C for 4 min., then 5°C/min to 200°C, then 10°C/min to 270°C, and hold for 1 minute
Injection volume:	1 μl
Injector temperature:	190°C
Detector (FID):	270°C

Approximate retention times

Pseudoephedrine-TMS	14.99 min.
Ephedrine-TMS	15.16 min.
Phenylbenzylamine-TMS (lS)	23.49 min.
Caffeine	26.22 min.
Ketamine-TMS	26.75 min.
2C-B-TMS	27.91 min.

Calculations

For routine analysis, the computer software will perform the calculations upon completion of the analytical run. The result will be automatically printed on a report and expressed as %w/w of an analyte as a base (i.e., weight of analyte, relative to sample weight).

Annex V. Validated HPLC method for quantitation of selected ATS

Below is a validated method for the HPLC quantitation of selected ATS solutes including amphetamine, methamphetamine, phentermine and MDMA.

HPLC Method for quantitation of ATS

Preparation of ATS standard and sample solutions

ATS standard solution

Weigh an appropriate amount of standard ATS(s) into a volumetric flask to obtain a final concentration of approximately 0.50 mg/ml. Dilute to volume with methanol.

ATS sample solution

Weigh an appropriate amount of sample into a volumetric flask so that the final phenethlamine concentration is approximately that of standard solution. Dilute to volume with methanol.

HPLC operating conditions

Column:	5 μm Luna C18 (Phenomenex, Torrance, CA, USA) 150 x 3.0 mm
Column temperature:	35°C
Injection:	5 μl
Mobile phase:	10% acetonitrile, 90% (50 mM phosphate + 50 mM triethanolamine, pH 2.2);[a] flow rate 1.0 ml/min
UV wavelength:	210 nm

[a]The buffer is prepared by dissolving 22.5 ml concentrated phosphoric acid into 4 liters of HPLC grade water. Approximately 25 ml triethanolamine is added slowly to adjust solution to pH of 2.2.

Approximate relative migration times

Nicotinimide	0.28
Phenethylamine	0.55
Phenylpropanolamine	0.56
Doxylamine	0.56
Procaine	0.62
Ephedrine	0.64
Pseudoephedrine	0.65
Amphetamine	0.82
Acetominiphen	0.93

Approximate relative migration times *(continued)*

Methamphetamine	1.00 (2.7 min)
MDA	1.00
Quinine	1.04
Chloroquine	1.09
Dimethylamphetamine	1.12
MDMA	1.18
Caffeine	1.48
Lidocaine	1.50
MDEA	1.55
Ketamine	1.95
Chlorpheniramine	1.99
P2P	3.17
Safrole	3.50
Quaifenesin	3.61
Aspirin	5.09

Calculations

The percentage ATS content of the sample is calculated from the ATS peak area, and the peak area and concentration of the relevant ATS standard.

Further reading

Malone, J.V. (1998). HPLC Quantification of Clandestinely Manufactured Mixtures of Amphetamine and Methamphetamine, *Microgram,* vol. 31, pp. 304-307

Annex VI. Validated CE method for quantitation of selected ATS

Below is a validated method for the CE quantitation of selected ATS solutes including amphetamine, methamphetamine, MDA, MDMA and MDEA on an Agilent HP^{3D} CE instrument. Note that conditions such as capillary length, capillary temperature, voltage, flush times and pressures and injection parameters could change with other instrument manufacturers.

Dynamically coated capillary method for quantitation of ATS

Preparation of ATS standard and sample solutions

Injection solvent

Weigh 1034 mg of sodium phosphate monobasic into 100 ml volumetric flask. Dilute to volume with HPLC grade water (adjust pH to approximately 2.6 using phosphoric acid and add dropwise). Transfer contents into a 2000 ml volumetric flask with aid of HPLC grade water. Dilute to volume with HPLC grade water. This final solution contains 3.75 mM phosphate, pH 3.2. Alternatively, transfer entire contents (with aid of HPLC grade water) of 250 ml bottle of injection solvent concentrate (MicroSolv, Eatontown, NJ, USA) into 5 litre flask. Dilute to volume with HPLC grade water.

ATS internal standard solution

Weigh an appropriate amount of N-butylamphetamine HCl (or an appropriate internal standard) into a volumetric flask to obtain a final concentration of approximately 1.0 mg/ml. Dilute to volume with injection solvent.

ATS standard solution

Weigh an appropriate amount of standard ATS(s) into a volumetric flask to obtain a final concentration of approximately 0.08 mg/ml. Pipette appropriate amount of internal standard solution to obtain a final concentration of 0.1 mg/ml. Dilute to volume with injection solvent.

ATS sample solution

Weigh an appropriate amount of sample into a volumetric flask so that the final phenethylamine concentration is approximately that of standard solution. Pipette appropriate amount of internal standard solution to obtain a final concentration of 0.1 mg/ml. Dilute to volume with injection solvent.

CE operating conditions (Achiral)

Capillary:	Bare silica 32 cm (23.5 cm to detector window) by 50 μm i.d.
Capillary temperature:	15°C
Conditioning:	1 minute 0.1N sodium hydroxide; 1 minute water; 1 minute CElixir A (MicroSolv); 2 minute CElixir B, pH 2.5 (MicroSolv).
Injection:	50 millibar x 2 second of sample followed by 35 millibar x 1 second of water
Run buffer:	CElixir B, pH 2.5
Voltage:	10 kV
UV wavelength:	195 nm

Approximate relative migration times

Doxylamine	0.76
Chlorpheniramine	0.78
Quinine	0.80
Beta-phenethylamine	0.81
Chloroquine	0.81
Nicotinimide	0.84
Amphetamine	0.87
Methamphetamine	0.88
Procaine	0.88
MDA	0.90
Norpseudoephedrine	0.91
MDMA	0.91
Norephedrine	0.92
Pseudoephedrine	0.92
Tetracaine	0.93
Ephedrine	0.93
Phenylephrine	0.95
MDEA	0.96
Ketamine	0.96
Phenyltoxylamine	0.97
N-Butylamphetamine (IS)	1.00 (4.6 min)
Methorphan	1.00
Lidocaine	1.03
Benzocaine	1.25
Acetominophen	2.11
Caffeine	2.14
Quaifenesin	2.14
P2P	2.24
DMSO (neutral marker)	2.40
Aspirin	2.71

Calculations

The content (%) of the unknown ATS is calculated from its peak area, and the peak area and concentration of the ATS standard, relative to the peak area of the internal standard (standard and sample).

CE operating conditions (Chiral)

Capillary:	Bare silica 32 cm (23.5 cm to detector window) by 50 μm i.d.
Capillary temperature:	15°C
Conditioning:	1 minute 0.1N sodium hydroxide; 1 minute water; 1 minute CElixir A (MicroSolv); 2 minute CElixir B, pH 2.5 + 50 mM 2-Hydroxypropyl-β-Cyclodextrin (MicroSolv).
Injection:	50 millibar x 2 second of sample followed by 35 millibar x 1 second of water
Voltage:	20kV
UV wavelength:	195 nm (bandwith 10 nm)

Approximate relative migration times

l-Norpseudoephedrine	0.81
d-Norephedrine	0.83
l-Norephedrine	0.83
l-Pseudoephedrine	0.83
l-Amphetamine	0.85
d-Ephedrine	0.86
d-Amphetamine	0.86
l-Ephedrine	0.87
l-Methamphetamine	0.87
d-Norpseudoepherine	0.88
d-Methamphetamine	0.89
d-Pseudoephedrine	0.90
N-Butylamphetamine[a]	1.00 (3.75 min)
N-Butylamphetamine[a]	1.02
MDA[a]	1.03
MDA[a]	1.04
MDMA[a]	1.05
MDMA[a]	1.07
MDEA[a]	1.10
MDEA[a]	1.12

[a]d or l-enantiomer

Further reading

Lurie, I. S., Hays, P. A. and Parker, K. P. (2004). Capillary Electrophoresis Analysis of a Wide Variety of Seized Drugs Using the Same Capillary with Dynamic Coatings, *Electrophoresis.*, vol. 25, pp. 1580-1591.

Lurie, I. S., Bethea, M. J., McKibben, T. D., Hays, P. A., Pellegrini, P., Sahai, R., Garcia, A. G. and Weinberger R. (2001). Use of Dynamically Coated Capillaries for the Routine Analysis of Methamphetamine, Amphetamine, MDA, MDMA, MDEA and Cocaine using Capillary Electrophoresis, *J. Forensic Sci.*, vol. 46, pp. 1025-1032.

Annex VII. Derivatizations

Derivatization of ATS is not mandatory, since the majority of ATS is amenable for GC analysis and thermally stable. However, derivatization of ATS (primary or secondary amines) improves their chromatographic properties by reducing undesirable and non-specific column adsorption as well as matrix interferences.

The derivatization methods recommended below work successfully for most of the commonly encountered ATS, however, in rare occasions, the reaction conditions such as reaction time or temperature have to be adjusted.

Analytical note

If derivatization is chosen as a method of sample preparation, sample extraction should be performed as described in the relevant sections above. Following the extraction, the solvent should be evaporated to dryness under a gentle stream of nitrogen at room temperature, or alternatively at 30ºC. In order to avoid loss of analyte by evaporation, this step should be performed very carefully, especially for quantitative ATS analysis. The most efficient way of preventing evaporation of analytes is to evaporate the solvent to approximately 1 ml and then add a few drops (50 μl) of a solvent with high boiling point (solvent keeper), for example, dimethylformamide. After addition of the solvent keeper, further evaporation should proceed carefully until only a thin film of solvent remains. The sample is now ready for derivatization using one of the recommended methods.

Derivatization procedures

Acetylations

Heptafluorobutyric anhydride (HFBA)

Procedure A (acetylation with anhydride)

Add 50 μl of HFBA to the dry residue of extracted ATS in a reacti-vial.* Cap vial, shake for 30 seconds and incubate for 20 min at 75ºC. Evaporate excess reagent. Reconstitute the dry residue in 50 μl of ethyl acetate, and inject 1-2 μl into the GC column.

Procedure B (acetylation with anhydride in presence of a basic catalyst)

Add 50 μl of 0.5M potassium hydroxide to the dry residue of extracted ATS followed by 500 μl of toluene. After mixing and centrifugation, transfer the organic layer into a clean

*Reacti-vials are screw-cap vials made of thick, temperature resistant glass, usually with a conically shaped bottom part inside the vial. In the absence of specialized vials, derivatization can be done in any Teflon-lined screw-cap test tube.

test tube, and add 50 μl of HFBA. Mix thoroughly and immediately add 500 μl of 10% w/v sodium bicarbonate with continuous mixing. Centrifuge the test tube until the upper toluene layer is separated. Inject 1-2 μl of toluene layer into the GC column.

Pentafluoroacetic anhydride (PFAA)

Add 50 μL of PFAA to the dry residue of extracted ATS in a reacti-vial. Cap vial, shake for 30 seconds and incubate for 20 min at 75ºC. Evaporate excess reagent. Reconstitute the dry residue in 50 μl of ethyl acetate, and inject 1-2 μL into the GC column

Trifluoroacetic anhydride (TFAA)

Add 100 μL of ethyl acetate and 50 μL of TFAA to the dry residue of extracted ATS in a reacti-vial. Cap vial, shake for 30 seconds and incubate for 20 min at 60ºC. Evaporate excess reagent. Reconstitute the dry residue in 50 μL of ethyl acetate, and inject 1-2 μl into the GC column.

N-Methylbis-trifluoroacetamide (MBTFA)

Add 500 μL MBTFA to the dry residue of extracted ATS in a reacti-vial. Cap vial, shake for 30 seconds and incubate for 30 min at room temperature. Evaporate excess reagent. Reconstitute the dry residue in 50 μL of ethyl acetate, and inject 1-2 μl into the GC column.

Since MBTFA elutes early in the GC analysis, evaporation of excess reagent may not be necessary if the analyte concentration is anticipated to be large enough for direct injection of the derivatizaion mixture.

Further reading

M. Doneke, *J. Chromatography,* vol. 78, p. 273, (1973)

Silylation

N-Methyl-N-tert.-butyl-dimethylsilyl trifluoroacetamide (MTBSTFA)

Add 100 μl of acetonitrile and 150 μL of MTBSTFA to the dry residue of extracted ATS. Cap the vial and heat for 15 min at 90ºC. Leave the sample at ambient temperature for another 2 h, or more, especially if hindered secondary amino groups are to be silylized. Add 500 μL of acetonitrile, mix well, and inject 1-2 μL into the GC column (if low analyte concentrations are anticipated, the sample may be injected directly without dilution with acetonitrile).

Alternatively, to avoid MTBSTFA peak early in the GC chromatogram, combine the dry residue of extracted ATS with 100 μL of acetonitrile and 100 μL MTBSTFA in a 3 ml vial. Seal vial and allow mixture to stand for 2 h. Add 100 μL of water to hydrolyse any unreacted MTBSTFA. Add 250 μL of n-hexane, mix vigorously and centrifuge. Decant upper hexane layer and inject 1-2 μl into the GC column.

Further reading

R. Melgar, R. C. Kelly, *J. Anal. Toxicol.*, vol. 17 (Nov./Dec., 1993), p. 399.

N,O-bis-[(trimethylsilyl)trifluoroacetamide] (BSTFA)

Add 50 μL of BSTFA and 100 μL of acetonitrile to the dry residue of extracted ATS in a reacti-vial. Cap vial, shake and incubate for 15 min at 70° C. Inject 1-2 μL into the GC column.

Chiral derivatization

ATS diastereoisomer substrates can be prepared using many different reagents such as acylchlorides, alkylsulphonates, isothiocyanates, chloroformates, but the two listed below are the most popular.

R(+)/S(-)-α-methoxy-α-(trifluoromethyl)-phenylacetic acid (Mosher's acid), or
R(+)/S(-)-α-methoxy-α-(trifluoromethyl)-phenylacetic acid chloride (Mosher's acid chloride)

Dissolve the dry sample residue of extracted ATS in 1 ml of THF and mix with 0.5 ml of 0.2 M solution of Mosher's acid in THF in a reacti-vial with a Teflon-lined screw cap. Add 0.5 ml of 10% w/v solution of sodium bicarbonate, cap the vial and heat at 65°C for 1 h. Extract the aqueous phase with chloroform, dry with magnesium sulphate, and evaporate to dryness. Reconstitute the residue in a suitable solvent for GC analysis (e.g., chloroform, see section on qualitative GC analysis, above).

Instead of Mosher's acid, the corresponding chloride can be used. It is commercially available, or can be prepared by refluxing the acid with thionyl chloride. Acid chlorides are usually more reactive, although Mosher's acid itself results in quantitative derivatization of most amines, with the exception of ephedrine and pseudoephedrine. Mosher's acid or its derivatives can be used as reagents for both GC and HPLC analysis.

N-trifluoroacetyl-L-prolyl chloride (TPC, or TFAP-Cl)

Dissolve the dry sample residue of extracted ATS in 2 ml of dry chloroform. Add 4 ml of TPC reagent. Stir or shake the mixture, and then add 40 mg of dry triethylamine. Continue to stir for 15 minutes. Wash with 3 ml of 6 N HCl and then with water. Add $MgSO_4$ and allow the mixture to stand for 15 minutes. Inject 1-2 μL into the GC column.

TPC is known to produce stable derivatives of almost all ATS including ephedrines. It is more amenable for GC analysis.

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