Guilty by Dissociation – Development of Gas Chromatography-Mass Spectrometry (GC-MS) and other rapid screening methods for the analysis of 13 diphenidine-derived New Psychoactive Substances (NPSs).

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Abstract

The prevalence of New Psychoactive Substance(s) (NPSs) in forensic science drug casework has increased markedly in recent years. This has given rise to both legal and analytical challenges in the identification of these substances. The requirement for validated, reliable and rapid testing methodologies for these compounds is obvious. This work reports the analysis of thirteen synthesised diphenidine derivatives encountered in casework using presumptive testing, thin layer chromatography and gas chromatography–mass spectrometry (GC-MS). Specifically, the validated GC-MS method provides, for the first time, both a general screening method and quantification of the active components for seized solid samples, both in their pure form and in the presence of common adulterants.

Keywords

New Psychoactive Substances; Characterisation (NMR, FT-IR); Diphenidine; Methoxphenidine; GC–MS; Triage

1 Introduction

In recent years, there has been a significant rise in the number of New Psychoactive Substances (NPS, formally known as *"legal highs"*) seized by law enforcement agencies globally [1]. New Psychoactive Substance(s) is the standardized terminology referring to substances which mimic the effects of common illicit materials (for example, methamphetamine and cannabis) but which are uncontrolled under drug legislation, e.g. the United Kingdom Misuse of Drugs Act (1971) [2]. Dissociative anaesthetics (e.g. dextromorphan, ketamine and phencyclidine) are substances that distort perceptions, produce feelings of detachment and induce a state of anaesthesia by antagonising ionotropic *N*-methyl-*D*-aspartate receptors (NMDAR) in the central nervous system [3]. The most recent class of NMDAR antagonists to emerge on the NPS market are the diarylethylamines: 1-(1, 2-diphenylethyl)piperidine (diphenidine, **3a**) [4] and 1-[1-(2-methoxyphenyl)-2-phenylethyl]piperidine (2-methoxphenidine, 2-MXP, **3b**) [5] which are marketed as *"research chemicals"* (Scheme 1). Though both the supply and production of diphenidine (and other NPS) are now controlled in the United Kingdom by the Psychoactive Substances Act (2016) [6], the global prevalence of novel diphenidine derivatives still raises considerable legal and analytical challenges in the forensic identification of these materials.

INSERT SCHEME 1

Diphenidine, which has been implicated in a number of fatalities both in Europe [7] and Asia [8 – 10] has been encountered in its pure form [4, 11, 12] and in combination with the synthetic cannabinoids AB-CHMINACA/5F-AMB [8] and 5F-AB-PINACA [13]. Wallach *et al.* [4] have recently published the comprehensive analytical characterisation of diphenidine using ^{1}H-NMR, ^{13}C-NMR, HR-ESI-MS, ESI-MS-MS, GC-(EI/CI)-MS and ATR-IR techniques. Qualitative forensic analysis of seized solid samples has been achieved by employing a range of spectroscopic (e.g. NMR [4, 10 – 12, 14], IR [14] and Raman [14]) and chromatographic (e.g. GC-MS [4, 11, 12, 15], HPLC [4], LC-HR-MS [11, 12], LC-MS-MS [4] and UPLC-MS [15]) techniques. Wink *et al* has studied the biotransformation of diphenidine in rats and pooled human microsomes [16] and additionally a number of researchers have recently reported the toxicological analysis of the substance in blood [7, 8, 10, 17], tissue [8, 9], gastric fluid [10], urine [7, 8, 10, 16] and hair [18]. In 2016, McLaughlin *et al.* reported the comprehensive analytical characterisation of the substituted derivative 2-methoxphenidine (**3b**), its regioisomers (**3c** and **3d**) and seized tablets using ^{1}H-NMR, ^{13}C-NMR, LC-ESI-MS, HR-ESI-MS, GC-(EI/CI)-MS, TLC, MAII-MS and ATR-IR techniques [5]. Additionally toxicological screening methods for 2-MXP in blood (using HPLC [19], LC-MS-MS [19, 20] or UPLC-QTOF-MS [19]) and urine (using HPLC [19], LC-MS [7], LC-MS-MS [19, 20] or UPLC-QTOF-MS [19]) have emerged in response to recently reported drug-related incidents [7, 19, 20]. The increased usage of diphenidine-derived dissociatives has required the development of rapid testing methods for both their identification and quantification. The lack of such methods – especially for new and emerging derivatives – is clearly apparent

in the literature. This paper seeks to address this deficiency and reports presumptive colour tests, thin layer chromatographic and GC-MS data for thirteen new diphenidine derivatives encountered by law enforcement. Specifically, the validated GC-MS method provides, for the first time, both a general screening method and quantification of the active components for seized solid samples, both in their pure form and in the presence of common adulterants. Additionally the Supplementary Information includes the characterization data (^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR (**3e – 3g**) and ATR-FTIR) for the synthesised compounds prepared and utilised in this study and serves as additional comparative information for laboratories engaged in the routine analysis of these compounds.

2 Materials and Methods

All reagents were of commercial quality (Sigma-Aldrich, Gillingham, UK or Fluorochem Limited, Hadfield, UK) and used without further purification. Solvents (Fisher Scientific, Loughborough, UK) were dried, where necessary, using standard procedures. ^{1}H-NMR (10 mg/600 μL in CD_2Cl_2) and ^{13}C-NMR spectra (20 mg/600 μL in CD_2Cl_2) were acquired on a JEOL AS-400 (JEOL, Tokyo, Japan) NMR spectrometer operating at a proton resonance frequency of 400 MHz and referenced to the residual solvent peak (^{1}H-NMR, δ = 5.32 [21]; ^{13}C-NMR, δ = 53.84 ppm [21] respectively). ^{19}F-NMR spectra (20 mg/600 μL in CD_2Cl_2 containing 0.03% *v/v* trifluoroacetic acid, TFA) for compounds (**3e – 3g**) were acquired on the same instrument and referenced to TFA (^{19}F-NMR, δ = -76.55 ppm [22]). All samples were filtered prior to analysis. Infrared spectra were obtained in the range 4000 – 400 cm^{-1} using a Thermo Scientific Nicolet iS10ATR-FTIR instrument (Thermo Scientific, Rochester, USA). Microanalysis was carried out using a PerkinElmer 2400 Series II elemental analyser (PerkinElmer, San Jose, USA). The procedures for the synthesis of diphenidine hydrochloride (**3a**) [4], 2-methoxphenidine hydrochloride (**3b**) [5], 3-methoxphenidine hydrochloride (**3c**) [5] and 4-methoxphenidine hydrochloride (**3d**) [5] have been previously reported.

2.1 Synthesis

The hydrochloride salts of diphenidine (**3a**) and its derivatives (**3b – 3m**) were prepared using an adaptation of the method reported by Le Gall *et al.* [23] with the following modifications: To a dried round-bottomed flask (100 mL) containing zinc dust (2.0 g, 30 mmol) suspended in acetonitrile (40 mL), was added benzyl bromide (0.4 mL) and trifluoroacetic acid (0.2 mL). The resulting solution was stirred for 5 min and then benzyl bromide (3.0 mL, 25 mmol), piperidine (0.99 mL, 10 mmol) followed by the pre-requisite benzaldehyde (11 mmol), were introduced to the mixture, and the solution was stirred at room temperature for an additional 1 h (CARE! Exothermic). The resulting solution was poured into a saturated aqueous NH_4Cl solution (150 mL) and extracted with dichloromethane (2×100 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to give a crude yellowish oil. The oil was dissolved in diethyl ether (150 mL) and concentrated sulphuric acid (0.75 mL) was added dropwise, to the vigorously stirred solution.

After five minutes, the precipitated ammonium salt was filtered, washed with diethyl ether (2×50 mL) and air-dried for 5 – 10 minutes. The ammonium salt was re-dissolved in aqueous sodium hydroxide (5% w/v, 150 mL) and then extracted with dichloromethane (2×150 mL). The combined organic fractions were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellowish oil. The oil was dissolved in diethyl ether (200 mL), treated with hydrogen chloride (4M in dioxane, 3.0 mL, 12 mmol) and left to stand for 5 minutes. The crystallized products were filtered and washed sequentially with the minimum volume of ice-cold acetone and ice-cold ethyl acetate-diethyl ether (1:5) to afford the corresponding hydrochloride salts as colourless to off-white powders (>99.5% by elemental analysis), which were fully structurally characterized by ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR (**3e** – **3g**) and ATR-FTIR (see Supplementary Information). Yields of products (after purification): Diphenidine hydrochloride (**3a,** 29%); 2-methoxphenidine hydrochloride (2-MXP, **3b,** 35%); 3-methoxphenidine hydrochloride (3-MXP, **3c**, 21%); 4-methoxphenidine hydrochloride (4-MXP, **3d**, 25%); 2-trifluoromethoxphenidine hydrochloride (2-TFMXP, **3e**, 41%); 3-trifluoromethoxphenidine hydrochloride (3-TFMXP, **3f**, 36%); 4-trifluoromethoxphenidine hydrochloride (4-TFMXP, **3g**, 64%); mescphenidine hydrochloride (3,4,5-TMXP, **3h**, 72%); 2,3-(methylenedioxy)diphenidine hydrochloride (2,3-MDDP, **3i**, 77%); 3,4-(methylenedioxy)diphenidine hydrochloride (3,4-MDDP, **3j**, 27%); 1-naphthenidine hydrochloride (1-NPD, **3k**, 55%); 2-naphthenidine hydrochloride (2-NPD, **3l**, 32%) and IAS-013 hydrochloride (**3m**, 46%).

2.2 Presumptive tests

Presumptive tests were carried out according to the United Nations recommended guidelines [24]. The following standard presumptive tests applied in this study: (i) Marquis; (ii) Mandelin; (iii) Scott's and (iv) Zimmerman's test(s). The preparation of the reagents and test procedure is detailed below. Six repetitive tests of each compound were conducted and negative control samples were used in all tests. Test solutions (10 mg mL^{-1}) were prepared by dissolving the required reference standards in deionised water.

Marquis Test: 1% formaldehyde (37% aqueous solution) in concentrated sulphuric acid (10 mL, d = 1.86 g mL^{-1}). 1 – 2 drops of each test sample in deionised water (10 mg mL^{-1}) was placed into a dimple well of a white spotting tile and 2 drops of the test reagent added. Any colour change or other noticeable effect occurring immediately on addition of the reagents was noted and observations were made again after 5 min.

Mandelin Test: 1% ammonium metavanadate in concentrated sulphuric acid (10 mL, d = 1.86 g mL^{-1}). 1 – 2 of drops each test sample in deionised water (10 mg mL^{-1}) was placed into a dimple well of a white spotting tile and 2 drops of the test reagent added. Any colour change or other noticeable effect occurring immediately on addition of the reagents was noted and observations were made again after 5 min.

Scott Test: 1% cobalt(II)thiocyanate in glycerol-deionised water (1:1, 10 mL). 1 – 2 drops of each test sample in deionised water (10 mg mL^{-1}) was placed into a dimple well of a white spotting tile and 2 drops of the test reagent added. Any colour change or other noticeable effect occurring immediately on addition of the reagents was noted and observations were made again after 5 min.

Zimmerman Test: Reagent 1: 1% solution 1,3-dinitrobenzene in methanol (10 mL); Reagent 2: 15% aqueous potassium hydroxide solution (10 mL); 1 – 2 drops of each test sample in deionised water (10 mg mL^{-1}) was placed into a dimple well of a white spotting tile and 2 drops of Reagents 1 and 2 were added sequentially with stirring after each addition. Any colour change or other noticeable effect occurring immediately on addition of the reagents was noted and observations were made again after 5 min.

2.3. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was carried out on aluminium-backed SiO_2 plates (Merck, Germany) and spots were visualised using ultra-violet light (254 nm). The mobile phase used was dichloromethane-methanol (9:1 *v/v*) containing 0.8% ammonia (7N in methanol) [5]. The developed plate was viewed under UV light (254 nm) and any spots noted. The plate was sprayed with modified Dragendorff-Ludy-Tenger reagent [5], the blood-red spots marked with a pencil and the Retention Factor (R_f) and Relative Retention Factor (RR_f, with respect to diphenidine, **3a**) calculated for each analyte. Six repetitive tests of all compounds were conducted and negative control samples were used in all tests. All thirteen diphenidine samples were run on the same plate in each case.

2.4 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed using an Agilent 6850 GC and a MS5973 mass selective detector (Agilent Technologies, Wokingham, UK). The mass spectrometer was operated in the electron ionisation mode at 70 eV. Separation was achieved with a capillary column (HP5 MS, 30 m Å~ 0.25 mm i.d. 0.25 μm) with helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature programme is detailed in Table 1. A 2 μL aliquot of the samples (qualitative analysis, calibration standards and test solutions) were injected (manually) with a split ratio of 100:1. The injector and the GC interface temperatures were both maintained at 280 °C respectively. The MS source and quadrupole temperatures were set at 230 °C and 150 °C respectively. Mass spectra were obtained in full scan mode (50–550 amu). All samples (qualitative analysis) were prepared as 1 mg mL^{-1} solutions in methanol with no derivatisation and analysed individually and in combination with three commonly encountered adulterants benzocaine, caffeine and procaine. Eicosane (1 mg mL^{-1}) was used as an internal standard and each sample was injected six times.

INSERT TABLE 1

2.5 Calibration standards

10.0 mg of analytes (**3a** – **3m**), benzocaine, caffeine and procaine were weighed accurately into a 20.0 mL clear glass volumetric flask and diluted to volume with methanol to give a solution containing all components at 500 μg mL^{-1}. This solution was then further diluted with methanol and 1 mL of eicosane (1.0 mg mL^{-1} in methanol) added (in each case) to give calibration standards containing 25.0 μg mL^{-1}, 50.0 μg mL^{-1}, 100.0 μg mL^{-1}, 200.0 μg mL^{-1} and 250.0 μg mL^{-1} of each analyte and the internal standard at 100 μg mL^{-1}.

2.6 Test solutions (Qualitative GC-MS analysis)

The street samples of diphenidine (1.0 g) and methoxphenidine (1.0 g) were obtained as off-white crystalline powders, from *"Buy Research Chemicals UK"* (http://www.brc-chemicals.com) and used without further purification. The individual samples were homogenized and arbitrarily labelled, SS-1 and SS-2, prior to analysis. 10.0 mg of the test substance was weighed accurately into a 50.0 mL clear glass volumetric flask and diluted to volume with methanol. This solution was then further diluted (1:2, 10 mL) with methanol to give a test solution containing 100.0 μg mL^{-1} of the sample. The test samples were injected in duplicate.

2.7 Test solutions (Quantitative GC-MS analysis)

10.0 mg of the test substance was weighed (in triplicate) accurately into a 50.0 mL clear glass volumetric flask and diluted to volume with methanol. This solution was then further diluted (1:2, 10 mL) with methanol and 1.0 mL of eicosane (1.0 mg mL^{-1} in methanol) added (in each case) to give a test solution containing 100.0 μg mL^{-1} of the sample and the internal standard at 100.0 μg mL^{-1}. The test samples were injected in duplicate.

2.8 GC-MS method validation

The GC-MS method was validated in accordance with the ICH guidelines [25] using the following parameters: linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). *Linearity, precision:* six replicate injections of the calibration standards were performed and the data analysed under the same conditions. The %RSD was calculated for each replicate test sample. *Accuracy (percentage recovery study):* determined from spiked samples prepared in triplicate at three levels over a range of 80-120% of the target concentration (100 μg mL^{-1}). The percentage recovery and %RSD were calculated for each of the replicate samples. *Limits of detection and quantification:* six replicate injections of the calibration standards were performed and the data analysed under the same conditions. The limits of detection and quantification were calculated based on the standard deviation of the response and the slope.

3 Results and Discussion

3.1 Synthesis

Samples of thirteen diphenidine derivatives (see Table 2) were prepared as their corresponding hydrochloride salts. The synthesis of the racemic target compounds was achieved using a modification of the previously reported methods [4, 5, 23] from prerequisite aromatic aldehydes in 21 – 77% overall yield, respectively as stable, colourless to off-white powders (Scheme 1). The hydrochloride salts (**3a** – **3m**) were determined to be soluble (10 mg mL^{-1}) in deionised water, methanol, dichloromethane and dimethylsulfoxide. To ensure the authenticity of the materials utilized in this study the synthesized samples were fully structurally characterized by ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR (**3e** – **3g**) and FTIR (see Supplementary information) and the purity of all samples was confirmed by elemental analysis (>99.5% in all cases).

3.2 Thin Layer Chromatography (TLC)

McLaughlin *et al.* [5] demonstrated that it was possible to establish a specific and sensitive test using TLC, for the isomers of MXP (**3b** – **3d**). When TLC was carried out on the thirteen substituted diphenidine derivatives, the spots produced by each compound gave identical colours (blood-red) when viewed with modified Dragendorff-Ludy-Tenger reagent. The examination of the Retention Factors (R_f) demonstrates separation of the compounds based upon this measure, particularly of the three MXP isomers (2-MXP (**3b**), 3-MXP (**3c**) and 4-MXP (**3d**): R_f = 0.76, 0.87 and 0.79 respectively) – which correlates with the data obtained by McLaughlin *et al.* [5]. Separation is slightly less clear-cut for other isomeric derivatives: 2,3-MDDP (**3i**, R_f = 0.78) *vs.* 3,4-MDDP (**3j**, R_f = 0.84); 1-NPD (**3k**, R_f = 0.91) *vs.* 2-NPD (**3l**, R_f = 0.85) and in the case of the TFMXP isomers (**3e** – **3g**) the three compounds co-eluted. The TLC data for each compound, including their Retention Factor (R_f) and Relative Retention Factor (RR_f, with respect to diphenidine, **3a**) are presented in Table 2.

INSERT TABLE 2

3.3 Presumptive tests

Presumptive colour tests were carried out according to the United Nations recommended guidelines [24]. As there are no reports regarding the presumptive testing of diphenidine and its substituted derivatives the following standard presumptive tests were applied in this study: (i) Marquis test; (ii) Mandelin test; (iii) Scott's test and (iv) Zimmerman test. The preparation of the reagents and test procedure is detailed in the Experimental section. A solution of each reference standard (10 mg mL^{-1}) was prepared in deionised water and 1–2 drops placed into a dimple well of a spotting tile. The required presumptive test reagent (1–2 drops) was then added and any colour change or other noticeable effect occurring immediately on addition of the reagents was noted and observations were made again after five minutes. The results (Table 3) indicated that all the derivatives (**3a** – **3m**), containing a tertiary amine, gave a positive reaction with the Marquis and Scott's reagents, however, a gradual loss of intensity of the initial colour with Marquis reagent was observed over the five minute period. In the case of the latter reagent, which is employed in the screening of cocaine

[26], the coloured products may result from the coordination of the tertiary amines to the pink $Co^{(II)}$ octahedral complex affording the blue $Co^{(II)}$ tetrahedral complex [26, 27]. The transient coloured products, observed in the Marquis test, may be rationalised by the reaction of the drug molecules with sulfuric acid in a mechanism analogous to that of the reaction of MDMA [26, 27], however, the gradual loss in colour may indicate that this product is not stable under the test conditions. Acidified ammonium metavanadate (Mandelin reagent) gave positive reactions with all the derivatives, except for 3-trifluoromethoxdiphenidine (**3f**) and 4-trifluoromethoxdiphenidine (**3g**). The reason for the lack of response of (**3f**) and (**3g**) is not readily explained, whilst the slightly different colored products obtained with the MXP isomers [2-MXP (**3b**, dark yellow); 3-MXP (**3c**, brown) and 4-MXP (**3d**, pale yellow) respectively] could be potentially used to discriminate between these three positional isomers. The Zimmerman reaction relies on the presence of an activated methylene group, which under alkaline conditions, reacts with 1,3-dinitrobenzene to give an intensely colored Meisenheimer complex [28] and has been used to determine the presence of barbiturates [26] and synthetic cathinones [28]. In the case of the substituted diphenidine derivatives (**3a** – **3m**), a white or pale yellow precipitate forms on the sequential addition the reagents. This precipitate is believed to be the free-base form of the corresponding tertiary amines, which are insoluble in water, rather than a positive reaction of the substrates with the Zimmerman test. The observed colour changes (Table 2) indicate that Scott's reagent could provide a simple and rapid test for these materials. Cocaine also forms a blue $Co^{(II)}$ tetrahedral complex with Scott's, however, it does not give a positive reaction with the Marquis reagent. The recommendation of this study is that two presumptive tests (Marquis and Scott's) are employed to discriminate between cocaine and these novel diphenidine-derived NPS.

INSERT TABLE 3

3.4 Gas Chromatography-Mass Spectrometry (GC-MS)

The qualitative GC-MS method used required an extremely straightforward solvation of the samples in methanol (1 mg mL^{-1}) followed by direct injection into the instrument. No derivatisation step was required. All thirteen diphenidine derivatives were resolved from each other and from three common adulterants: caffeine, benzocaine and procaine. An exemplar chromatogram is presented in Figure 1. The use of GC-MS also facilitated the visualization of the mass spectral data for each individual compound and these are presented in Figure 2 and Figure 3.

INSERT FIGURE 1

INSERT FIGURE 2

INSERT FIGURE 3

A number of researchers have reported utilising GC-MS to separately determine diphenidine (**3a**) and the three methoxphenidine regioisomers (**3b** – **3d**) in seized solid samples [4, 5, 11, 12, 15] and the toxicological screening of the analytes in biological matrices [10, 16]. However, no validated quantitative chromatographic methods (or limits of detection and quantification) which provides both a general screening method and quantification of the psychoactive components in forensic bulk samples have, to-date, been reported.

Calibration standards were prepared and all thirteen substituted diphenidines demonstrated a linear response (r^2 = 0.996 – 0.998) over a 25.0 – 250.0 μg mL^{-1} range with satisfactory repeatability (RSD = 1.29 – 14.02 %, n = 6). The limits of detection and quantification for the analytes were determined, based on the standard deviation of the response and the slope, as being 4.23 – 5.99 and 12.83 – 17.51 μg mL^{-1} correspondingly. The method was also suitable for the detection and quantification of the three common adulterants (benzocaine, caffeine and procaine), demonstrating linear response (r^2 = 0.996 – 0.998) over the same concentration range with reasonable repeatability (RSD = 1.80 – 10.41%, n = 6). The limits of detection and quantification were also determined, for the adulterants, and found to be 5.97 – 11.82 μg mL^{-1} and 18.10 – 35.82 μg mL^{-1} respectively. The validation parameters for the method are summarised in Table 4. The accuracy (percentage recovery study) of the assay was determined from spiked samples prepared in triplicate at three levels over a range of 80 – 120% of the target concentration (100 μg mL^{-1}). Though the repeatability (%RSD) of the method was significantly lower than expected, which we believe is a result of the manual injection of the calibration standards, the percentage recovery (% assay) and %RSD calculated for each of the three replicate samples demonstrated excellent recoveries for all thirteen analytes within the desired concentration range. All results are within acceptable limits (100 ± 2%, see Supplementary Information; Tables S1 – S13) and the validated GCMS method was deemed suitable for the analysis of the two street samples.

INSERT TABLE 4

3.5 Forensic Application

The diphenidine (SS-1) and methoxphenidine (SS-2) samples obtained from *"Buy Research Chemicals UK"* (http://www.brc-chemicals.com, September 2015) were both purported to be >99% pure and to contain 1 g of either diphenidine (**3a**, Figure 4a) or 2-methoxphenidine (**3b**, Figure 4d). Preliminary presumptive colour tests were carried out according to the procedures reported herein. The diphenidine sample (SS-1) gave positive reactions with Marquis (orange), Mandelin (dark yellow) and Scott (blue) reagents indicating the presence of diphenidine (**3a**). The second sample (SS-2), which was alleged to be 2-methoxphenidine, also

gave positive reactions with the three test reagents, however in this case a pink colour was obtained with the Marquis reagent signifying the presence of 2-methoxphenidine (2-MXP, **3b**). Thin Layer Chromatographic (TLC) analysis of the two samples utilizing a silica gel stationary phase and a mobile phase consisting of dichloromethane-methanol (9:1 *v/v*) containing 0.8% ammonia (7N in methanol) indicated that both samples contained single components (SS-1, R_f = 0.84 and SS-2, R_f = 0.77). Comparison of the samples with the reference materials confirmed the presence of diphenidine (**3a**, R_f = 0.85) and 2-methoxphenidine (**3b**, R_f = 0.76) correspondingly.

INSERT FIGURE 4

Qualitative GC-MS analysis confirmed the presumptive tests and indicated that the samples contained diphenidine (SS-1: t_R = 23.72 min, *m/z* (base peak) = 174 $[M+H]^+$, **3a**, Figure 4b and 4c) and 2-methoxphenidine (SS-2: t_R = 28.06 min, *m/z* (base peak) = 204 $[M+H]^+$, **3b**, Figure 4e and 4f) respectively, with no apparent adulteration, as indicated on the packaging. ^{1}H-NMR (400 MHz, CD_2Cl_2) analysis and comparison with standards of diphenidine (**3a**) and 2-methoxphenidine (**3b**) showed that the samples were essentially pure and confirmed the absence of any adulterants or diluents within the samples (Figure 5).

INSERT FIGURE 5

With substantial evidence, supporting a quantitative GC-MS approach for detecting various substituted diphenidine derivatives in street samples, the viability of the proposed protocol was tested. The samples were reanalysed (in triplicate) using the validated GC-MS method at a concentration of 100 μg mL^{-1}. The GC-MS results (Figure 6), confirm that the samples only contained the two alleged components (SS-1: t_R = 23.72 min, **3a**, 100.3% w/w, %RSD = 0.21%, *n* = 3) and SS-2: t_R = 28.06 min, **3b**, 99.5% w/w, %RSD = 1.37%, *n* = 3). Unlike other NPS products encountered on the recreational drugs market [25 – 27], both these samples appeared to both comply with the vendors claims (in terms of principal ingredient), be of high purity (>99% w/w) and there was no evidence (confirmed by ^{1}H-NMR, *vide supra*) that either contained any additional NPSs or commonly used diluents and/or adulterants.

INSERT FIGURE 6

4 Conclusion

We have presented the results of analysis of thirteen substituted diphenidine samples commonly encountered as NPSs in casework. All analyses were undertaken on known provenance samples prepared in-house and then cross-validated with two samples obtained from internet vendors. The presumptive (colour) tests provided a simple and rapid *"yes/no"* determination for these materials and some separation of the

compounds was possible using conventional thin layer chromatographic analysis. The developed method for GC–MS analysis provides a screening method, which facilitates the separation and identification of all thirteen diphenidine derivatives. The validated method has the added advantage of a rapid single step extraction in methanol with no necessity for derivatisation and acts as both an ideal triage method for seized samples and quantitative analysis of the psychoactive ingredients. Additionally the Supplementary Information provided herein acts as an important repository of characterization data (^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR [**3e** – **3g**] and FTIR) for the compounds utilised in this study and serves as a resource for laboratories engaged in the routine analysis of these types of compounds.

5 Conflict of interest

The authors declare no conflict of interest.

6 Compliance with ethical standards

This study did not involve research on human participants or animals.

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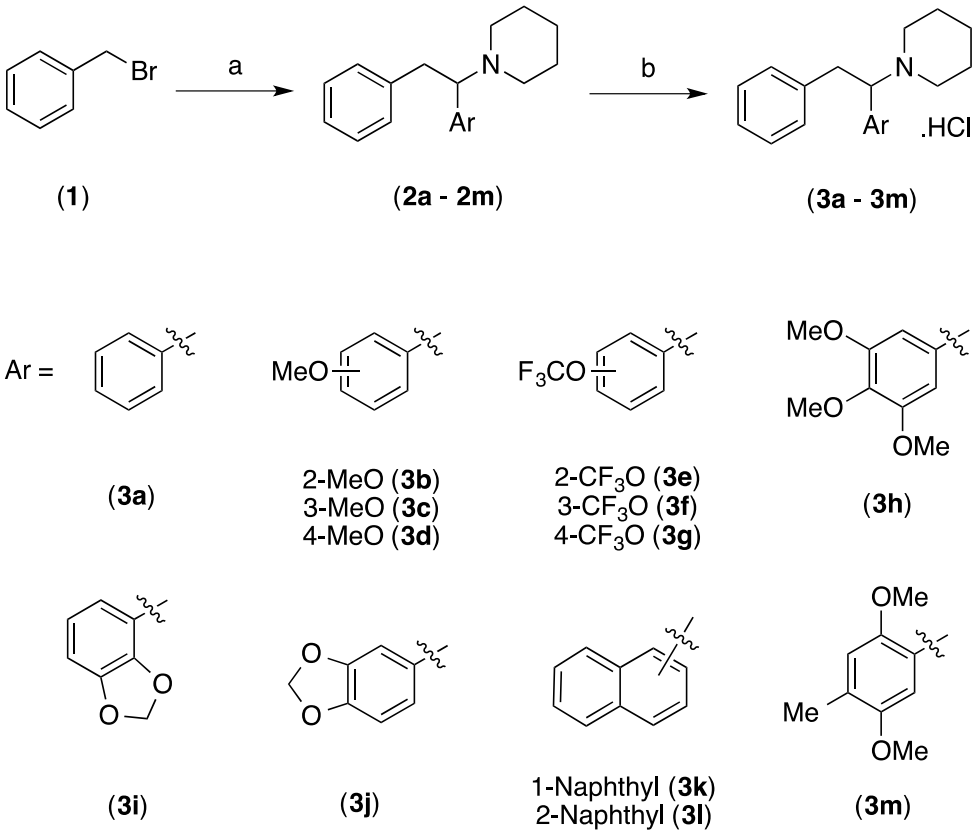


Scheme 1. *Reagents and Conditions:* (a) Zn/TFA/ArCHO/piperidine; (b) HCl (4M solution in dioxane)/Et_2O (21 – 77% yield). See Materials and Methods (Section 2.1) for experimental details.

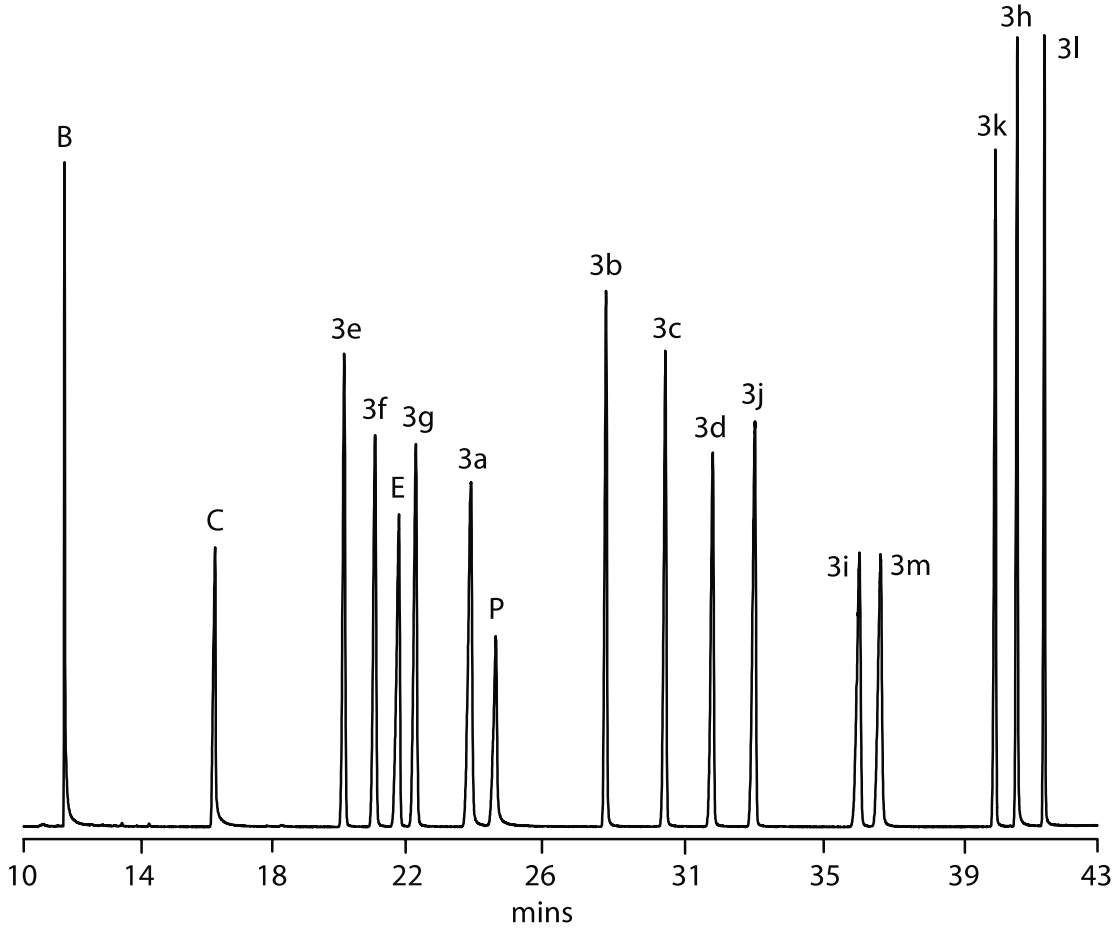


Figure 1. Exemplar chromatogram demonstrating separation of all thirteen substituted diphenidines and relevant adulterants: benzocaine (B); caffeine (C); 2-trifluoromethoxphenidine (2-TFMXP, **3e**); 3-trifluoromethoxphenidine (3-TFMXP, **3f**); eicosane (internal standard, E); 4-trifluoromethoxphenidine (4-TFMXP, **3g**); diphenidine (**3a**); procaine (P); 2-methoxphenidine (2-MXP, **3b**); 3-methoxphenidine (3-MXP, **3c**); 4-methoxphenidine (4-MXP, **3d**); 3,4-(methylenedioxy)diphenidine (3,4-MDDP, **3j**); 2,3-(methylenedioxy)diphenidine (2,3-MDDP, **3i**); IAS-013 (**3m**); 1-naphthenidine (1-NPD, **3k**); mescphenidine (3,4,5-TMXP, **3h**) and 2-naphthenidine (2-NPD, **3l**) [Underlined compounds are common adulterants]. See Materials and Methods (Section 2.4) for experimental details.

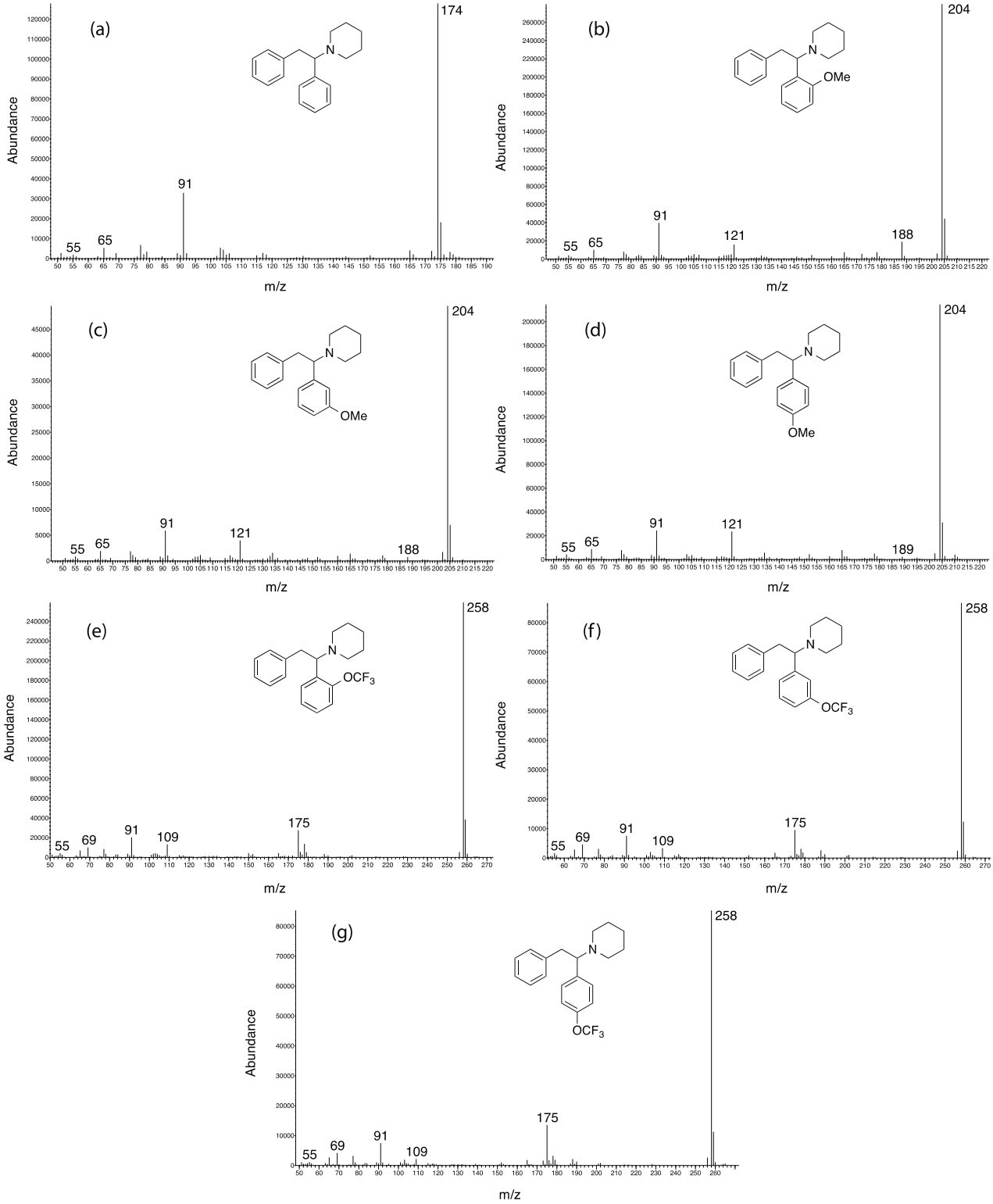


Figure 2. EI-MS spectra of (a) diphenidine (**3a**) and its substituted derivatives (b) 2-methoxphenidine (2-MXP, **3b**); (c) 3-methoxphenidine (3-MXP, **3c**); (d) 4-methoxphenidine (4-MXP, **3d**); (e) 2-trifluoromethoxphenidine (2-TFMXP, **3e**); (f) 3-trifluoromethoxphenidine (3-TFMXP, **3f**) and (g) 4-trifluoromethoxphenidine (4-TFMXP, **3g**).

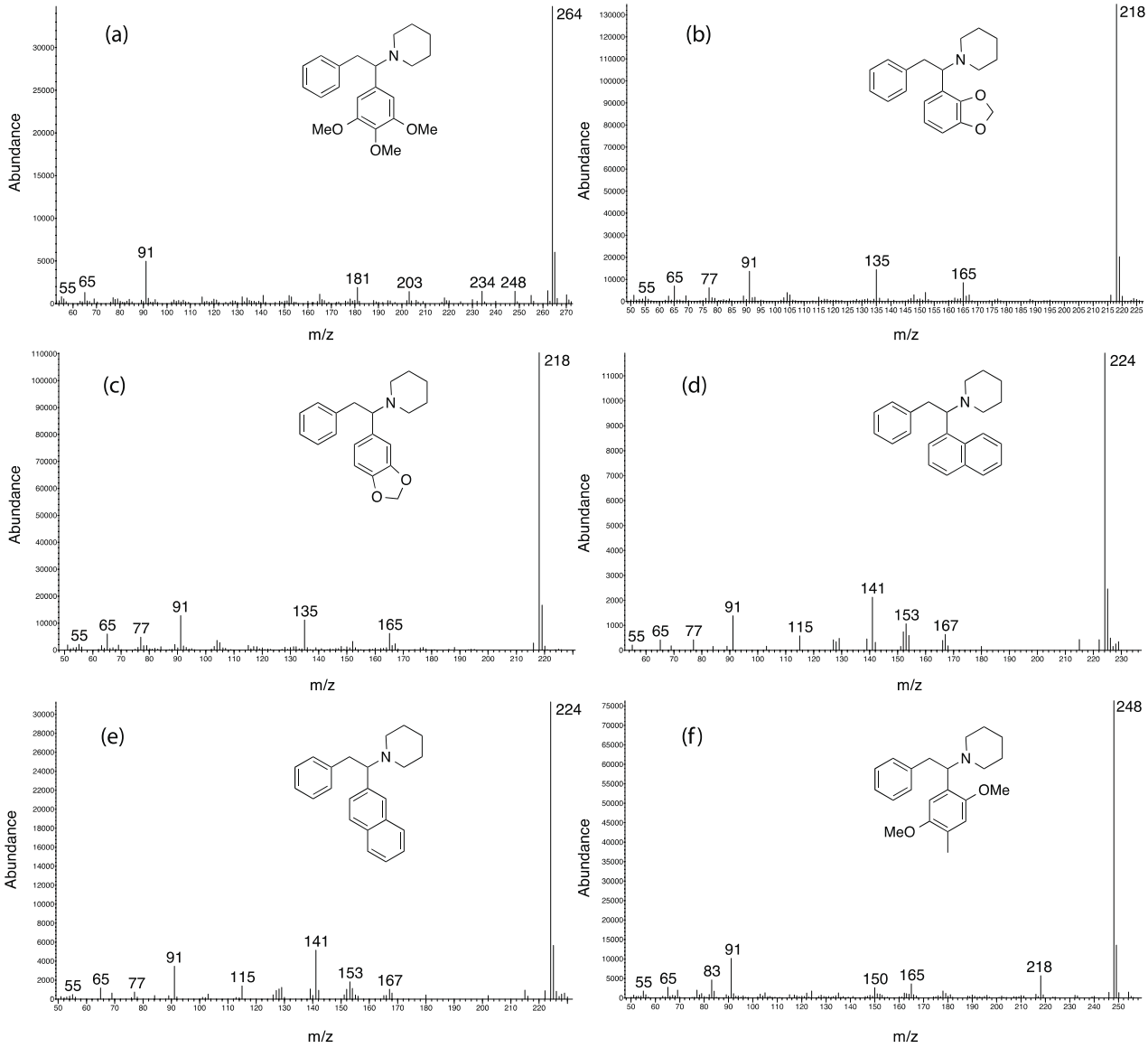


Figure 3. EI-MS spectra of (a) mescphenidine (3,4,5-TMXP, **3h**); (b) 2,3-(methylenedioxy)diphenidine (2,3-MDDP, **3i**); (c) 3,4-(methylenedioxy)diphenidine (3,4-MDDP, **3j**); (d) 1-naphthenidine (1-NPD, **3k**); (e) 2-naphthenidine (2-NPD, **3l**); (f) IAS-013 (**3m**).

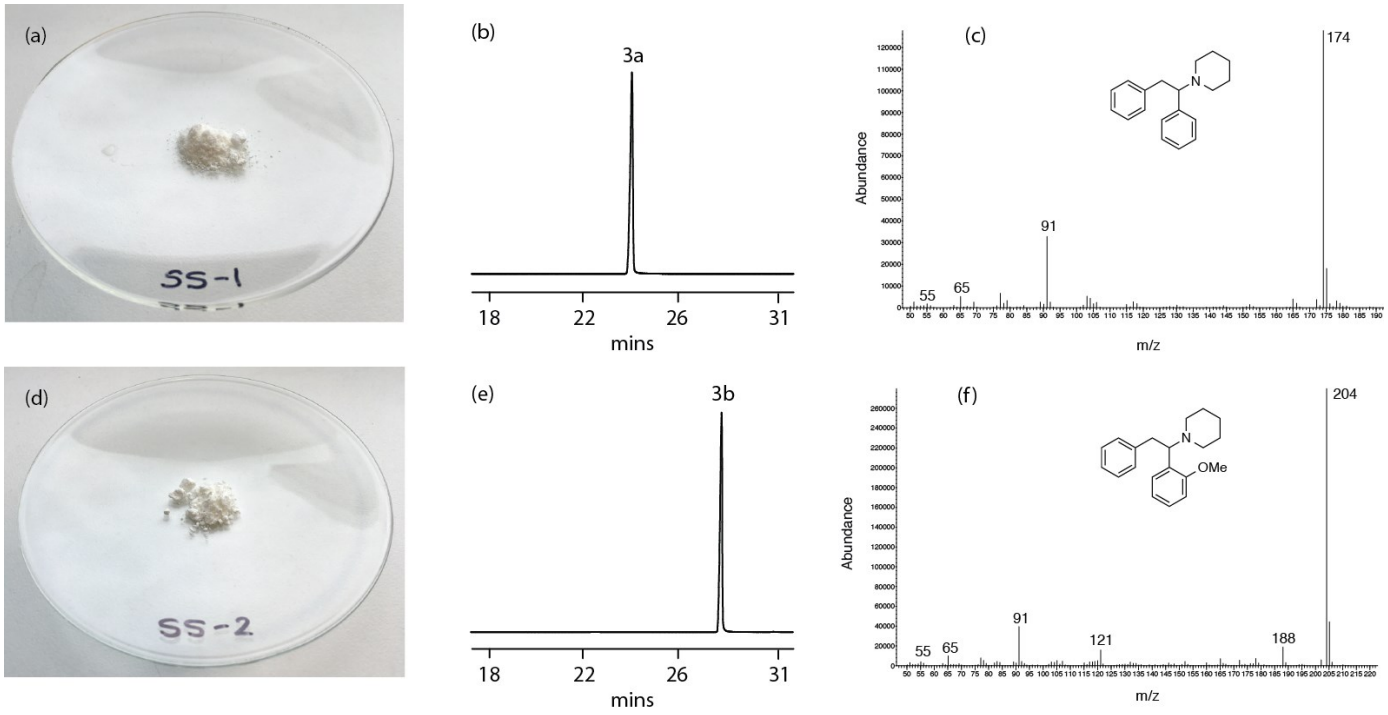


Figure 4. Qualitative GC–MS analysis of the diphenidine (SS-1, 0.1 mg mL^{-1} in methanol) and methoxphenidine (SS-2, 0.1 mg mL^{-1} in methanol) products: (a) diphenidine sample (1 g, SS-1) obtained from *"Buy Research Chemicals UK"* (http://www.brc-chemicals.com); (b) GC chromatogram for SS-1; (c) EI-MS spectrum (+ve ion mode) of peak (t_R = 23.72 min) corresponding to diphenidine (**3a**, $[M+H]^+$ = 174, base peak); (d) methoxphenidine sample (1 g, SS-2) obtained from *"Buy Research Chemicals UK"* (http://www.brc-chemicals.com); (e) GC chromatogram for SS-2; (f) EI-MS spectrum (+ve ion mode) of peak (t_R = 28.06 min) corresponding to 2-methoxphenidine (**3b**, $[M+H]^+$ = 204, base peak). See Materials and Methods (Section 2.4 and 2.6) for experimental details.

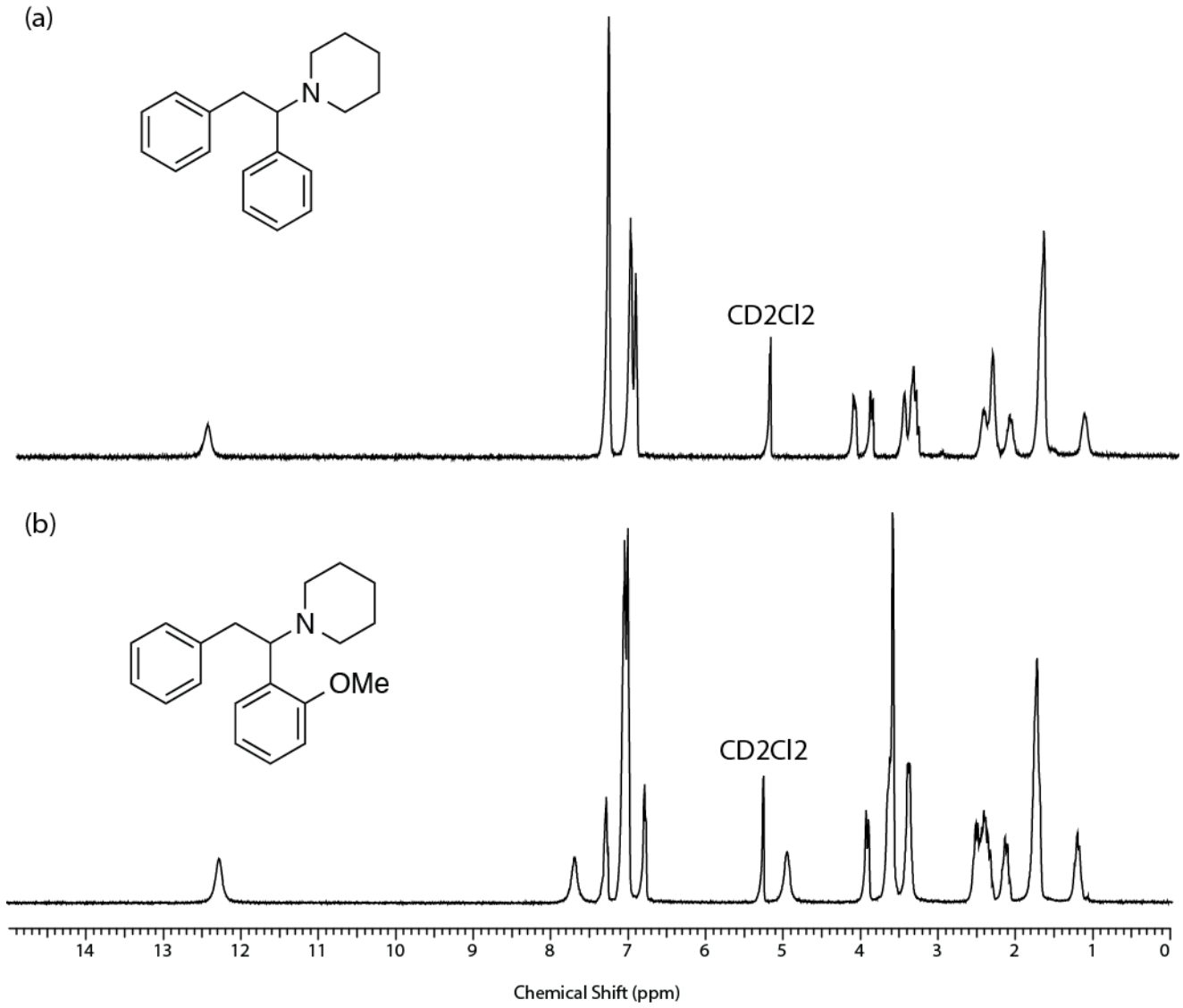


Figure 5. ^{1}H-NMR (400 MHz, CD_2Cl_2) analysis of (a) diphenidine (SS-1, 10 mg/600 μL) and (b) methoxphenidine (SS-2, 10 mg/600 μL) products obtained from *"Buy Research Chemicals UK"* (http://www.brc-chemicals.com).

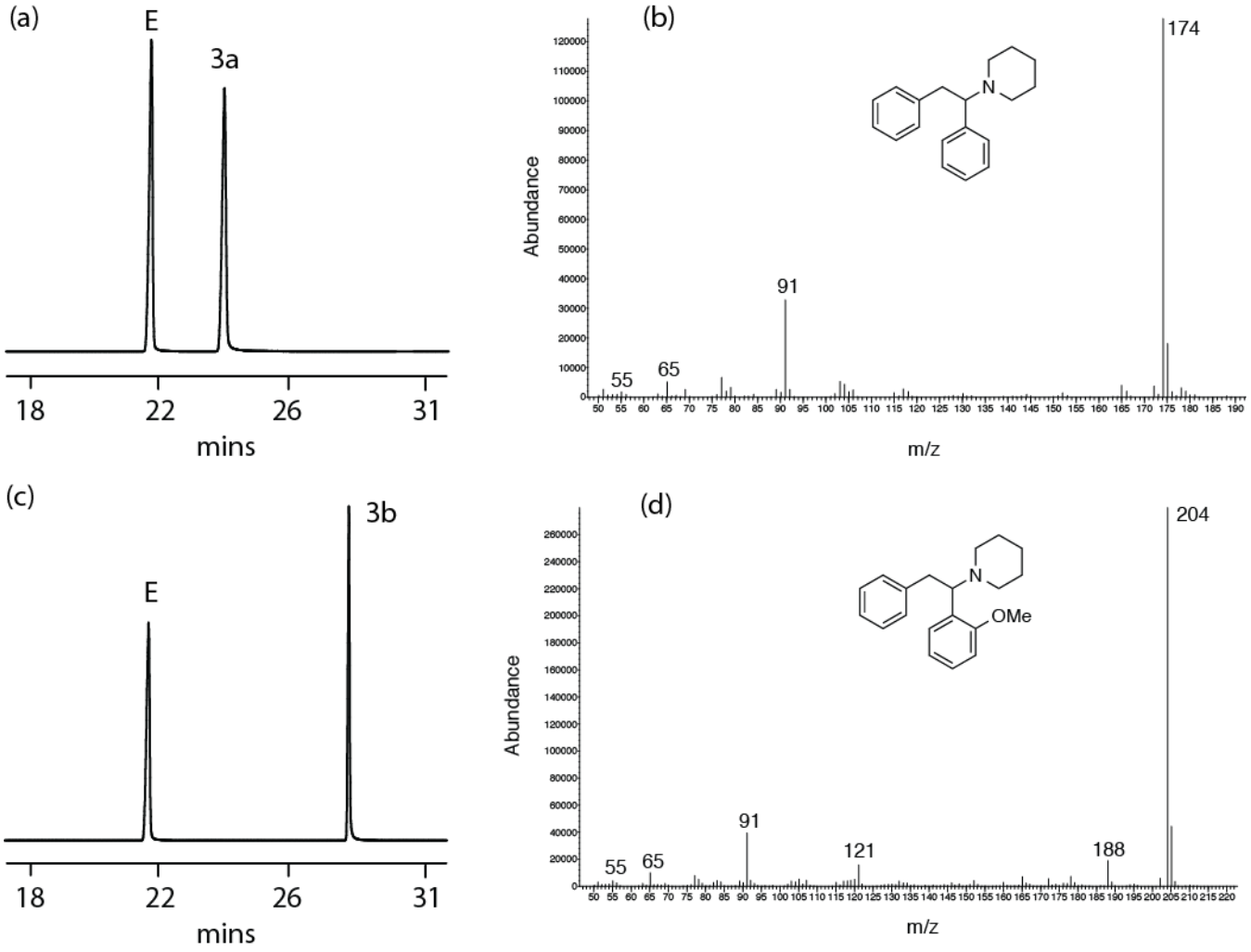
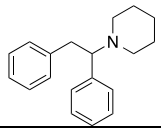
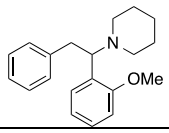
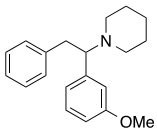
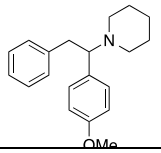
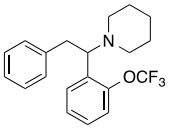
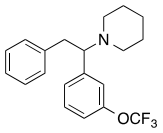
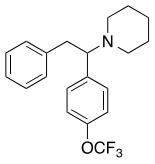
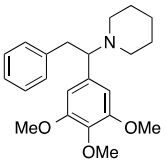
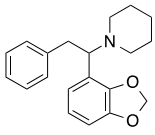


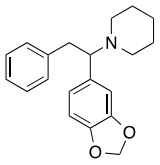
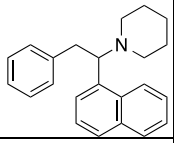
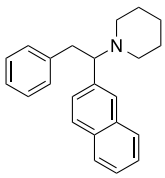
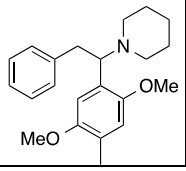
Figure 6. Quantitative GC–MS analysis of the diphenidine (SS-1, 0.1 mg mL^{-1} in methanol containing 0.1 mg mL^{-1} eicosane, E) and methoxphenidine (SS-2, 0.1 mg mL^{-1} in methanol containing 0.1 mg mL^{-1} eicosane, E) products: (a) GC chromatogram for SS-1; (b) EI-MS spectrum (+ve ion mode) of peak (t_R = 23.72 min) corresponding to diphenidine (**3a**, $[M+H]^+$ = 174, base peak); (c) GC chromatogram for SS-2; (d) EI-MS spectrum (+ve ion mode) of peak (t_R = 28.06 min) corresponding to 2-methoxphenidine (**3b**, $[M+H]^+$ = 204, base peak). See Materials and Methods (Section 2.4 and 2.7) for experimental details. NB. t_R (eicosane) = 21.55 min.

Table 1. Temperature programme for GC-MS analysis of diphenidine (**3a**) and its substituted derivatives (**3b** – **3m**).

Time (min)	Temperature (^{o}C)	Rate (^{o}C min^{-1})	Hold Time (min)
0	60 – 170	10	13
24	170 – 200	20	12
37.5	200 – 260	10	0
43.5	260 – 280	20	1

Table 2. Diphenidine (**3a**) and its substituted derivatives (**3b** – **3m**), their structures and thin layer chromatography (TLC) data. See Materials and Methods (Section 2.3) for experimental details.

	Chemical Name	Common Name and/or Abbreviation	Structure	Mol. Wt.	Base Peak (m/z)	Spot colour under UV light (254 nm)	Spot colour after staining with modified Dragendorff-Ludy-Tenger Reagent	R_f value	RR_f[a]
3a	1-(1,2-Diphenyl-ethyl)piperidine	Diphenidine		265.4	174	Black spot	Blood-red spot	0.85	1.00
3b	1-(1-(2-Methoxyphenyl)-2-phenylethyl)piperidine	2-Methoxphenidine (2-MXP)		295.4	204	Black spot	Blood-red spot	0.76 (0.57)[b]	0.89
3c	1-(1-(3-Methoxyphenyl)-2-phenylethyl)piperidine	3-Methoxphenidine (3-MXP)		295.4	204	Black spot	Blood-red spot	0.87 (0.77)[b]	1.02
3d	1-(1-(4-Methoxyphenyl)-2-phenylethyl)piperidine	4-Methoxphenidine (4-MXP)		295.4	204	Black spot	Blood-red spot	0.79 (0.60)[b]	0.93
3e	1-(2-Phenyl-1-(2-(trifluoromethoxy)-phenyl)ethyl)piperidine	2-Trifluoromethoxphenidine (2-TFMXP)		349.4	258	Black spot	Blood-red spot	0.94	1.11
3f	1-(2-Phenyl-1-(3-(trifluoromethoxy)-phenyl)ethyl)piperidine	3-Trifluoromethoxphenidine (3-TFMXP)		349.4	258	Black spot	Blood-red spot	0.93	1.09
3g	1-(2-Phenyl-1-(4-(trifluoromethoxy)-phenyl)ethyl)piperidine	4-Trifluoromethoxphenidine (4-TFMXP)		349.4	258	Black spot	Blood-red spot	0.92	1.08
3h	1-(2-Phenyl-1-(3,4,5-trimethoxyphenyl)ethyl) piperidine	Mescphenidine (3,4,5-TMXP)		355.5	264	Black spot	Blood-red spot	0.84	0.99
3i	1-(1-(Benzo[*d*][1,3]dioxol-4-yl)-2-phenyl-ethyl)piperidine	2,3-(Methylene-dioxy)diphenidine (2,3-MDDP)		309.4	218	Black spot	Blood-red spot	0.78	0.92

3j	1-(1-(Benzo[*d*][1,3]dioxol-5-yl)-2-phenyl-ethyl)piperidine	3,4-(Methylene-dioxy)diphenidine (3,4-MDDP)		309.4	218	Black spot	Blood-red spot	0.84	0.99
3k	1-(1-(Naphthalen-1-yl)-2-phenylethyl)piperidine	1-Naphthenidine (1-NPD)		315.5	224	Black spot	Blood-red spot	0.91	1.07
3l	1-(1-(Naphthalen-2-yl)-2-phenylethyl)piperidine	2-Naphthenidine (2-NPD)		315.5	224	Black spot	Blood-red spot	0.85	1.00
3m	1-(1-(2,5-Dimethoxy-4-methylphenyl)-2-phenylethyl)piperidine	IAS-013		339.5	248	Black spot	Blood-red spot	0.74	0.87

Key: [a]Relative Retention Factor (with respect to diphenidine, **3a**); [b]Retention Factor reported in McLaughlin *et al.* [5]

Table 3. Reactions of diphenidine (**3a**) and its substituted derivatives (**3b** – **3m**) with the Marquis, Mandellin, Scott and Zimmerman tests. See Materials and Methods (Section 2.2) for experimental details.

	Marquis		**Mandelin**		**Scott**		**Zimmerman**	
	Immediate colour change	Colour after 5 minutes	Immediate colour change	Colour after 5 minutes	Immediate colour change	Colour after 5 minutes	Immediate colour change	Colour after 5 minutes
3a	+ (orange)	pale brown[a]	+ (dark yellow)	+ (yellow green)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3b	+ (pink)	colourless[a]	+ (dark yellow)	+ (dark yellow)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3c	+ (red brown)	colourless[a]	+ (brown)	+ (brown)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3d	+ (pale pink)	colourless[a]	+ (pale yellow)	+ (pale yellow)	+ (blue)	+ (blue)	- (pale yellow ppt)	- (pale yellow ppt)
3e	+ (pale yellow)	colourless[a]	+ (light yellow)	+ (light yellow)	+ (blue)	+ (blue)	- (white ppt)[b]	- (white ppt)[b]
3f	+ (orange)	colourless[a]	-	-	+ (blue)	+ (blue)	- (white ppt)[b]	- (white ppt)[b]
3g	+ (orange)	colourless[a]	-	-	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3h	+ (pale orange)	pale pink[a]	+ (pale green)	+ (pale green)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3i	+ (pale brown)	colourless[a]	+ (brown)	+ (brown)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3j	+ (purple)	colourless[a]	+ (brown)	+ (brown)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3k	+ (beige)	pale brown[a]	+ (dark yellow)	+ (brown)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3l	+ (grey blue)	colourless[a]	+ (brown)	+ (brown)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]
3m	+ (pale orange)	colourless[a]	+ (green/yellow)	+ (green/yellow)	+ (blue)	+ (blue)	- (pale yellow ppt)[b]	- (pale yellow ppt)[b]

Key: [a]A gradual loss of intensity of the initial colour with Marquis reagent was observed over the five minute period; [b]Formation of water insoluble, tertiary amine free-base observed.

Table 4. Summary of GC-MS validation data for the quantification of diphenidine (**3a**) and its substituted derivatives (**3b** – **3m**). See Materials and Methods (Sections 2.4, 2.5 and 2.7) for experimental details. NB. t_R (eicosane) = 21.55 min. See Figure 1 for representative chromatogram.

						Precision (%RSD, *n* = 6)				
Analyte	t_R (min)	RRT[a]	Regression co-efficient	LOD[r] (μg mL^{-1})	LOQ[s] (μg mL^{-1})	25 μg mL^{-1}	50 μg mL^{-1}	100 μg mL^{-1}	200 μg mL^{-1}	250 μg mL^{-1}
Benzocaine	10.98	0.46	0.996[b]	5.97	18.10	7.81	7.81	5.25	4.13	2.19
Caffeine	15.68	0.66	0.998[c]	11.82	35.82	10.41	5.54	4.79	2.99	3.16
3e	19.80	0.83	0.998[d]	4.23	12.83	3.53	1.29	1.79	2.25	2.47
3f	20.77	0.88	0.998[e]	4.61	13.97	4.30	1.52	2.16	2.69	2.77
3g	22.02	0.93	0.997[f]	5.31	16.10	5.82	2.44	2.63	3.01	3.09
3a	23.72	1.00	0.997[g]	5.08	15.39	4.39	2.34	2.33	3.49	3.00
Procaine	24.25	1.02	0.996[h]	6.24	18.92	9.05	3.30	5.29	1.80	2.86
3b	28.06	1.18	0.997[i]	5.22	15.81	9.36	3.85	3.68	3.22	3.52
3c	29.94	1.26	0.998[j]	4.58	13.88	9.06	3.73	3.52	2.92	2.77
3d	31.40	1.32	0.996[k]	5.71	17.30	9.88	3.60	3.52	3.37	3.69
3j	32.76	1.38	0.997[l]	4.86	14.74	8.15	3.29	3.11	3.57	2.82
3i	36.03	1.52	0.996[m]	5.99	18.16	11.20	4.41	4.35	3.27	4.08
3m	36.70	1.55	0.996[n]	5.70	17.29	13.12	5.98	3.92	4.12	3.00
3k	40.43	1.70	0.997[o]	5.13	15.54	9.83	4.06	3.79	3.66	3.02
3h	41.13	1.73	0.996[p]	5.78	17.51	14.02	5.99	4.63	4.31	3.31
3l	42.00	1.77	0.997[q]	4.81	14.57	12.72	3.54	4.19	3.98	2.17

Key: [a]Relative Retention Time (with respect to diphenidine, **3a**); [b]y = 2.2474x – 0.0053; [c]y = 2.6993x – 0.0373; [d]y = 4.487x – 0.0137; [e]y = 4.3581x – 0.014; [f]y = 4.6764x – 0.0147; [g]y = 5.1585x – 0.0081; [h]y = 2.5746x – 0.055; [i]y = 4.2365x – 0.00274; [j]y = 4.7439x – 0.0266; [k]y = 4.6776x – 0.0254; [l]y = 5.1358x – 0.0329; [m]y = 4.1666x – 0.0301; [n]y = 4.1262x – 0.0375; [o]y = 5.0733x – 0.0456; [p]y = 5.1602x – 0.0631; [q]y = 4.7472x – 0.0475; [r]Limit of Detection (calculated based on the standard deviation of the response and the slope); [s]Limit of Quantification (calculated based on the standard deviation of the response and the slope).