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Development and validation of a comprehensive method for detection of eighty stimulants, narcotics & other drugs by gas chromatography nitrogen phosphorous detector/mass spectrometry in sports doping analysis

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ABSTRACT

The use of stimulants and narcotics in human sports is prevalent since ancient times. These drugs are prohibited for use only "in competition" in sports by World Anti-Doping Agency (WADA), due to relatively short onset of action and if taken immediately prior to the sport event, will cause a performance enhancing effect. Doping control involves initial screening of suspicious samples for a prohibited drug followed by more specific confirmatory method. The dope testing has to be accomplished in a defined time period while fulfilling relevant technical criteria, therefore a simple, high throughput & open analytical method allowing detection of maximum number of analytes is choice of every anti-doping laboratory. The present work provides a comprehensive, sensitive and selective GC-NPD/MSD method for the detection of 80 stimulants, narcotics & few other drugs of abuse excreted in free form in human urine. The method utilizes the feasibility of combining both the detectors (MS & NPD) with one GC producing dual data in a single run for fast & more reliable identification. The sample preparation was performed by liquid-liquid extraction of alkalinised urine. The limit of detection (LOD) for all substances was between 25-100 ng/ml. The method has been successfully utilized for the testing of more than nineteen thousand samples since 2009.

KEYWORDS

Stimulants;
Narcotics;
Sports dope testing;
GC-NPD/MSD;
WADA.

INTRODUCTION

Stimulants represent one of the oldest classes of doping agents which have a direct stimulating effect on the central nervous system (CNS) and have been used to increase performance, endurance, and stamina for centuries. In addition they may improve the faculty to exercise strenuously or produce a decreased sensitivity to pain[1]. The class of stimulants is prohibited by the World Anti-Doping Agency

(WADA) which contains various agents with different structural features[2]. Since 19th century, stimulants have been a major problem in elite sports and numerous adverse analytical findings (AAFs) has been annually reported by doping control laboratories worldwide[3].

The narcotic analgesics, banned in sports by WADA, are represented by morphine and its chemical and pharmacological analogues. They are derived from opium, which in turn is derived from the poppy plant (papaver somniferum). They act on the CNS & surroundings tissues by stimulating opioid receptors and reduce feelings of pain[4]. Due to the short & rapid onset of action of stimulants and narcotics, they are listed as banned substances in competition but are permitted out of competition. Stimulants and narcotics in general were among the first analytes to be tested in systematic doping controls. In the late 1950s, the capability of gas chromatography (GC) to separate compounds relevant for doping controls was recognized and introduced into sports drug testing to measure various classes of analyte[5-9]. Analyzers such as flame ionization and nitrogen–phosphorus detectors (FID and NPD, respectively) as well as ionization β-ray (strontium 90) or electron capture detectors were used. The enormous complexity of biologic matrices and the continuously increasing number of drugs in the prohibited list, however, necessitated more specific and unequivocal analyzers than for instance NPD and FID alone.

This resulted in the frequent use of GC equipped with NPD and mass spectrometer (GC-MSD/NPD), a combination that allows the exploitation of advantages provided by both analytical techniques simultaneously. The considerable proton affinity of stimulants and narcotics enabled the use of robust and sensitive instruments composed of liquid chromatography (LC) combined with (tandem) mass spectrometers (LC-MS/MS) to detect and quantify stimulants and narcotics in doping controls[10, 11]. Yet, the most popular screening methods for these drugs are on gas chromatography coupled with mass spectroSmeter or NP detector[12-14]. This is because most of the stimulants and narcotics are volatile and contains nitrogen in their structure hence are highly amenable for GC-MSD/NPD analysis. In addition, the analysis on mass spectrometer is performed in full scan mode which is a vital tool for retrospective analysis.

Anti-doping analysis is conducted in two steps. Initially, screening of samples is performed, in the case of a suspicious result; an additional selective confirmation is carried out using different chromatographic, mass spectrometric and immunological methods[11,15-17]. Moreover, separate analytical procedures required for different classes of drugs makes the doping control process more complex, time-consuming and laborious. Therefore, the doping control laboratories strive to have least number of analytical methods, without the probability of false reporting.

Until the year 2009, the dope testing of freely excreted stimulants and narcotics at National Dope Testing laboratory (NDTL), India consisted of alkaline liquid-liquid extraction of urine sample followed by analysis on GC-NPD and further re-injection on GC-MSD for a suspicious sample. The re-injection prior to confirmatory analysis provides additional structural information but requires more time[18]. Hence, a simple & open method was developed on GC-NPD/MSD (dual detector) for detection of volatile stimulants & narcotics prohibited in sports and few analgesics, sedatives etc. which are relevant in drug of abuse testing.

EXPERIMENTAL

Chemicals and reagents

All chemicals and reagents were of analytical or reagent grade. Tertiary butyl methyl ether (TBME) and potassium hydroxide were purchased from Merck, Mumbai, India. The certified reference standards of stimulants & narcotics and/or their metabolites were obtained from established sources like Sigma-Aldrich, USA, National Measurement Institute, Australia, Cerilliant, USA. Few standards were generously provided by anti-doping laboratories of Cologne, Italy and Montreal. The purified water using a Milli-Q water purification system installed in the laboratory (Millipore, Bedford, USA) was used.

Sample preparation

The sample extraction method involved addition of 2 µg/ml of internal standard (diphenylamine & N-methyl phenol thiazine) to 5ml of alkalinized urine sample (pHH"14 with 500 µl of 5N Potassium Hydroxide)). Liquid-liquid extraction was performed with 2 ml of TBME after adding 3 grams (approx.) of sodium sulfate to the samples for salting out effect. After mixing for 20 minutes on horizontal shaker and centrifugation for 5 minutes at 3000 rpm, the ether layer was separated and directly transferred into auto sampler glass vials for analysis on GC-NPD/MSD.

Preparation of standard solutions& quality control samples

The stock solutions of all the reference standards were prepared in ethanol at the concentration of 1.0 mg/ml. Standard mixtures were prepared at two different concentrations (50 µg/ml & 100 µg/ml) for each compound from stock solutions in ethanol. The urinary quality control (QC) samples were prepared with every batch at a concentration levels of 500 ng/ml & 200 ng/ml for stimulants & narcotics, respectively. The solution (1 mg/ml) of diphenylamine (DPA) & 10-N-methylphenothaizine (NMPZ) was prepared and diluted at 200 µg/ml in ethanol to use as internal standard (IS). All standard solutions prepared were stored at 4ºC.

Instrumentation

GC–MS analysis in scan mode was performed on an Agilent 7890A gas chromatograph equipped with Agilent 7683B automatic liquid sampler and interfaced to an Agilent 5975C inert mass-selective detector(70 eV, electron impact) and installed with an Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase;12.5m×0.20mm I.D., 0.33µm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of injector, interface and ion source were 280°C, 300°C and 230°C, respectively. Helium was used as carrier gas at a flow rate of 1.2 ml/min (at 100°C) in constant pressure mode. Sample (4µl) was introduced into the inlet in pulsed split mode (split ratio 5:1; pulse pressure) and the column temperature was set initially at 100°C (0 min) programmed to final 300°C at a rate of 20°C/min (4.5min). The scan mass range was kept 40–450 amu. The electron multiplier voltage (EMV) was kept 1750 V with a gain factor of 1.5. At least three characteristic ions per analyte were used for peak identification. Analyte peaks in the samples were identified by comparing the ion area ratios & retention times with those of the direct standards.

The NPD was connected to the GC-MS using a dedicated micro channel splitter (MCS) installed in the oven compartment and supplied with helium through auxiliary pneumatic control. The column effluent was split in to two outflows at 1:1 in MCS, each entering the ion chamber (MS) and the NPD detector. The NPD detector was operated at 320°C with constant flows of fuel gas (Hydrogen) (3 ml/min), reference gas (Air) (85 ml/min) and makeup (Helium) (5 ml/min). The detector signal was set at 40 pA.

Method development and validation

The analytical method was developed and validated as per the WADA guidelines for the anti-doping laboratories[19]. For validation the parameters specificity, selectivity, linearity, intra and inter-day precision, recovery, limit of detection (LOD), and robustness were determined.

Recovery

The recovery of analytes for which reference standards were available was estimated by spiking five replicates (for 3 days) of blank urine with each analyte at a concentration of 500 & 200ng/ml for stimulants and narcotics, respectively. The peak area ratios of analytes to IS of extracted *vs* an unextracted sample were calculated.

Specificity

Evaluation of specificity was carried out by analyzing six different spiked and six different blank urine samples collected from healthy volunteers for significant interfering peaks in the MSD & NPD output data at expected retention times of the analytes.

Linearity & precision

The linearity of the method was determined by

injecting unextracted standards of each analyte at five concentrations in the range of 25-1000 ng/ml (25, 50, 100, 500 & 1000) and the correlation coefficient was calculated by extrapolating the concentration ratio against response ratio. Intra-day precision was determined at MRPL for each compound using five replicates of spiked urine samples. The corresponding inter-assay precision was calculated from samples prepared and analyzed at three different days (n=5/day during 10 days). The precision of the method was determined by calculation of the relative standard deviations (RSD %) of the mean of ion peak area ratio of the analytes to internal standard. The precision of retention time was calculated as RSD% of relative retention time (RRT) of each analyte of IS for both NPD & MS data (n=d/day for 3 days).

Limit of detection (LOD)

The LOD was defined as the lowest concentration of analyte that can be reliably identified, measured with a signal-to-noise ratio (S/N>3) greater than 3. The S/N of the least abundant diagnostic ion (preferably molecular ion) was calculated using ten blank samples and ten fortified samples at concentration levels from 25 to 250 ng/ml.

Applicability to excretion study samples/routine doping control samples

A total of 19564 doping control samples received in National Dope Testing Laboratory (NDTL), India from 2009 to 2014 were analyzed by the developed method for stimulants & narcotics, including samples of mega events *viz* Youth Olympic Games (2010), XIX Commonwealth Games (2010) & 1st Asian beach games (2011).

RESULTS

Method development & optimization

The method could analyze eighty compounds of different chemistries prohibited in sports (stimulants and narcotics) and several other drugs of abuse like sedatives, anti-histamines and analgesics. The method was validated for all analytes except 22 compounds for which reference material was not available; however these substances could be identified using respective positive control samples. The short column allowed separation of most of the analytes in a run of 14.5 minutes. The use of structural analogue as an internal standard is mandatory only for quantitative confirmatory analysis[19]; however DPA and NMPZ were used in the developed method to monitor the extraction reproducibility. Due to the extraction at a high pH level the chromatographic background was significantly reduced allowing better correlation of data of both the detectors.

Most of the CNS stimulants are derived from the basic phenylalkyamine structure. Modifications involved are substitution at alkyl chain (e.g. Amfepramone by oxidation of alkyl chain &bis substituted methylation of amino function; ephedrines by hydroxylation of methylene moiety), amino functional group (e.g. N-ethyl amphetamine after mono substituted ethylation of amino function), and rarely at aryl moiety (e.g. fenfluramine by trifluoro methylation of aryl ring and methylation of amino group).

Consequently, many analogues show an identical fragmentation pattern resulting in similar base peak in mass spectra. For instance, methamphetamine & phentermine showed identical base peak at m/z 58 (Figure-1), likewise amphetamine & heptaminol had a base peak of m/z 44 (Figure 2). Chemical modification in such cases may improve the quality & information of mass spectra, however it does not eliminate the limitation of similar fragmentation pathways. Nevertheless, these substances could be identified by RT based separation and considering other more significant but less abundant ions (Figure-3). The molecular ion of underivatized stimulants & narcotics are not always prominent in +EI ionization due to very excessive fragmentation of molecular radical cation ($M^{+\cdot}$). However, many of the phenylalkylamine & alkylamine stimulants generate molecular ion at detectable abundance providing more reliability in MS analysis.

Many stimulants are excreted in urine as metabolites apart from parent. For example, metabolites of few drugs like normethadone & EDDP (metabolites of methadone), N-desmethylselegiline (metabolite of seleigiline), norpethidine (pethidine metabolite), cotinine (nicotine by product), etc are excreted

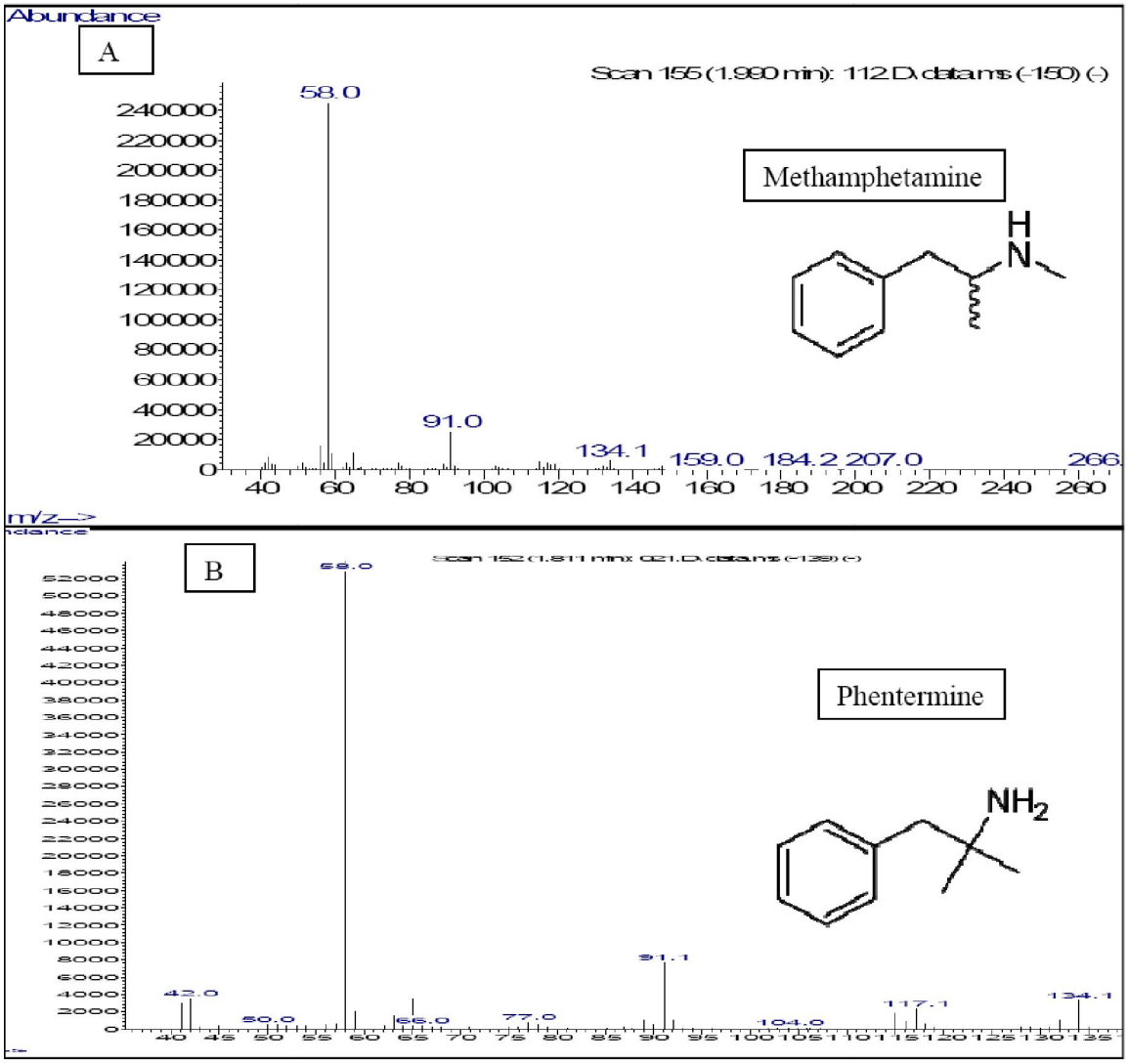

Figure 1 : Methamphetamine (A) and phentermine (B) showing identical base peak but different diagnostic ions

unconjugated in human urine. The method could be successfully validated & applied for detection of these metabolites. The method was validated and utilized in routine after parallel analysis to old method for one month.

The RT reproducibility was determined for both NPD & MS peaks by calculating relative retention times (RRT) to the IS. The RT & diagnostic ions (m/z) along with molar mass & molecular ion are provided in TABLE 1. It was difficult to have diagnostic ions of relative intensity of >10% in early eluting amphetamine type stimulants (ATS) due to structural limitation as most of these analytes had a base peak of m/z 44 or m/z 58, with other ions having abundances less than 5%. The mass ions with relative intensities below 10% are generally produced of molecular radical cation or further loss of alkyl or hydroxyl moiety. Such ions are fragmented instantly & intensively under high ionization energies and so the intensities of intact ions are relatively uncertain & less reproducible. Nevertheless, a screening method is meant for preliminary identification & isolation of suspicious samples for confirmatory analysis.

The GC method was capable of detecting highly volatile & low molecular weight compounds (amphetamine & isomethepthne) as well as less volatile & higher molecular weight substances (strychnine). All compounds were identified within 14.5 minutes of GC elution with solvent delay of 1.0 minute. The column was injected with 4 µl of sample volume through split liner at the split ratio of 5:1 at

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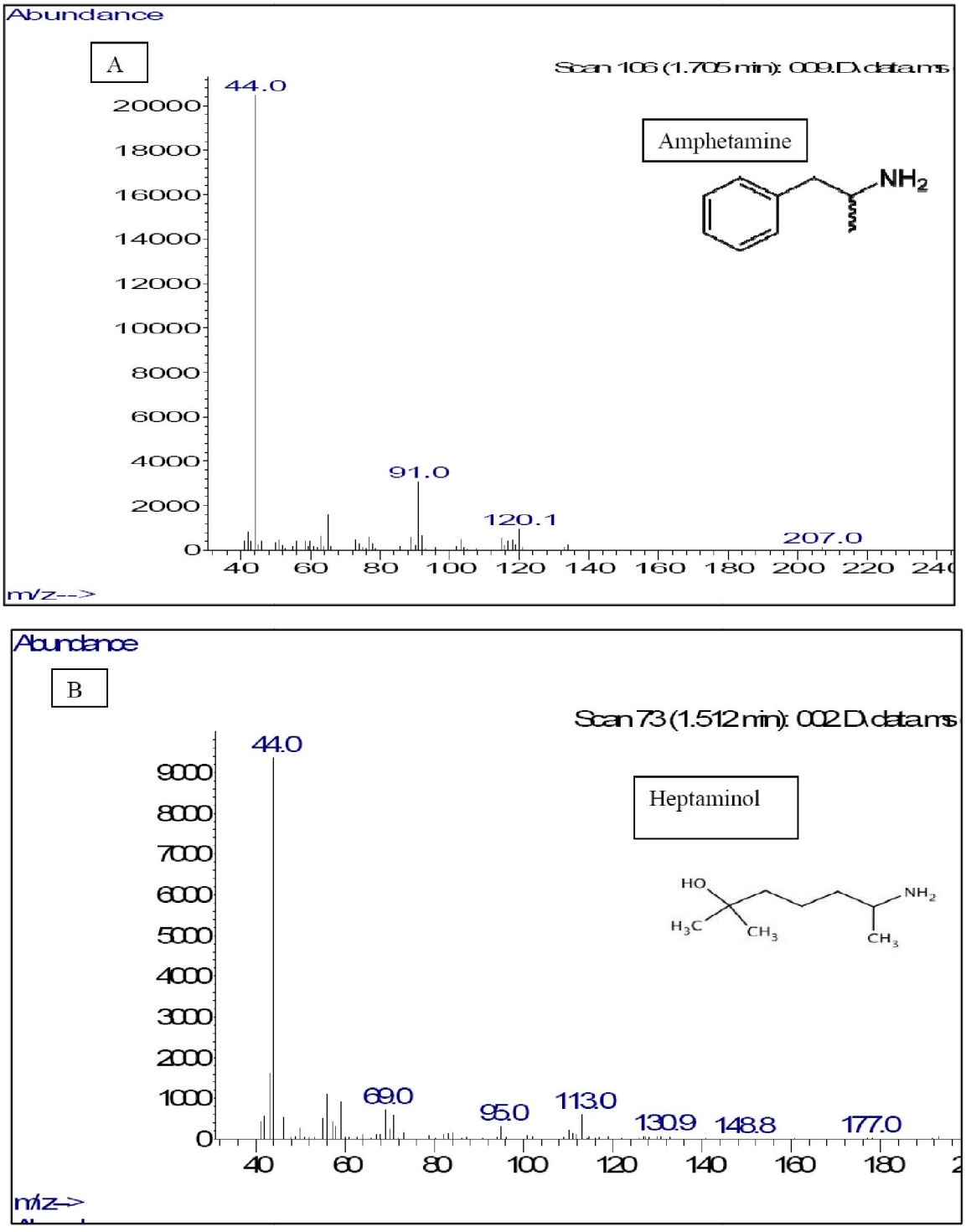

Figure 2 : Amphetamine (A) and Heptaminol (B) showing identical base peak but different diagnostic ions

280°C to avoid saturation of liner (leading to overloading of column) and ensuring vaporization of all the analytes of interest. Good chromatographic resolution was achieved for most of the compounds. The initial column temperature was set at 100°C to avoid ghost peak arising from solvent & related volatile impurities. A linear increment of column temperature (100°C to 300°C @20°C) facilitated separation of analytes of different volatility & masses in the column. The final column temperature was held for 4.5 minutes to avoid retention of non-volatile or active species on to the column.

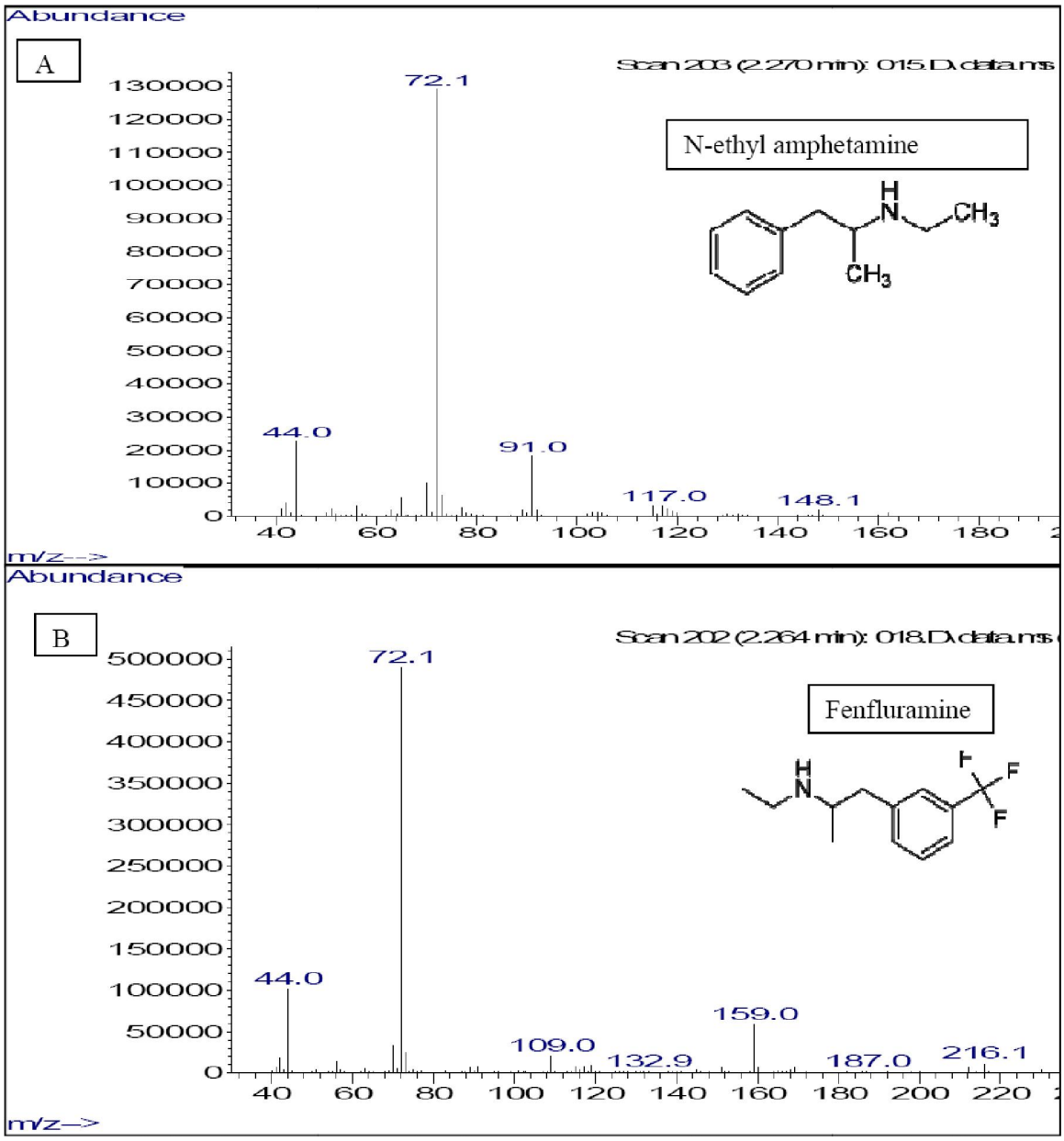

Figure 3 : Co-eluting compounds ethyl amphetamine (A) and fenfluramine (B) showing common base ion (m/z 72) and different diagnostic minor ions (*m/z* 159 and 220)

As the minimum required performance limit (MRPL) of stimulants & narcotic drugs are higher than other classes of substances[20], significant detection levels were achieved while operating the mass spectrometer in full scan mode in this method. The combination of NPD and MS (scan mode) provided sensitive and selective identification, and structural information of analytes.

Method validation

Precision

Repeatability of retention time exhibits the robustness of the analytical instrument operated under certain parameters over a period of time. Analysis of five replicates of QC samples for three days yielded stable retention times (CV < 2%) for all of the compounds except amphetamine, p-methyl amphetamine & isomethepthne which were eluted near to solvent front (within 0.7 minutes post solvent delay of 1.0 minutes) and accounted with CV% in the range of 2.0 to 2.2. No significant matrix effect on RT was observed because of very high pH used during extraction which eliminated most of the endogenous amine interferences.

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TABLE 1 : Retention time (RT), base peak, molecular ion and other fragments of compounds analyzed by GC-NPD/ MSD

S.No.	DRUG	RT (min)	CHARACTERSITIC MASS IONS (m/z)		
			Base Peak	Mol. Ion	Other Fragments
	Diphenylamine(IS)	4.55	169	169	170
	NMPZ(IS)	7.02	213	213	198
	Acetophenone	1.9	109	151	43, 80, 53, 108
	Amitryptylline	7.83	58	277	59, 30, 275, 217
	Amphetamine	1.66	44	135	120,134
	Benfluorex	1.72	105	350	192,159,149,216
	Benzphetamine	5.91	91	239	65,56148
	1-Benzylpiperazine	3.78	91	176	134,176
	Brompheneramine	7.36	247	*	248, 167, 180, 58, 194, 318
	Bupropion	4.47	44	239	111,100,139
	Caffeine	5.89	194	194	109,165
	Cathine	2.76	44	151	77,132
	Chlobenzorex	6.6	125	259	125,91,168
	Chlorpheneramine	7.0	203	274	58, 205, 168, 42, 167
	Codeine	8.81	299	299	162,229,282,214
	Cotinine	5.1	98	176	118, 119, 147, 99
	Cropropamide	5.23	100	240	115,168,195
	Crotethamide	4.94	86	226	69,154,181
	Cyclobenzaprine	8.03	58	*	215, 202, 189, 275
	3,3 diphenpropylamine	5.69	194	211	165,116,179,152,211
	Diazepam	9.14	256	284	283, 255, 221, 165
	Diclofenac	7.6	214	277	242, 179, 178, 151
	Desmethylsellegiline	2.9	82	*	91, 115, 172
	Dextromoramide	11.0	100	*	56, 128, 265
	Diethyl Propion	3.85	100	205	77,115,56
	Dimethyl amphetamine	2.27	72	163	,73,148,133
	Diphenhydramine	6.08	58	*	165, 152, 227, 167, 255
	Dextyromethorphen	7.55	59	271	150, 171, 214, 256
	Ephedrine	3.07	58	165	105,117,132
	EDDP Perchlorate	7.03	276	277	220, 262, 278, 56
	Ethyl Amphetamine	2.21	72	163	162,148,103
	Fluoxetine	6.14	44	*	104, 91, 78, 148
	Fancamfamine	4.95	215	215	98,215,186
	Fenetylline	10.57	250	341	250,70,181
	Fenfluramine	2.25	72	231	109,44,159
	Fenproporex	4.48	97	188	56,132,187
	Fentanyl	10.11	245	336	245,189,146
	Furfenorex	4.93	81	229	138,53
	Heptaminol	1.6	44	145	113,128,59
	Hydroxy cotinine	5.5	106	192	135, 119, 93, 78
	Isometheptene	1.21	58	141	95,126,84,71

S.No.	DRUG	RT (min)	CHARACTERSITIC MASS IONS (m/z)		
			Base Peak	Mol. Ion	Other Fragments
	Ibuprofen	4.4	161	206	163, 119, 118, 164
	Lamotrigene	9.06	185	255	187, 157, 114, 87
	Lidocaine	6.28	86	234	58, 87, 56, 77
	Mefenorex	4.36	120	211	120,122,84,196
	Meperidine/Pethidine	5.39	71	247	172,247,218
	Methamphetamine	1.92	58	149	91,134,
	Mephentermine	2.34	72	163	148,117
	MDA	3.69	44	179	77,105,179,136
	MDMA	4.02	58	193	135,77,105
	Methadone	7.54	72	309	294,165,309
	Methoxyphenamine	3.1	58	179	121,178,164
	Methyl Ephedrine	3.33	72	179	77,105,
	Nicotine	2.8	84	*	133, 161
	Nikethamide	4.07	106	178	177,78,149
	Nor nicotine	3.3	119	148	70, 147, 105, 120
	Neonicotine	3.7	84	162	105, 133, 162
	Norfluoxetine	6.03	30	*	134, 103, 191, 91
	Nortryptylline	7.92	44	263	202, 203, 204, 191
	Norfenfluramine	1.72	44	203	109,184,159
	Ortetamine	2.22	44	149	105,115,148
	Oxycodone	9.4	315	315	315,230,258
	Paroxitine	9.5	44	329	192, 70, 41, 109
	P-Methyl Amphetamine	2.17	44	149	105,134,117
	Pentetrazole	4.28	55	138	82,138,41
	Pentazocine	8.17	217	285	217,284,270
	Phentermine	1.82	58	149	91,134,117
	Phendimetrazine	3.63	57	191	70,191,85,191
	Phenpromethamine	2.01	44	*	77,91,105,128
	Pipradol	7.6	84	267	105,248,182
	Prenylamine	9.44	58	*	238,167,152,91,115
	Prolintane	4.6	126	217	174,91,70
	Propylhexedrine	1.83	58	155	140,155
	Propoxyphene	7.75	58	339	208,115
	Pseudoephedrine	3.33	58	*	105,117,132
	Ketamine	6.04	180	*	209, 152, 166, 194, 237
	Selegiline	3.37	96	187	56,91
	Strychnine	12.81	334	334	334,319,162
	Tramadol	6.52	58	*	263, 135
	N-desmethyl tramadol	6.7	44	*	188, 249
	O-desmethyltramadol	6.9	58	249	46, 59, 55, 121
	Tryptamine	5.2	130	160	131, 103, 51

***No molecular ion observed in the spectra**

The method precision was estimated on QC samples spiked in negative urines of different pH

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TABLE 2 : Method validation results showing recovery percentage, precision and LOD of the compounds analyzed by GC-NPD/MSD method

S. No.	Compound	WADA MRPL (ng/ml)	LOD (ng/ml)	Recovery (%)	RRT- precision (RSD%) (n=5)	Inter-day Precision (RSD%) (n=5X3)	Intra-day precision (RSD%) (n=5)
	Diphenylamine (ISTD)	NA*	NA	-	1.1	6.2	3.2
	NMPZ(ISTD)	NA	NA	-	1.3	5.5	1.2
	Amphetamine	500	50	91	2.1	8.8	5.1
	Benfluorex	500	100	76	1.9	10.2	6.3
	Benzphetamine.	500	50	98.8	1.8	5.4	4
	1-Benzylpiperazine	500	50	95.8	1.7	6.9	4.6
	Bupropion	500	50	89.5	1	3.8	1.2
	Caffeine	500	50	82	1.9	11.2	8.3
	Cathine	500	100	94	1.5	2.4	1.1
	Chlobenzorex	500	50	97	0.9	5.9	4.1
	Codeine	NA	50	88	1.0	8.1	7.6
	Cotinine	50	20	92.5	1.1	7.6	4.9
	Cropropamide	500	50	103	1.6	9.2	7.8
	Crotethamide	500	50	97.3	1.6	10.2	5.6
	3,3 diphenpropylamine	500	50	98.5	1.2	8.6	6.4
	Desmethylsellegiline	500	50	79	1.4	11.6	8.9
	Dextromoramide	200	50	101	1.7	10.8	6.7
	Diethylpropion	500	50	97.5	1	5.5	3.6
	Dimethyl amphetamine	500	50	96.5	1.1	7.4	4.9
	Ephedrine	500	100	99.5	1.5	3.6	2.2
	EDDP Perchlorate	200	50	69	0.7	12.1	8.7
	Ethyl Amphetamine	500	50	97.6	1.9	6.5	5.5
	Fancamfamine	500	50	99.2	1.8	8.0	5.9
	Fenetylline	500	100	89	1.9	4.7	3.1
	Fenfluramine	500	50	91.6	1.8	8.3	6.9
	Fenproporex	500	50	102	0.6	6.7	5.3
	Fentanyl	10	50	88.8	0.9	10.0	8.8
	Furfenorex	500	50	98.3	1.2	4.9	3.7
	Heptaminol	500	100	94.6	1.6	11.1	8.4
	Isometheptene	500	100	97.6	2.2	11.5	9.2
	Mefenorex	500	50	107	1.9	6.1	4.8
	Meperidine/Pethidine	200	50	87	1.1	6.6	3.8
	Methamphetamine	500	50	84.8	1.9	9.7	6.6
	Mephentermine	500	50	109	1.7	10.3	7.7
	MDA	500	50	105.1	0.9	7.6	4.1
	MDMA	500	50	104.7	0.8	8.2	6.4
	Methadone	200	50	92.2	1.4	6.4	4.5
	Methoxyphenamine	500	50	88.2	1.3	9.4	5.9

S. No.	Compound	WADA MRPL (ng/ml)	LOD (ng/ml)	Recovery (%)	RRT- precision (RSD%) (n=5)	Inter-day Precision (RSD%) (n=5X3)	Intra-day precision (RSD%) (n=5)
	Methyl Ephedrine	500	100	102	1.2	3.1	2.5
	Nicotine	50	20	88.9	1.2	7.2	5.1
	Nikethamide	500	50	89.8	1.1	4.8	3.3
	Nor nicotine	50	20	82.6	1.8	7.6	6.9
	Norfenfluramine	500	50	95.8	2.1	10.9	7.1
	Norfentanyl	200	100	88	0.8	11.8	9.4
	Ortetamine	500	50	81.2	2.0	11.6	6.8
	Oxycodone	200	100	102.8	1.3	6.9	5.8
	P-Methyl Amphetamine	500	50	92.6	2.1	10.7	8.4
	Pentetrazole	500	50	79	1.5	8.8	4.1
	Pentazocine	200	100	80.8	0.9	6.9	4.8
	Phentermine	500	50	89.3	1.8	9.9	5
	Phendimetrazine	500	50	74.2	1.3	7.2	5.6
	Phenpromethamine	500	50	77.8	1.9	9.7	8
	Pipradol	500	50	90.8	1.3	10.2	6.9
	Prenylamine	500	50	93	1.6	11.1	9.9
	Prolintane	500	50	100	1.1	7.6	6
	Propylhexedrine	500	50	100	1.8	10.4	8.3
	Propoxyphene	NA	100	93.3	1.1	10.9	9.7
	Pseudoephedrine	500	100	100.1	0.9	2.9	1.8
	Selegiline	500	50	94.3	1.5	7.2	5.6
	Strychnine	200	50	87.5	1.2	5.5	4.9

***NA: not applicable (indicates substances which are either not prohibited or included in WADA monitoring program; hence MRPL is not applicable.**

(5.5-8.5) and specific gravity (1.004-1.032 g/ml). The intermediate precisions (intra-and inter-day) showed coefficients of variation less than 15% for all analytes. The method was found to be repeatable with CV of < 10% over the entire range of substances (TABLE 2).

Limit of detection (LOD)

The LOD of different compounds in the developed method is listed in TABLE 2. All stimulants were detected at concentrations far below WADA MRPL as well as LOD's for narcotics were found to be at or below 50% of WADA MRPL with signal to noise ratio above 3 using two charaterstics ions. The detection limits of several other analytes not prohibited in sports were 250 ng/ml.

Linearity

The linearity was evaluated for stimulants & narcotics from 25-1000 ng/ml (25, 50, 100, 500 & 1000). The correlation coefficients (R^2) ranging from 0.986 to 0.999 showed the method linearity for all analytes over the specified concentrations.

Recovery (%)

The recovery percentage for all the analytes was found to be between 69-109%, (TABLE 2). The recoveries were sufficient to reliably identify the analytes at or below the levels prescribed by WADA.

Specificity

An analytical method without any significant interference at retention times of analytes of interest as well as absence of ions coming from interferences or background is proposed as specific. No interferences were observed at the retention time of analytes of IS in all the blank urines analyzed.

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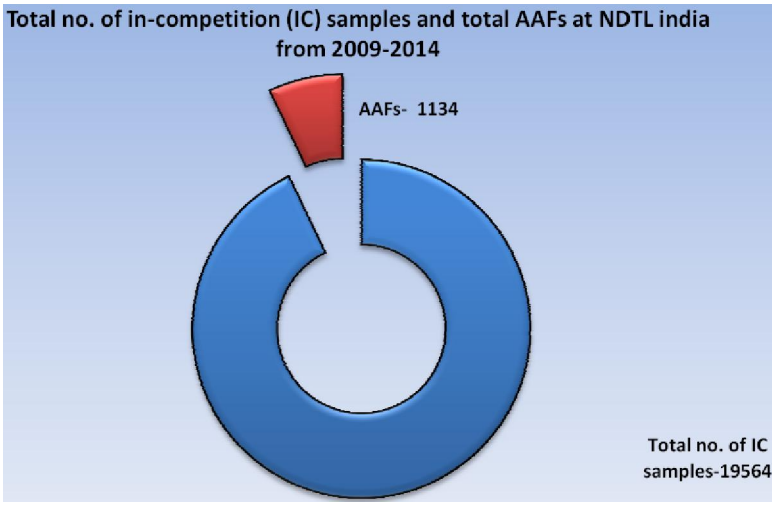

Figure 4 : Total number of in-competition samples tested and AAFs reported at NDTL, India from 2009-2014

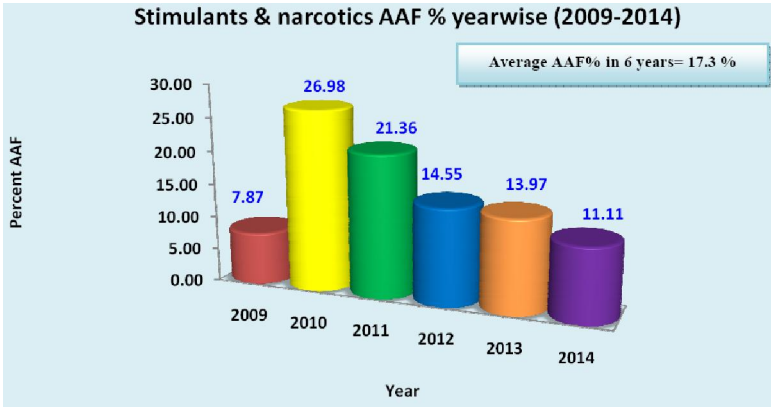

Figure 5 : Year wise distribution of AAFs for stimulants & narcotics reported using GC-NPD/MSD method in NDTL, India (2009-2014)

Applicability to routine analysis

The method was successfully applied to the analysis of 19564 in-competition routine sample received in NDTL from 2009 to 2014. A total of 1134 (5.8 %) adverse analytical findings (AAFs) for various drugs of abuse were reported during the period (Figure 4). Out of the total adverse analytical findings, 17.3 % of AAFs were accounted for stimulants & narcotics (Figure 5). The breakup of 5 major analytes reported as AAF (2009-14) using this method is illustrated in figure 6.

DISCUSSION

Stimulants & narcotics include drugs which produce alertness & analgesia, respectively. Both the classes have relatively short onset of action and taken just prior to the competition. As a result, both the classes are forbidden in sports only during competition[19]. Apart from pharmacological factors, various physical & chemical challenges like wider chemistries, pKa, polarity & structural specificities limits use of a universal method for detection of all stimulants & narcotics. Even the metabolism and elimination properties vary extensively resulting in different urinary by-products of stimulants & narcotics, though the MRPLs are sufficient to detect their abuse by athletes. As these drugs are abused at high dose to produce the ergogenic effect in-competition; a sample showing presence of a stimulant or narcotic at levels below 10 % of MRPL should not be declared positive[20].

Immediately after introduction of stimulants & narcotics as forbidden substances in sports, attempts were made to invent a systemic detection method; the capability of chromatography to separate components was utilized to detect sympathomimetic

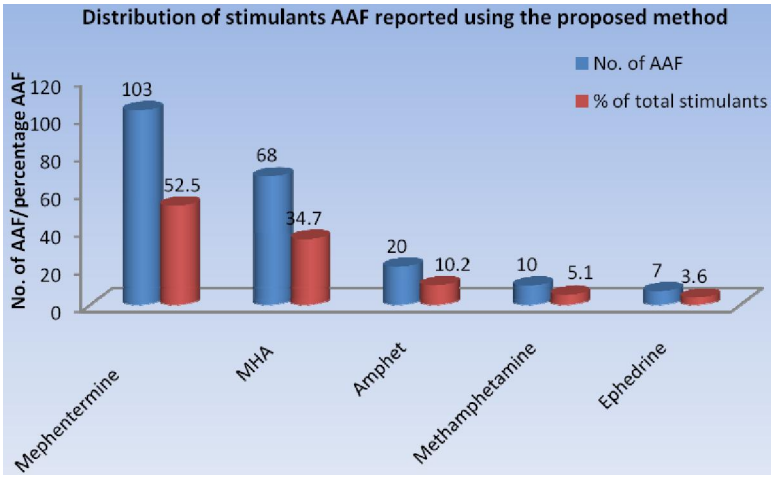

Figure 6 : Drug wise distribution of AAFs for stimulants in NDTL (2009-2014)

agents on GC[7, 8]. The detectors like FID, NPD or electron capture were used with GC. Later during 1970s, mass spectrometer interfaced with GC emerged with larger role in doping control. Further, combination of GC with NPD & MS allowed utilization of capacities of both the analyzers, simultaneously[12, 13]. Although, LCMS/MS based methods are now available for sensitive & trace level detection of polar, non-volatile & heat labile substances[14-16], the role of GC-NPD/MS remains indispensible for comprehensive & sensitive analysis of volatile stimulants, narcotics & other drugs of abuse.

The present method has been successfully used in screening & confirmation of stimulants & narcotics on GC-NPD/MSD. The current method is capable of analysing 1 sample in 14.5 min. for 80 analytes against the two separate run (each 15 min.) on the traditional GC-NPD & GC-MS method. The advanced GC system equipped with fast electronics & pneumatic control was used for faster temperature ramps, high capillary flows & signal processing. The inert ionization source of mass spectrometer facilitated effective ionization of analytes minimizing noise ions coming from active surfaces of ion chamber. The triple axis mass detector (TAD) used in MS ensured capturing maximum electrons coming from multiplier thus enhancing the sensitivity of analysis.

The separate injections on GC-NPD & GC-MS require additional sample extract, equipment, and manpower instrument and data handling; which limits use of such analytical methods for screening analysis particularly during major events testing. The method developed has proven as high throughput & comprehensive during the testing of major events viz. I Singapore Youth Olympic Games and XIX Commonwealth games where a turnaround time of 24 hours was required. The method has been found to be simple, robust and reliable with easy operation & low maintenance. Since then, it has been used in the laboratory for in-competition testing for more than 6 years and over 19000 urine samples have been analysed.

CONCLUSION

A rapid, comprehensive and sensitive method was developed utilizing dual detector technology for the analysis of 80 stimulants & narcotics in sports drug testing. The experiments were carried out under standard mass spectrometric conditions for +EI analysis on GC-NPD/MS. The method was validated according to the International Standard for Laboratories[19] as per World Anti-Doping Agency enforcements. The analytical procedure enabled detection and identification of many drugs and their metabolites, including most of the stimulants, 6-adrenergic agents and narcotics (methadone, pentazocine and pethidine). In addition it is possible to detect other nitrogen-containing drugs such as anti-histaminics, benzodiazepines, tricyclic antidepressants and local anesthetics. A robust and reliable method is presented which can readily be used in forensic, toxicological, work place and clinical testing.

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