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Analysis and Validation of GC-FID Method for the Determination of Amphetamine-Type Stimulants (ATS) Drugs in Human Nail Samples

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Blood and urine are the common and conventional biological samples to determine drug abuse. Drug analysis in nail samples has rapidly emerged as an alternative to normal biological specimens to detect drugs. The objective of this study is to determine the presence of Amphetamine-Type Stimulants (ATS) drug from the nail samples and to validate the method of ATS drug analysis in nail samples of drug abusers by using Gas Chromatography-Flame Ionization Detector (GC-FID). 88 male nail samples were collected from Narcotic Addiction Relief Centre in Sabah and Rehabilitation Centre in Johor. The identification of ATS drugs in the nail samples were analysed by using alkaline digestion method with GC-FID. Method for nail analysis were successfully analyzed and validated with the assessment of the following parameters: accuracy linearity of calibration curve, limit of detection (LOD) and limit of quantification (LOQ). This study indicates that the highest content of ATS drug detected in nail samples was MDA analyte which was detected in 83 out of 88 subjects. The second most detected ATS drug was AMP analyte, followed by MDEA, MBDB and MAMP. The least amount of ATS drug detected in nail samples was the MDMA analyte detected only in 18 out of 88 subjects. MDMA showed the lowest LOD and LOQ which are 0.0823 ppm and 2.4943 ppm respectively. While AMP shows the highest LOD and LOQ with the concentration of 0.1566 ppm and 4.7465 ppm respectively. The application of alkaline digestion method was effective in quantifying ATS drugs in nail samples. Furthermore, the nail samples also very stable and can be kept for a longer time period and difficult to alter its composition. There is no doubt that nail analysis can provide valuable and valid information on previous drug use in the fields of forensic toxicology.

Keywords: ATS drugs; drug abusers; nail samples; GC-FID; LOD; LOQ

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Nail analysis serves as a valuable technique in the identification of drug use and abuse. Nails, like hair are keratinised biological structures that have the capacity to retain substances over time, so they have the potential to serve as an alternative to conventional matrices such as blood and urine [1]. Nail specimens retain drugs for months to a year. As compared to hair, nails are less prone to drug destruction from exposure to sunlight, heat, and water [2]. Nail analysis is also an optimal alternative to hair testing in circumstances where hair removal is impracticable, such as when the donor lacks hair or for religious obligations or hair samples are not available due to decomposition of the dead body [3]. This makes nail samples more reliable option for forensic and legal purposes [4].

A fingernail regenerates in four to six months, meanwhile toenails regenerate in eight to twelve months or more [5]. In general, the growth of fingernail of normal human is around 0.1 mm per day, 3.0 mm per month and 38.1 mm per year [6]. Research has reported that the human fingernail growth is in average of 3.0 mm per month and regeneration time is 3 to 5 months while for toenail the growth rate in the range of 1.1 mm per month and regeneration time is 8 to 16 months respectively [7].

Despite, numerous researchers conducting drug analysis in hair samples, very few studies are reported on the use of nail samples as biological specimens in drug analysis. Although limited in number, these nail studies proved that nails can be used for detecting drug exposure [8]. Based on [9], the most investigated analysis of nail clippings that had been done, found the results were similar to those obtained from hair analysis. It can be seen as nail matrix can detect up to 89 different analytes for example antidepressants, drugs of abuse and antihypertonics even though incorporated with a large number of substances. Researchers [3] obtained nail samples from individuals with documented history of illicit drug use in five different parts of China. A grand number of 294 nail samples were gathered to screen for 106 various forms of drug abuse. Out of 294 nail samples examined, 213 tested positive for at least one substance. Therefore, this study is carried out to determine and quantify the

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amount of ATS drugs and their metabolites in nail samples obtained from drug abusers in Malaysia by using Gas Chromatography with Flame Ionization Detector (GC-FID).

EXPERIMENTAL

Chemicals and Materials

The chemicals used in this study were methanol, dichloromethane, sodium hydroxide (NaOH), ethyl acetate and pentafluoropropionic anhydride (PFPA). Six amphetamine-type stimulants (ATS) standards were used in this study which were amphetamine (AMP), methamphetamine (MAMP), N-methyl-1-(1,3-benzo-dioxol-5-yl)-2-butanamine (MBDB), 3,4 methylene-dioxyamphetamine (MDA), 3,4-Methylenedioxy- N-ethylamphetamine (MDEA), 3,4-methylenedioxy-methamphetamine (MDMA). All standards were purchased from Cerilliant (USA) with a concentration of 1000 ppm for each standard. All glassware's used in this study were sterilized with an autoclave machine (Hirayama HVE-50). The apparatus used for sample preparation were weighing balance (A&D HR-250AZ), shaking water bath (Memmert WNE 14) and Eppendorf AG22331 centrifuge.

Instrument

The analytical instrument used to analyze ATS drugs in nail samples was Gas Chromatography (Agilent Technologies 7890A) equipped with Flame Ionization Detector (FID) and HP-5MS capillary column (Agilent J&W Scientific, Folsom, CA, USA).

Validation of Analytical Method

A method validation was established and evaluated according to the guidelines of Standard Practices for Method Validation in Forensic Toxicology by Scientific Working Group for Forensic Toxicology (SWGTOX) [10] before being applied to the real samples. Accuracy, linearity of calibration model, limit of detection (LOD) and limit of quantification (LOQ) were the validation parameters that have been evaluated to validate the analytical method of drug analysis in nail samples which were conducted in triplicate. The accuracy of the method was calculated as percentage recovery (R%) and was assessed in concentration levels of 30 ppm. About 300 µL of each ATS standard was spiked into drug-free samples (blank nail samples) and extracted for the accuracy determination. The percentage recoveries found for each standard were 97% for AMP, 88% for MAMP, 95% for MDA, 79% for MDMA, 78% for MDEA and 92% for MBDB.

The calibration curve of peak area of blank or drug-free samples (x-axis) versus concentration of ATS drugs in ppm (y-axis) was plotted based on five concentrations levels of standard ATS drugs that had been injected into GC-FID. Five levels of concentrations that begin with 2, 5, 10, 30 and 50 ppm of each ATS drug were established with three replicates at each concentration. The linear regression of equation $y = mx + c$ and coefficient of determination (R^2) were established based on the calibration curve. Limit of detection (LOD) is the lowest concentration of the analyte that can be distinguished from the background noise. In this study, the method was validated by using Signal-to-Noise (S/N) ratio technique. In chromatography, the LOD is the injected amount that results in a peak with a height at least three times higher as the baseline noise level (S/N: 3/1). Limit of quantification (LOQ) is the lowest concentration of an analyte in the sample that can be determined with acceptable precision and accuracy under stated conditions of test. Both LOD and LOQ are two basic elements of method validation that define the limitations of an analytical method. LOQ was determined based on the standard concentration of ATS drugs with a peak height of ten times higher than the baseline noise level (S/N: 10/1).

Standard Preparation

All six ATS standards were prepared as 1000 ppm in methanol and stored at 4°C. A mixture of ATS standard stock solution (100 ppm) was prepared from six individual ATS standards each with 1000 ppm. The spiking samples were prepared from standard mixture stock solution (100 ppm) by spiking 20 µL, 50 µL, 100 µL, 300 µL and 500 µL to prepared calibration standards of 2, 5, 10, 30 and 50 ppm, respectively. The extraction step was performed immediately after the spiking procedure. Finally, a calibration curve was plotted to quantify the amount of ATS drugs present in drug abusers' nail samples.

Sample Collection and Storage

Blank nail or drug-free nail samples were obtained from ten drug-free volunteers by using sterilized nail clippers for control and quantification purpose. 88 nail samples were collected from male drug abusers with ages of 19 to 44 years that consumed the illicit ATS drugs. The nail samples were obtained from Narcotic Addiction Relief Centre in Sabah and Rehabilitation Centre in Johor. The nail samples were stored and wrapped in aluminium foil to maintain the integrity of the samples [11]. Nail samples were stored under dry conditions at room temperature. Nail sample preparation was initially performed with decontamination or washing method, followed by extraction of ATS drugs and finally with derivatization step. The samples then were ready to be injected into analytical instrument of GC-FID.

Decontamination of Nail Samples

Decontamination process is the first step of drug analysis in nail samples. The purpose of decontamination step is to remove external interferences without extracting the analyte of interest from samples matrix [12]. The nail samples (Figure 1) were first washed

in 5 mL of distilled water for 2 minutes, and again washed with 5 mL of methanol for 2 min. Then, the samples were left to be dried at room temperature. Next, approximately 25 mg of decontaminated nail samples were transferred to another test tube for extraction process.

Extraction of ATS Drug in Nail Samples

After decontamination step, extraction of ATS drug in nail samples were carried out using the method described by [6]. 25mg of nail samples weighed and put into 10 mL glass tubes. Next, 1 mL NaOH at 1M was introduced into the tubes and incubated in an oven at 95°C for 30 min. Then nail samples were cooled to room temperature and extracted with 3 mL ethyl acetate (Figure 2). After 10 mins, the samples were centrifuged at 3000 rpm for 10 min. The upper organic layer was transferred to a new test tube. Next, the extract was concentrated to dryness under nitrogen steam followed by the derivatization process.

Derivatization of Extracted Nail Samples

The extracted nail samples were derivatized by adding 50 µL of ethyl acetate and 50 µL of PFPA as the derivatizing agent. The samples were then heated in water bath for 30 min at 50°C. Next the samples were dried under nitrogen stream and reconstituted with 50 µL of ethyl acetate. Finally, 1 µL of aliquot was transferred into 1 mL vial and injected into the GC-FID.

Quantitative Analysis of Nail Samples

The presence of ATS drugs and metabolites in nail samples were identify by using GC-FID. Six ATS drugs including AMP, MAMP, MDA, MDMA, MDEA and MBDB were identified based on the retention time of each analyte eluted in standard ATS mixture. Detection of six analytes of ATS drugs in nails samples were identified based on the eluted retention time of ATS standards in standard solution mixture. The extracted nail samples were analysed using Gas Chromatography (Agilent Technologies 7890A) equipped with Flame Ionization Detector (FID). Chromatographic separation was carried out on HP-5MS capillary column (Agilent J&W Scientific, Folsom, CA, USA) with helium as carrier gas at a flow rate of 3.1599 mL/min. Splitless injection mode was used with injector temperature at 250°C. The oven temperature was set at 90°C for 2.0 min, and then increased to 150°C at a rate of 20°C /min for 17 min.

Figure 1. Nail Sample.

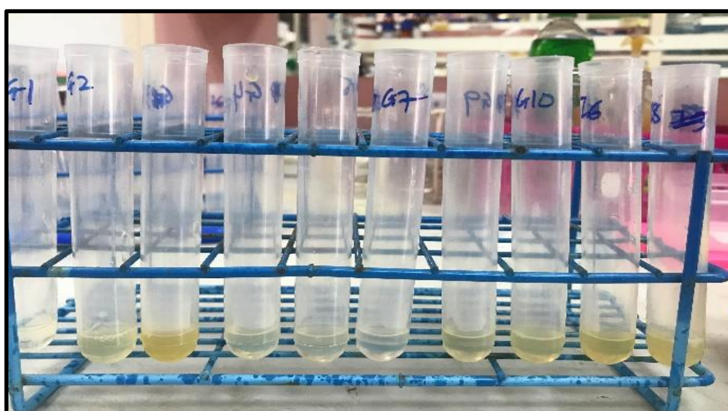

Figure 2. Nail samples extraction using ethyl acetate.

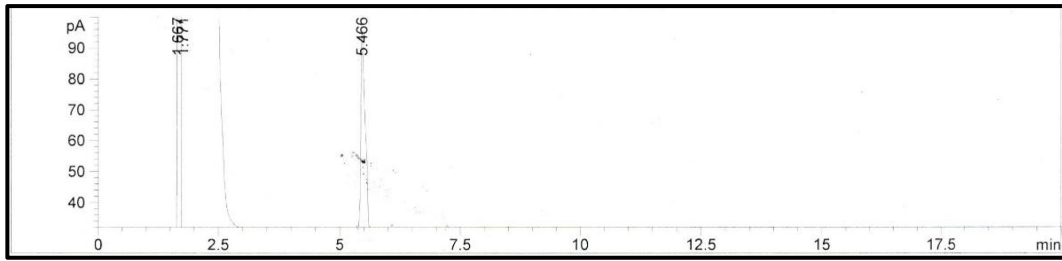

Figure 3. Chromatogram of Individual Standard AMP.

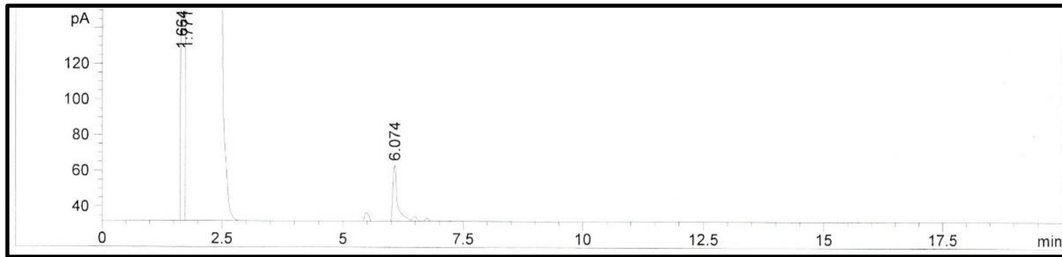

Figure 4. Chromatogram of Individual Standard MAMP.

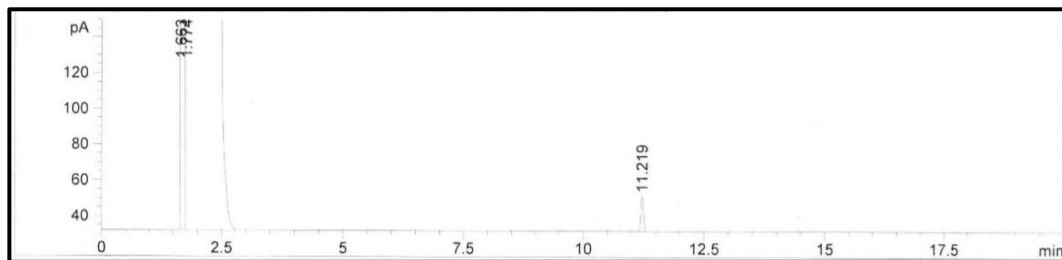

Figure 5. Chromatogram of Individual Standard MDA.

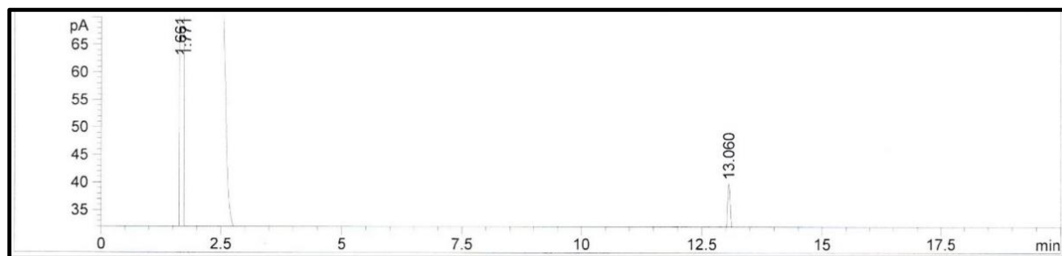

Figure 6. Chromatogram of Individual Standard MDMA.

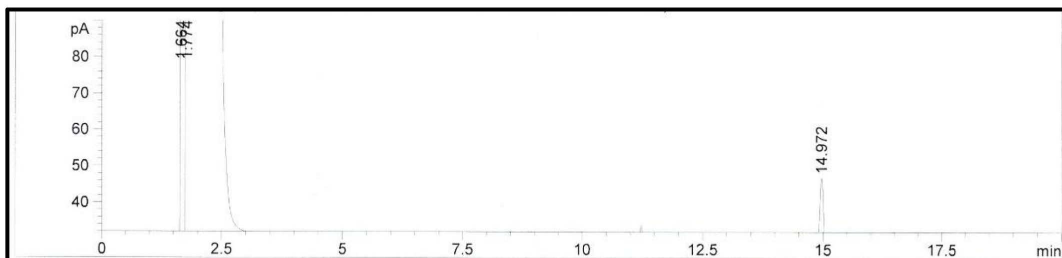

Figure 7. Chromatogram of Individual Standard MDEA.

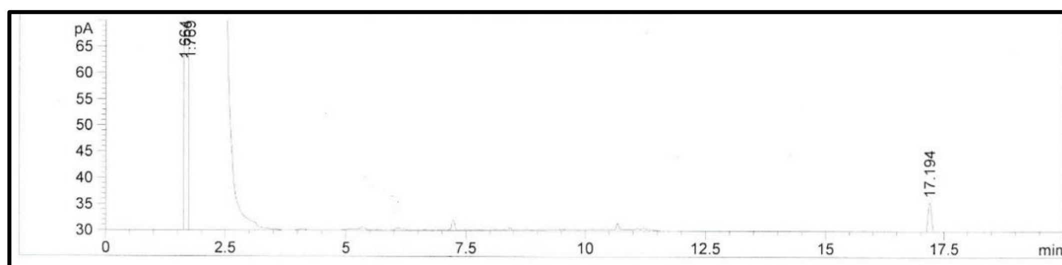

Figure 8. Chromatogram of Individual Standard MBDB.

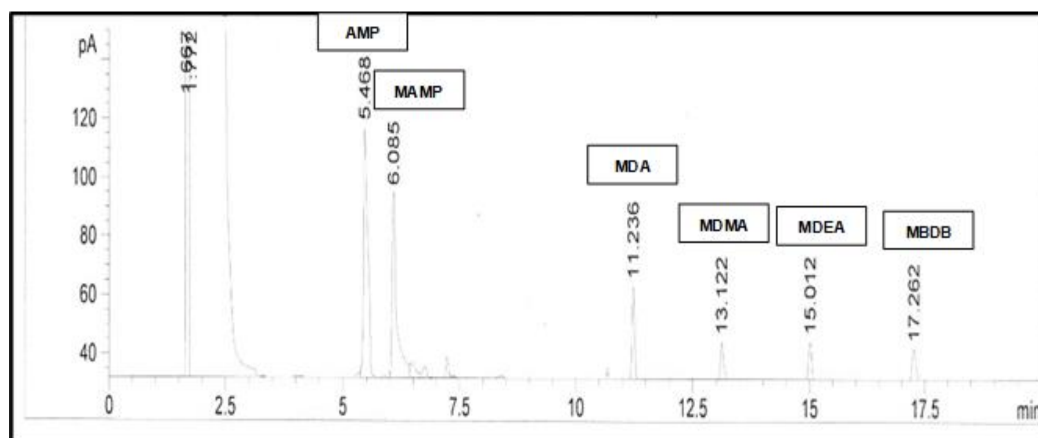

Figure 9. Chromatogram of Standard Mixture of ATS Drugs.

Table 1. Retention Time and Molecular Weight for Each Analyte in Mix Standard of ATS Drugs.

Analytes	Retention time, RT (min)	Molecular weight (amu)
AMP	5.468	374.22
MAMP	6.085	149.23
MDA	11.236	72.06
MDMA	13.122	193.25
MDEA	15.012	119.16
MBDB	17.262	207.27

The retention time of each ATS analyte obtained from standard mixture solution was recorded to construct the calibration curve of peak area of blank samples (x-axis) versus concentration of ATS drugs in ppm (y-axis). Each sample was injected in triplicate to get the average peak area. The calibration curve then was used to quantify the amount of ATS drugs present in drug abuser's nail samples.

RESULTS AND DISCUSSION

GC-FID Analysis of Individual Standard of ATS Drugs

The individual standard of ATS drugs was injected in triplicate into the GC-FID to determine the average retention time of each analyte. There were six retention times recorded for six different analytes of standard ATS drugs. The retention times for individual standard were 5.466 min (AMP), 6.074 min (MAMP), 11.219 min (MDA), 13.060 min (MDMA), 14.972 min (MDEA) and 17.194 min (MBDB) as shown in Figure 3 to 8.

GC-FID Analysis of Mix Standard of ATS Drugs

Standard mixture of ATS drugs was established by mixing all six individuals standards of ATS drugs into one vial. The chromatogram of standard mixture of ATS drugs is shown in Figure 9 and Table 1 shows the retention time for six standard ATS drugs. The retention time of each ATS drug in standard

mixture solution is slightly different from the individual retention time due to differences in matrix concentration. Chromatogram of ATS drugs analysed in this study show a typical chromatogram separation pattern type of ATS drugs in nail samples. The retention time was used to analyse the ATS drugs content in drug abuser's nail samples. The first analyte eluted was standard AMP which was at 5.468 min, followed by standard MAMP at 6.085 min. At retention time 11.236 min, standard MDA was eluted, MDMA appeared at 13.122 min, followed by MDEA at 15.012 min. Finally, standard MBDB was the last analyte to be eluted with a retention time of 17.262 min.

Calibration Curve of ATS Drugs in Blank Nail Samples

The calibration curve of peak area of blank nail or drug-free samples (x-axis) versus concentration of ATS drugs in ppm (y-axis) was plotted to quantify the amount present in nail samples of drug abusers. Five-point calibration curves were constructed for six different analytes of ATS drugs. Five levels of 2, 5, 10, 30 and 50 ppm concentrations were established to construct a calibration curve with three replicates at each concentration. The calibration curve of standard AMP showed a good linear regression with the equation of y = 0.5324x + 9.7152. Good linearity with correlation coefficient (r) of 0.9553 was obtained from the calibration curve as indicated in Figure 10. Figure 11 reveals the calibration curve of standard MAMP with linear regression and r value of y = 0.2832x + 11.2380 and 0.9611 respectively, while Figure 12 showed the linear regression of MDA is y = 0.047x + 3.1901 and the r value is 0.9556. Calibration curve of standard MDMA (Figure 13) also established a good linearity regression and r value of y = 0.0497x + 1.0990 and 0.9870, respectively. The linearity regression and r value of standard MDEA in Figure 14 was y = 0.1146x + 4.2359 and 0.9799, respectively. Finally, Figure 15 indicates that calibration curve of standard MBDB also showed good linearity regression with equation of y = 1.7607x - 0.7221 and r value of 0.9869.

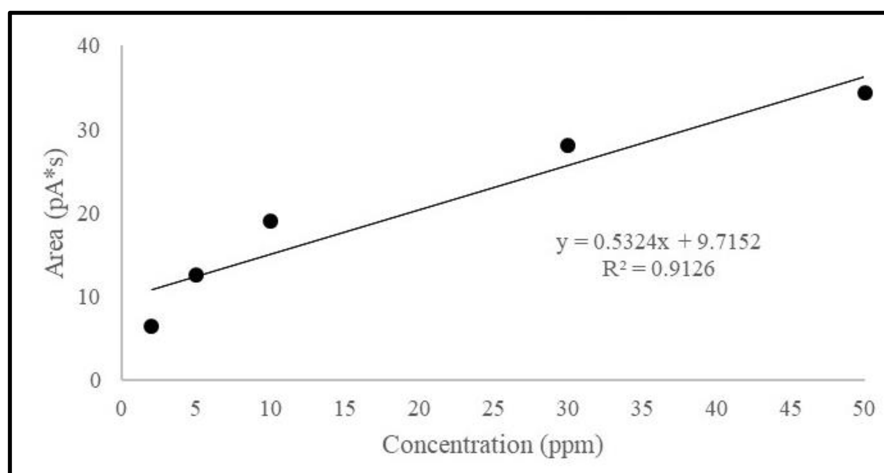

Figure 10. Calibration Curve of Standard AMP in Blank Nail Samples.

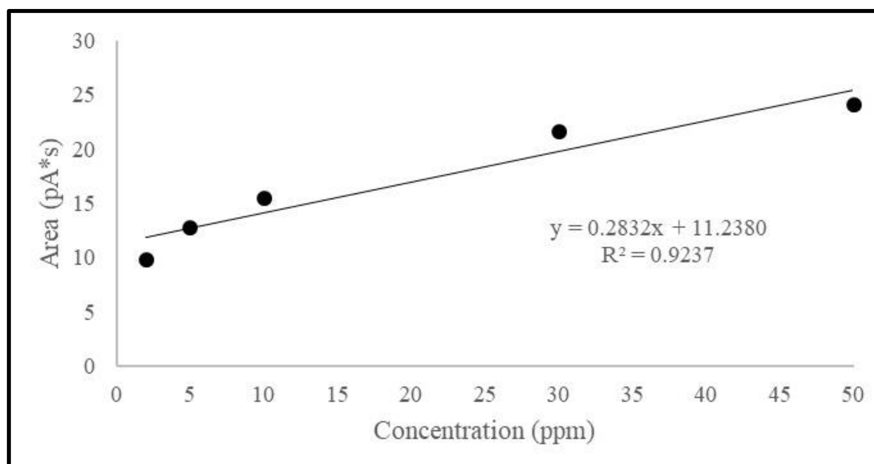

Figure 11. Calibration Curve of Standard MAMP in Blank Nail Samples.

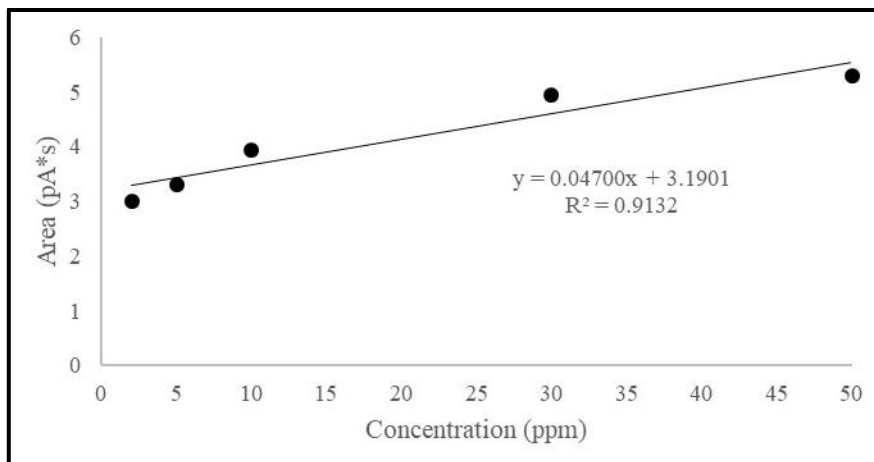

Figure 12. Calibration Curve of Standard MDA in Blank Nail Samples.

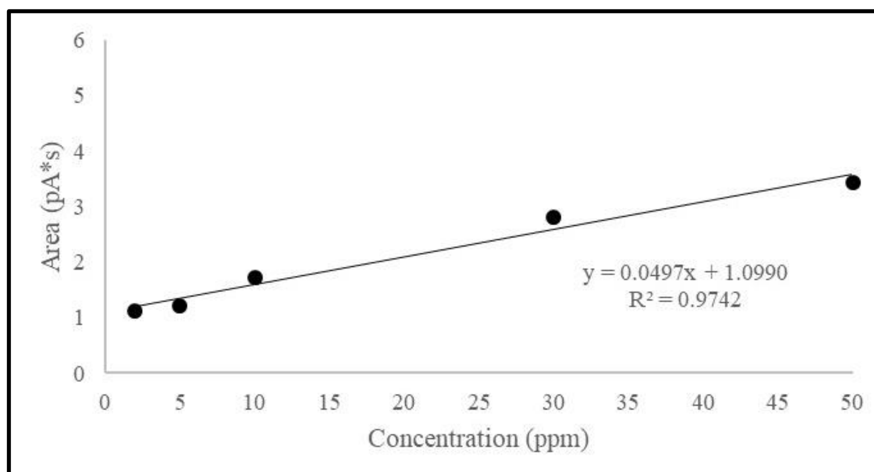

Figure 13. Calibration Curve of Standard MDMA in Blank Nail Samples.

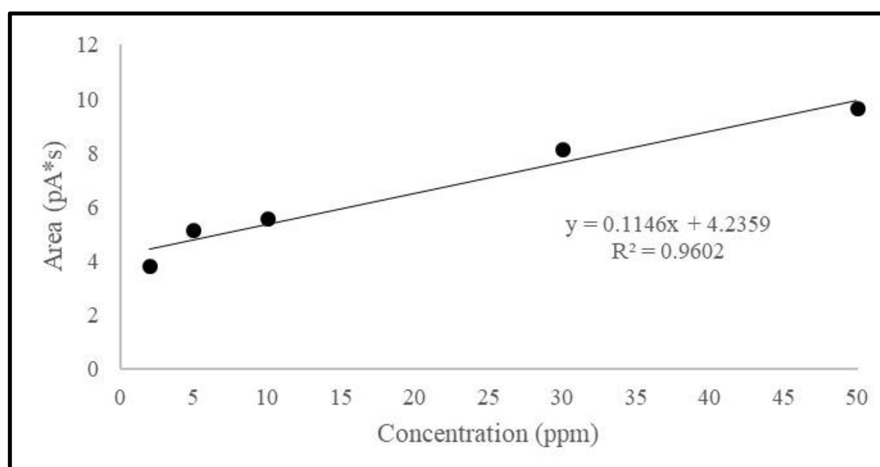

Figure 14. Calibration Curve of Standard MDEA in Blank Nail Samples.

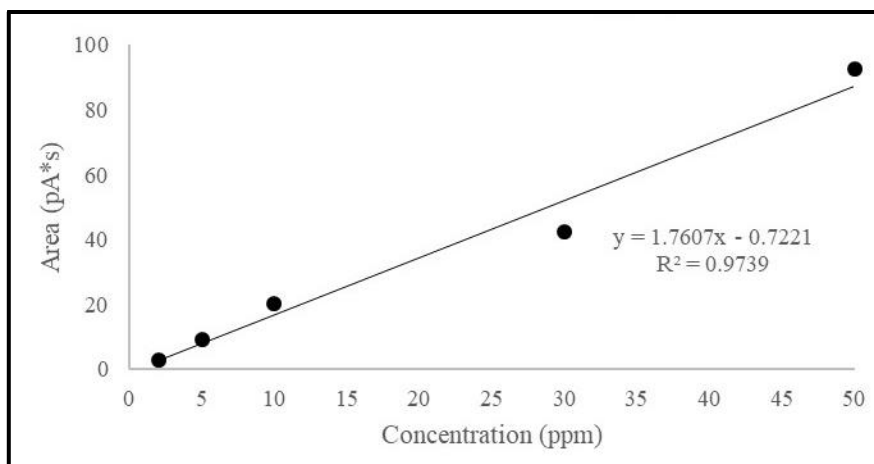

Figure 15. Calibration Curve of Standard MBDB in Blank Nail Samples.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) is the lowest concentration of the analyte that can be distinguished from the background noise while Limit of quantification (LOQ) is the lowest concentration of an analyte in the sample that can be determined with acceptable precision and accuracy under stated conditions of test. The LOD and LOQ for drug analysis in nail samples were determined and tabulated in Table 2. Table 2 indicates MDMA showed the lowest LOD and LOQ which were 0.0823 ppm and 2.4943 ppm respectively. While AMP shows the highest LOD and LOQ with the concentration of 0.1566 ppm and 4.7465 ppm respectively. The sensitivity of this method was relatively lower compared to another study done by [13] which showed LOD of 5.0 ppm while [14] indicated LOD of 10 ppm for MDA.

Table 2. Calibration Curve Details, LOD and LOQ for Nail Analysis.

Analyte	Slope	y-intercept	Coefficient of determination (R^2)	LOD (ppm)	LOQ (ppm)
AMP	0.5324	9.7152	0.9126	0.1566	4.7465
MAMP	0.2832	11.2380	0.9237	0.1454	4.4070
MDA	0.0470	3.1901	0.9132	0.1560	4.7275
MDMA	0.0497	1.0990	0.9742	0.0823	2.4943
MDEA	0.1146	4.2359	0.9602	0.1031	3.1234
MBDB	1.7607	0.7221	0.9739	0.0829	2.5129

Table 3. Number of ATS Drug Detected and Not Detected in Nail.

ATS Drug	AMP	MAMP	MDA	MDMA	MDEA	MBDB
Detected	76	28	83	18	46	44
Not Detected	12	60	5	70	42	44

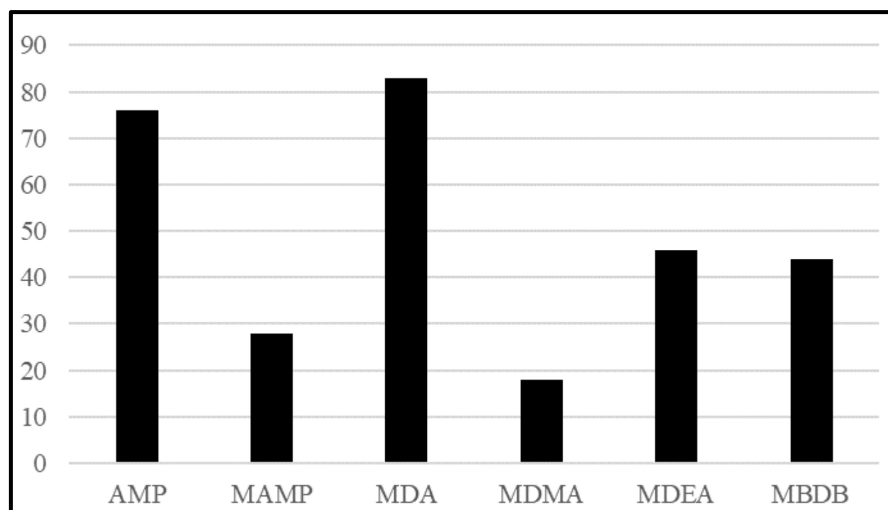

Figure 16. Number of ATS Drug Detected in Nail Samples.

Table 4. Range Concentration of ATS Drug Contents in Nail Samples.

ATS drug	Range concentration in nail samples (ppm)
AMP	0.6871 – 14.6647
MAMP	0.3056 – 35.5641
MDA	0.0969 – 16.3393
MDMA	3.6445 – 58.6617
MDEA	1.0448 – 58.3390
MBDB	2.1563 – 9.6628

ATS Drug Contents in Nail Samples of Drug Abusers

The analytical data obtained from nail samples of 88 subjects are recorded in Table 3 as well as in Figure 16. This present study indicates that the highest content on ATS drug detected in nail samples was MDA analyte which was detected in 83 out of 88 subjects. The second most detected ATS drug was AMP analyte, followed by MDEA, MBDB and MAMP. The least amount of ATS drug detected in nail samples was from MDMA analyte only in 18 out of 88 subjects. This is because not all the individuals consume the same drug with the same amount. Some of the drug with low concentration will not be detected using GC-FID.

The concentration of ATS drug in nail samples was calculated by using the calibration curve slope equation. Table 4 details the range concentration of each analyte of ATS drug quantified in nail samples of drug abusers. The range concentration for AMP analyte was between 0.6871 – 14.6647 ppm while for MAMP analyte was in the range 0.3056 – 35.5641 ppm. MDA was in the range concentration of 0.0969 – 16.3393 ppm. As for MDMA and MDEA analytes, the range of concentrations were between 3.6445 – 58.6617 ppm and 1.0448 – 58.3390 ppm respectively. While MBDB analyte was quantified between the range concentration of 2.1563 – 9.6628 ppm.

Previous study done by [6] indicated that the amount of ATS drug in nail samples was in the range of 0.063 – 2.090 ppm, while in this present study the data on range concentration is between 0.0969 – 58.3390 ppm was slightly higher. The factors that probably affect the interpretation of the present result might be the differences in sample numbers and populations. The 88 nail samples investigated in this present study were much wider compared to only 7 nail samples used in study done by [6] and the usage of different instruments contributed to the differences in the results. Different instruments used for drug analysis in nail samples can indeed produce varying results due to differences in sensitivity and selectivity. Instruments with higher sensitivity are capable of detecting lower concentrations of substance, potentially leading to different outcome compared to less sensitive instruments.

Therefore, continuous research should be conducted using a variety of analytical techniques to ensure comprehensive analysis such as liquid chromatography-mass spectrometry (LC-MS) [15,16], gas chromatography-mass spectrometry (GC-MS) [6], gas chromatography with tandem mass

spectrometry (GC- MS-MS) [14], ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) [17], liquid chromatography with tandem mass spectrometry (LC-MS-MS) [18]. These instruments offer different levels of sensitivity and selectivity, making them suitable for analyzing drugs even at low concentrations in nails. Enhanced sample preparation techniques are also crucial to improve detection limits and ensure reliable results. By employing a combination of these advanced instruments and optimizing sample preparation, researchers can achieve better outcomes in drug analysis from nail samples, contributing to more accurate forensic and clinical investigations.

Nail samples have many advantages compared to other biological samples like the nail specimen collection is non-invasive, making sample collection easier without causing discomfort to the individuals being tested. Moreover, nail samples utilized in drug testing can be obtained from nail clippings of both fingernails and toenails [1]. Additionally, artificial manicure applications, such as gels, polish, acrylics, and nail extensions, must be eliminated from the nails before testing to qualify the samples to be employed [19]. Preserving nail samples at room temperature is critical for drug analysis [20] because drug compounds in nail samples does not substantially deteriorate when kept at room temperature for extended period, guaranteeing the accuracy of the drug analysis results. Keeping the nail samples dry helps to prevent the growth of fungi or other microorganisms that could potentially contaminate the sample and interfere with the drug analysis [21].

CONCLUSION

Nails have the potential to serve as an alternative to conventional matrices such as blood and urine. The proposed Gas Chromatography-Flame Ionization Detector (GC-FID) method for the ATS drugs determination from nail samples of drug abusers were successfully analysed and validated with the assessment of the following parameters: linearity of calibration curve, limit of detection (LOD) and limit of quantification (LOQ). The proposed method was applied for ATS drugs analysis in nail samples of 88 drug abusers in Malaysia. The results showed that the proposed methods can be used for ATS drug determination in human nail samples. There is no doubt that nail analysis can provide valuable and valid information on previous drug use in the fields of forensic toxicology, especially at the drug rehabilitation and treatment centres. Furthermore, this project will be the first study conducted using drug abusers' nail samples as part of an ongoing effort to combat drug abuse in Malaysia.

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