Chemical profiling of the street cocktail drug ‘nyaope’ in South Africa using GC–MS III: A validated method for drug extraction and GC – MS analysis for the identification and comparison of ‘nyaope’ samples.

P.M. Mthembia,b,\*, E.M. Mwenesongolea,c, M.D. Coled

a Department of Genetics, University of the Free State, P.O. Box 339, Bloemfontein, 9300, South Africa

b South African Police Services Forensic Science Laboratory, Chemistry Section, Private Bag X620, Pretoria, 0001, South Africa

c Department of Chemical and Forensic Sciences, Botswana International University of Science and Technology, Palapye, Botswana

d Faculty of Science & Engineering, Anglia Ruskin University, Cambridge, United Kingdom

\* Corresponding author.

\*Email: [mthembim@saps.gov.za](mailto:mthembim@saps.gov.za) Tel.: +27 12 401 3319

**Abstract**

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Nyaope, a Tswana word for a mixture, or “mish-mash” describes a drug cocktail comprising heroin, cannabis, in addition, on occasion, to other controlled substances and to warfarin. It is highly addictive with extremely unpleasant side effects caused by withdrawal from the drug. It is a problem drug especially in the townships in South Africa. However, it’s prevalence in neighbouring Southern African states or further afield is not yet known. There is currently no validated method for the analysis and comparison of nyaope. This paper describes a validated method for the gas chromatography-mass spectrometry analysis of nyaope so that within batch and between batch comparisons of nyaope can successfully be made for the first time. The validated method managed an accuracy within the range 80% - 120%, the precision was less than 20% for all analytes and managed linearity with R2 ≥ 0.99. The detection limits for diamorphine, efavirenz, nevirapine and Δ9-tetrahydrocannabinol were 14.2, 18.6, 18.7 and 9.94 pg on column, respectively and the limit of quantitation were 43.1, 56.3, 56.6 and 30.1 pg on column, respectively.The simulated and case work samples were successfully discriminated into original batches using the identified nyaope components, the unsupervised chemometric methods principal component analysis and hierarchical clustering, as well as chromatographic profiles.

**Significance:**

This allows for data exchange between law enforcement agencies in South Africa and, provided the appropriate quality control measures are in place, between South Africa, neighbouring states and countries further afield.

Additionally, public health measures can be put in place now that it is possible to use a validated methods to determine the contents of nyaope.

**1. Introduction**

Despite being reported in the early 2000’s1,2 there remains a dearth of analytical chemistry and forensic science literature on the drug ‘nyaope’. Nyaope, the name of which is derived from the Tswana word for mixture or “mish-mash”2 is reported to be a mixture of powdered heroin, herbal cannabis and prescription anti-retrovirals. It has also been reported to contain methamphetamine and warfarin although some of these claims are disputed. An unpublished study reports a much wider range of drugs found in nyaope samples3. In parts of South Africa it is known as “Woonga” (spelled “Wunga” in Zulu2). It has no single composition and mixtures follow trends4 although the predominant drug by mass is cannabis, followed by heroin. It is typically smoked after being mixed with tobacco.

Nyaope contains substances that are controlled by the South African Drug and Drug Trafficking Act which have been controlled from as far back as 1992. The name nyaope itself is a street name and therefore cannot be listed in the Act. It is inexpensive with a single dose costing R20.00 – R30.00 (circa US$ 2)4 and is widely used by the Coloured and Black communities in the townships, by addicts who are sometimes as young as 12 or 13 years old4. Although the drug is widely reported in South Africa, nothing is known of it’s distribution in neighbouring Mozambique, Zimbabwe, Botswana and Namibia, although there are anecdotal reports that it may have been reported in Angola5. Due to the severe form that the addiction to nyaope can take and the intensity and nature of the withdrawal symptoms, the social cost of this drug is enormous6 and addicts, including children, adopt chaotic lifestyles, dropping out of school and engaging in criminal activity including theft and prostitution to fund their drug taking4.

The health risks associated with using Nyaope are not well investigated but reports include restriction of growth and development of neo-nates7 and infective endocarditis8 which had been misdiagnosed elsewhere as pulmonary tuberculosis or pneumonia9. The vast majority of users are HIV positive8. Other problems associated with nyaope use include damaged and infected veins, damaged heart valves, tissue infections, liver failure, kidney disease and lung problems10. It therefore represents a significant public health risk.

In South Africa, at present, the Criminal Law punishes drug related offences by a fine or imprisonment. The scale of the nyaope problem is difficult to quantify. At the present time there are few representative surveys on drug use and abuse in South Africa. A recent policy brief summarises the rise in trafficking and abuse of heroin in South Africa11. However, there is a growing thesis that to tackle the drug problem in South Africa, including that associated with nyaope, a number of approaches need to be taken4. This includes (i) punishment of those manufacturing, trafficking and distributing the drug. Additionally, a forensic care process is proposed towards addressing the nyaope problem4 where (ii) drug users are properly catered for by rehabilitation schemes, (iii) the social circumstances of the drug users are changed and (iv) implementation of a Public Health Awareness Scheme. Of course, this approach could be applied to many drug problems across the globe.

In order that (i) and (iv) can be achieved and supported it is necessary to prove that the drug is identified as nyaope and then to identify and quantify the drug contents. In order to achieve this a validated analytical method for the analysis of the contents of and comparison of nyaope is required – to date no such method has been developed. This is further exacerbated by the fact that the usual methods for cannabis analysis cause heroin to break down and conversely those used for heroin cause cannabis to break down. Our previous studies have identified that the forensic science question needs to be identified before police operations involving nyaope commence12, how nyaope should be stored post seizure12, and how the drug should be prepared prior to analysis by gas chromatography – mass spectrometry (GC – MS)13. In this paper we present, for the first time, a validated analytical method for the identification of nyaope and quantification of the drug components which addresses the analysis of heroin and cannabis when contained in the same drug sample. We further demonstrate that with correct collection, storage and sample preparation it is possible to compare nyaope samples, identifying those which are related and discriminating between those that are not, using, for the first time, chemometric clustering techniques. How this method will assist law enforcement and public health officials in South Africa, and further afield, is also discussed.

**2. Materials and methods.**

**2.1 Chemicals**

Tertiary butyl alcohol (t-BuOH) was purchased from Merck, tetracosane (TC)-99% was purchased from Sigma-Aldrich, isopropanol (i-PrOH)-AR grade was purchased from Associated Chemical Enterprise. Representative compounds, identified in casework samples of nyaope by the South African Police Service (SAPS), were used to validate the GC – MS method. Certified reference standards of Δ9-Tetrahydrocannabinol (Δ9-THC) and diamorphine (both 1 mg/mL) were purchased from Ceriliant-Sigma Aldrich. Caffeine and phenacetin were purchased from the US-Pharmacopeia as an USP powder reference standards while efavirenz and nevirapine were purchased from WHO International Chemical Reference Substances as ICRS powder reference substances.

**2.2 Preparation of internal standards**

The internal standard solution (ISTD), tetracosane (C24), was prepared at a final concentration of 0.02 mg/mL in tertiary butyl alcohol. Tertiary butyl alcohol has previously been shown to be the solvent of choice for presenting nyaope extracts to the GC-MS13. The internal standard solution was used to dilute the certified reference standards, and other samples, before GC-MS analysis.

**2.3 Preparation of calibration standards**

Stock solutions (1 mL at 1 mg/mL) of Δ9-THC in methanol and diamorphine in acetonitrile were placed in an amber GC-MS vial, evaporated to dryness under nitrogen and then re-dissolved in 1 mL of the ISTD to give stock solutions at 1 mg/mL. Phenacetin, caffeine, efavirenz and nevirapine were dissolved at concentrations of 1.03, 1.00, 0.998 and 1.05 mg/mL, respectively in the ISTD solution. From these, 14 standards in the concentration range 0 – 1.0 mg/mL, at notional concentrations of 0, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/mL were prepared.

**2.4 Instrumentation**

GC-MS analysis was carried out using an Agilent Technologies system (Chemetrix, RSA) consisting of a gas chromatograph (GC), Agilent 7890A, and mass selective (MS) detector (Agilent 5975 CVL MSD) with an auto sampler 7683 B series (1 µL injection). Chromatographic separation was performed using a computer controlled auto sampler with a fused-silica capillary column HP-5MS (30 m x 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Folsom, CA, USA). Splitless injection was used at 280 oC. The GC oven temperature programme consisted of an initial temperature of 100 oC for 0.4 min, raised to 290 oC at a rate of 60 oC/min, held at 290 oC for 2.4 min then raised to reach 316 oC at 60 oC/min and held for 3 min. The total run time was 9.40 minutes. High-purity helium (99.9995%) was used as the carrier gas, at flow rate of 1 mL/min. The MS parameters used was performed as follows: the interface temperature was 280 oC, the inlet temperature 250 oC, the ion-source temperature 230 oC, electron ionization (EI) was achieved with a source voltage of 70 eV and the mass spectrometer (quadrupole) was used in scan mode. The spectra were recorded in the scan range (m/z) 35 to 550 amu, at a scan rate of 1 scan/sec.

**2.5 Method validation.**

The method was validated by determining the precision of the retention index of each compound, the linearity of detector response, the limit of detection (LOD) and of quantitation (LOQ), repeatability and the reproducibility of the measurements14,15. The precision of the retention index was obtained for each compound (phenacetin, caffeine, efavirenz, nevirapine, Δ9-THC and diamorphine) by calculating the mean, standard deviation and relative standard deviation of the retention index, relative to tetracosane, for 10 replicate analyses.

Linearity of the detector response to the exemplar drugs was determined by preparation of calibration curves for samples in the concentration range 0.00 – 1.00 mg/mL. The regression equations for detector response relative to the internal standard, the value of R2 and residual plot analysis was used to confirm linearity of detector response.

Limit of detection (LOD) and limit of quantitation (LOQ) were determined using the calibration curve slope using reference sample solutions with concentrations in the vicinity of the LOD16, namely 0.000, 0.001, 0.0025, 0.005, 0.01 and 0.05 mg/mL and the equations:

Where σ = standard error of the measured response

S = slope of the regression line

CIS= concentration of the internal standard = 0.018 mg/mL

To measure the accuracy of the method (closeness to true concentration), 10 replicate measurements of standards of known concentrations were made, the experimental concentrations determined and the equation

applied.

Precision is a measure of the closeness of the analytical results obtained from a series of replicate measurements of the same measure under the conditions of the method. Intra-assay precision (repeatability) and inter-assay precision (reproducibility) were assessed using drug standard mixtures of phenacetin, caffeine, efavirenz, nevirapine, Δ9-THC and diamorphine at three concentration levels (0.01, 0.1 and 1.00 mg/mL). Repeatability was assessed by making ten replicate analyses of the drug standards at three concentration levels and calculating the mean, standard deviation and relative standard deviation of the relative response to the internal standard.

Reproducibility was assessed by making five replicate analyses of the drug standards over five consecutive days at the three concentration levels and calculating both within group (W) and between group (B) precision using one-way ANOVA (Group = Day) the equations17:

If MSB < MSW, set %RSDB = 0

Where,

*X* = Grand mean of all observations

*n* = Number of observations in group

*MSW* = mean of squares within group

*MSB* = mean of squares between groups

**2.6 Nyaope sample profiling and comparison**

In order to investigate the validity of the method for nyaope sample identification and comparison, both simulated samples and casework samples of nyaope were investigated.

Street cannabis and heroin samples seized by the SAPS were used to prepare simulated nyaope samples. Three blind simulated samples were prepared by mixing a heroin street sample, a cannabis street sample, efavirenz tablets and nevirapine tablets, in different combinations and proportions, to mimic as closely as possible a typical street nyaope sample. The three mixtures were homogenised by grinding using a mortar and pestle. The samples were then further divided into six blind sub-samples each to give a total of 18 samples marked S1 – S18. Homogenised samples which had mass ranging between 12 and 14 mg were mixed with 3 mL of the internal standard solution (0.02 mg/mL tetracosane in tertiary butyl alcohol) in a 20 mL head space vial. The mixture was sonicated for 15 minutes13,18,19, filtered into amber GC-MS vials and analysed in triplicate. Each of the extracts of the simulated samples S1 – S18 was analysed at 0, 24, 48 and 72 hours in order to confirm the stability of the extract once prepared13. Additionally, chromatograms of members of each of the three groups were compared at the same time intervals to determine whether samples from the same parent batch could be discriminated after these time intervals. Finally chromatograms from one member of each of the three groups were compared at these time intervals to demonstrate whether or not it is possible to discriminate between groups.

Five casework samples of nyaope were ground into a fine powder using a mortar and pestle. Sub samples (circa 12 – 14 mg) of these street samples were placed in a 20 mL vial and extracted with 3 mL of the internal standard solution prior to analysis. Each of the casework samples were analysed in triplicate by GC-MS at t=0 after extraction. The chromatograms were compared to determine whether it is possible to discriminate between street samples. Each extract was then analysed after 24, 48 and 72 hours to demonstrate stability of extracts of such samples. Semi quantitation was conducted on caffeine, diamorphine and Δ9-THC for each of the five casework samples.

Two unsupervised chemometric methods, agglomerative hierarchical analysis (HCA) and principal component analysis (PCA), was performed on both the blind simulated and case work nyaope samples using the XLSTAT statistical and data analysis solution2019 version . The HCA and PCA analysis was conducted for the samples analysed at 0, 24, 48 and 72 hours.

**3. Results and discussion**

**3.1 Compound identification - stability of retention indices.**

It is important that retention indices are stable for a given analytical method if drug comparisons are to be made. The retention indices were evaluated over five days for each component. The stability data for representative components of nyaope are given in Table 1. The ANOVA test (single factor) demonstrated (Fcalc = 0.029, Fcrit = 4.965) that there was no significant difference between days and that the retention indices were stable.

**Table 1: Stability data for retention indices of representative components of nyaope measured by GC – MS.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **Average** | **SD** | **%RSD** |
| **Phenacetin** | 0.666 | 0.000148 | 0.022 |
| **Caffeine** | 0.835 | 0.000169 | 0.020 |
| **Efavirenz** | 0.835 | 0.000169 | 0.020 |
| **Tetracosane** | 1.000 | 0.000000 | 0.000 |
| **Nevirapine** | 1.096 | 0.000209 | 0.019 |
| **Δ9-THC** | 1.178 | 0.000137 | 0.012 |
| **Diamorphine** | 1.361 | 0.000332 | 0.024 |

The relative standard deviation (Table 1) for the retention indices of these compounds caffeine, diamorphine, efavirenz, nevirapine phenacetin and ∆9-THC were all below 0.025% further illustrating the stability of this parameter. Identification of components of nyaope can therefore be made on the basis of retention index and the mass spectrum of each separated compound.

**3.2 Linearity of detector response.**

The detector response to standard compounds was linear over the concentration range investigated.The regression equations and R2 values are given for each of the components of nyaope measured in Table 2. All of the R2 values are above 0.99. This and analysis of residuals demonstrate that the detector response to these drugs is linear.

**Table 2: Regression equation, R2, LOD and LOQ values for the exemplar drugs in nyaope**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Drug** | **Regression equation** | **R2 value** | **LOD(pg)** | **LOQ (pg)** |
| **Caffeine** |  | 0.9975 | 21.0 | 63.6 |
| **Diamorphine** |  | 0.9981 | 14.2 | 43.1 |
| **Efavirenz** |  | 0.9995 | 18.6 | 56.3 |
| **Nevirapine** |  | 0.9987 | 18.7 | 56.6 |
| **Phenacetin** |  | 0.9970 | 39.1 | 118 |
| **Δ9-THC** |  | 0.9951 | 9.94 | 30.1 |

**3.3 Limits of detection and quantitation**

The detection limits and the limits of quantitation using this analytical method were determined as mass of the free drug on column and are given in Table 2. The detection limits varied between 9.94 – 39.1 pg on column and the limit of quantitation between 30.1 – 118 pg on column. The method is sufficiently sensitive to both detect these drugs in nyaope and quantitate them in a street sample.

**3.4 Accuracy**

Ten replicate analyses were performed for each drug at each of three known concentrations and the percentage accuracy of the measurement determined. Accuracy figures were found to lie between 82% and 112%. These lie between the limits of 80% and 120% and are therefore considered accurate, further validating the method21-23.

**3.5 Repeatability and reproducibility**

Repeatability was assessed through ten replicate analyses of the drugs at each of three different concentrations. The relative standard deviation of all analyses were found to lie below or at 15% (with nevirapine just above 15%, 0.01 mg/mL, RSD = 15.78%) demonstrating that the method is repeatable24,25.

Reproducibility was assessed through five replicate analyses of the drug standards. The relative standard deviation for both within-group and between group precision were found to lie below 15% (with caffeine and nevirapine just above 15%, 0.01 mg/mL, RSD = 15.01% and 15.19% respectively) demonstrating that the method is reproducible15,21,22.

On the basis of the data described above, and the recommendation of the UNODC that cannabis and heroin can be analysed by GC-MS, without derivatisation19,26, the method was deemed suitable for the analysis of the principal drug types in nyaope.

**3.6 Nyaope sample profiling and comparison.**

Each of the simulated samples was analysed after 0, 24, 48 and 72 hours. The average peak area ratio (PAR) was determined for each of the simulated samples. The ANOVA statistical analysis of one of the samples gave Fcalc = 0.0106 < Fcrit = 2.798 demonstrating that there were no significant difference between the PAR over the 72 hours. Retention time data is provided in Table 3. Pooled average response ratios for each of the batches were determined by averaging the PAR at t = 0, 24, 48 and 72 hours. ANOVA statistical analysis sample of the samples from one of the batches using the F-test (single factor) gave Fcalc = 0.0268 < Fcrit = 2.342 demonstrating that there were no significant difference amongst the peak area ratios for the samples belonging to the same batch over the 72 hours autosampler storage.

**Table 3: Retention time and relative retention time for the compounds identified in the analysis of simulated and case work nyaope samples. Identifications were made on the basis of retention indices and mass spectra of standards and casework samples (data available from corresponding author)**

|  |  |  |
| --- | --- | --- |
|  | **Retention time** | **Relative retention time** |
| Nicotine | 2.411 | 0.444 |
| Caryophyllene | 2.582 | 0.476 |
| Bulnesol | 3.098 | 0.571 |
| Phenacetin | 3.133 | 0.577 |
| Acetaminophen | 3.194 | 0.589 |
| Caffeine | 3.535 | 0.651 |
| Efavirenz | 4.545 | 0.837 |
| Methaqualone | 4.748 | 0.875 |
| Cocaine | 4.891 | 0.901 |
| Tetrahydrocannabivarin | 5.241 | 0.966 |
| Tetracosane | 5.427 | 1.00 |
| Cannabivarin | 5.573 | 1.027 |
| Cannabichromene | 5.731 | 1.056 |
| Cannabidiol | 5.754 | 1.060 |
| Nevirapine | 5.949 | 1.096 |
| Δ9-THC | 6.234 | 1.149 |
| Cannabigerol | 6.414 | 1.182 |
| Acetylcodeine | 6.501 | 1.198 |
| Cannabinol | 6.518 | 1.201 |
| 6-Monoacetylmorphine | 6.571 | 1.211 |
| Diamorphine | 7.020 | 1.293 |
| nonacosane | 7.517 | 1.385 |
| Vitamin E | 8.686 | 1.601 |

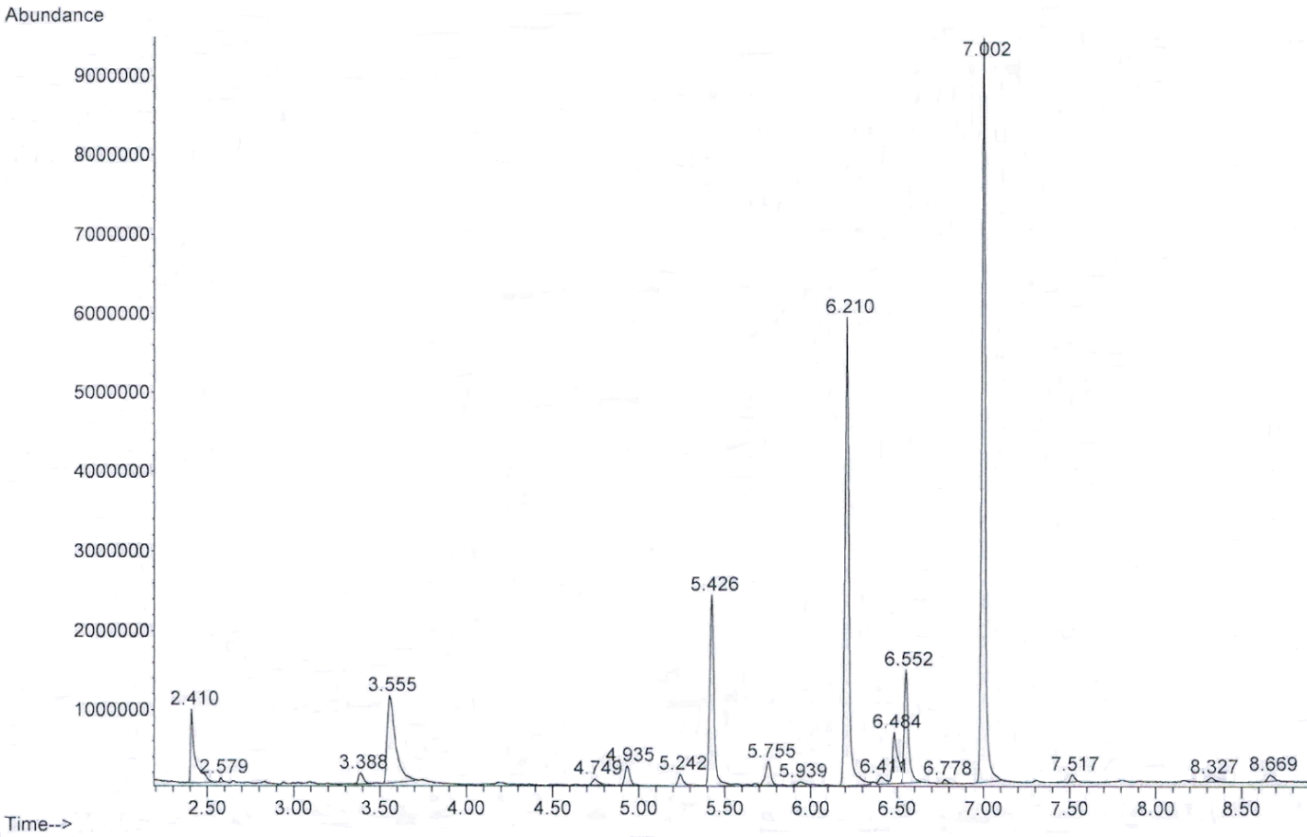
From the data obtained it can be demonstrated that each compound of interest can be identified and that their relative proportions do not change over a 72 hour period once extracted from nyaope, into tertiary butyl alcohol, confirming previous work13. When each of the six samples for the three different simulated samples were analysed it was found that the PAR, relating to each batch, could not be separated. It is therefore now possible to relate samples of nyaope to each other when they have come from a once larger parent batch as demonstrated by the PAR.

When separated by GC-MS it was possible to differentiate between the three batches of simulated nyaope (Figure 1) on the basis of chromatographic profile. This method of extraction and analysis allows, for the first time, analysis and comparison of nyaope samples by a forensic science laboratory.



**Figure 1: Exemplar chromatograms obtained by GC – MS analysis of the three batches of simulated nyaope showing that it is possible to differentiate different batches of the drug where identifications were (2.582) caryophyllene, (3.135) phenacetin, (3.536) caffeine, (4.543) efavirenz, (5.242) tetrahydrocannabivarin, (5.429) tetracosane IS, (5.733) cannabichromene, (5.948) nevirapine, (6.234) Δ9-THC, (6.411) cannabigerol, (6.520) cannabinol, (7.020) diamorphine, (7.520) nonacosane, (9.454) unknown. In the first chromatogram.**

To demonstrate that the method could be applied to casework samples, five samples of nyaope (denoted as 2514202B, 2520902B, 3400002B, 37959902B and 50390902B) were analysed. Figure 2 is a typical total ion chromatograph for one of the casework samples (37959902B). The components identified in the other casework samples are summarised in Table 4. The components, cocaine, diamorphine, methaqualone and Δ9-THC were identified on the basis of their retention time and mass spectral data using certified reference material. Caffeine was identified on the basis of their retention time and mass spectral data using USP reference standards.Acetaminophen, acetylcodeine, bulnesol, cannabichromene, cannabicoumaronone, cannabidiol, cannabigerol, cannabinol, cannabivarin, codeine, caryophyllene, 6-monoacetylmorphine, nicotine, nonacosane, tetrahydrocannabivarin and vitamin E were identified by comparing the experimental mass spectral data with the NIST mass spectral library NIST 14.From the components identified it can be seen that it is possible to discriminate between street samples of nyaope and that the method described can be applied to forensic casework.



**Figure 2: A typical total ion chromatograph for one of the casework samples, 37959902B where (2.410) nicotine, (2.579) caryophyllene, (3.388) unknown, (3.555) caffeine, (4.749) methaqualone, (4.935) unknown, (5.242) tetrahydrocannabivarin, (5.426) tetracosane IS, (5.765) cannabidiol, (5.939) codeine, (6.210) Δ9-THC, (6.411) cannabigerol, (6.484) acetylcodeine, (6.552) 6-monoacetylmorphine, (7.002) diamorphine, (7.517) nonacosane, (8.327) unknown, (8.669) vitamin E.**

**Table 4: Components identified in the five casework samples.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample Component** | **2514202B** | **2520902B** | **3400002B** | **37959902B** | **50390902B** |
| **bulnesol** | × | √ | × | × | × |
| **acetaminophen** | √ | × | × | × | × |
| **acetylcodeine** | × | × | √ | √ | × |
| **caffeine,** | √ | √ | √ | √ | √ |
| **cannabichromene** | √ | √ | √ | × | × |
| **cannabicoumaronone** | × | √ | √ | × | × |
| **cannabidiol** | × | × | × | √ | × |
| **cannabigerol** | √ | √ | × | √ | × |
| **cannabinol** | √ | √ | × | × | × |
| **cannabivarin** | × | √ | × | × | × |
| **cocaine** | × | × | √ | × | × |
| **codeine** | × | × | × | √ | × |
| **diamorphine** | √ | × | √ | √ | √ |
| **methaqualone** | × | √ | × | √ | √ |
| **6-monoacetylmorphine** | √ | × | √ | √ | √ |
| **nicotine** | × | √ | × | √ | √ |
| **nonacosane** | √ | √ | × | √ | × |
| **tetrahydrocannabivarin** | √ | √ | √ | √ | × |
| **Δ9-THC** | √ | √ | √ | √ | √ |
| **Vitamin E** | × | × | × | √ | × |

Semi quantitation of the five casework samples is summarised in Table 5 showing concentrations of the components caffeine, diamorphine and Δ9-THC. The caffeine concentration for samples 2514202B and 2520902B was below the limit of quantitation (63 pg on column). The pooled average concentration for each time interval of the three sub-samples in a batch was used to calculate the average %RSD, shown in Table 5, in order to determine if the concentrations are significantly different. The average %RSD was found to be < 10% for all the components (caffeine, diamorphine and Δ9-THC) in the samples. This indicates that there is no significant difference between the concentrations of a particular component, over a period of 72 hours once extracted into tertiary butyl alcohol. This suggests that all the components were stable for the 72 hours of autosampler stability confirming the previous finding13.

**Table 5: Concentration of actual street nyaope samples (mg/mL x 1000)**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Caffeine** | | | |  | **Diamorphine** | | | |  | **∆9-THC** | | |  |  |
|  | **0** | **24** | **48** | **72** | **%RSD** | **0** | **24** | **48** | **72** | **%RSD** | **0** | **24** | **48** | **72** | **%RSD** |
| 2514202B | < QL | < QL | < QL | < QL | **< QL** | 0.21 | 0.20 | 0.21 | 0.21 | **4.00** | 5.48 | 4.97 | 5.21 | 5.25 | **4.00** |
| 2520902B | < QL | < QL | < QL | < QL | **< QL** | nd | nd | nd | nd | **nd** | 1.44 | 1.39 | 1.32 | 1.32 | **4.34** |
| 3400002B | 0.50 | 0.50 | 0.49 | 0.49 | **1.47** | 2.53 | 2.42 | 2.44 | 2.38 | **2.52** | 0.32 | 0.33 | 0.31 | 0.30 | **3.92** |
| 37959902B | 0.44 | 0.42 | 0.42 | 0.42 | **2.19** | 2.10 | 2.08 | 2.11 | 2.03 | **1.67** | 0.87 | 0.85 | 0.78 | 0.84 | **4.70** |
| 50390902B | 0.82 | 0.80 | 0.81 | 0.81 | **0.94** | 2.87 | 2.82 | 2.82 | 2.79 | **1.10** | 0.11 | 0.10 | 0.10 | 0.10 | **6.36** |

< QL = below quantification limit

nd = not detected

To demonstrate that the extracts of casework samples are stable for up to 72 hours after extraction, chromatographic analysis of the five casework samples was undertaken.The ANOVA statistical analysis of the PAR of one of the casework samples (37959902B) using F-test (single factor) gave, Fcalc = 0.0429 < Fcrit = 3.285 demonstrating that there were no significant difference between the PAR over the 72 hours. ANOVA statistical analysis of the pooled average response ratios gave Fcalc = 0.0429 < Fcrit = 3.285 demonstrating that there were no significant difference amongst the peak area ratios for the samples belonging to the same group over the 72 hours autosampler storage. From this data it can be concluded that, as with simulated nyaope samples, casework samples of nyaope are stable up to 72 hours after the preparation of drug extracts in tertiary butyl alcohol.

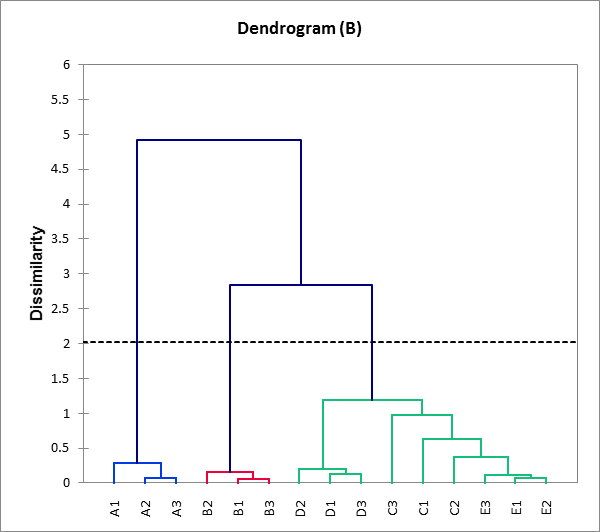
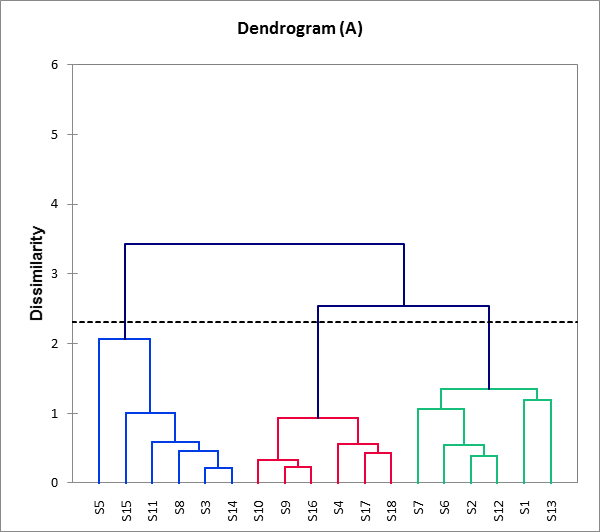
**3.7 Hierarchical Cluster Analysis**

HCA, using agglomerative clustering and unweighted linkage, was conducted on the average concentrations of CAFF, DAM, EFV, NVP PNT and Δ9-THC for each of the 18 blind simulated and the 5 case work nyaope samples. The matrices generated for the HCA clustering indicated in Table 6 for the time interval t = 72 hours, demonstrate that the HCA method was suitable in discriminating the samples into different classes27. The matrices demonstrate that there was a maximum distance between Class 2 and Class 3 for the simulated samples and between class 1 and class 3 for the case work samples. It further demonstrate that there was a minimum distance between Class 1 and Class 3 for the simulated samples and between class 2 and class 3 for the case work samples.

The results of the hierarchical clustering analysis performed on both the simulated and case work nyaope samples is also indicated in the dendrograms in Figure 3 for the time interval t = 72 hours. The results demonstrate that HCA has successfully discriminated both the simulated and case work samples into three and five different batches respectively. The HCA further demonstrated that both simulated and case work nyaope samples can still be discriminated even after 72 hours of autosampler storage.

**Table 6: Matrices showing distances between central objects at t = 72 hours for the simulated nyaope samples S2, S3 and S18 and case work samples A2, B3 and C1.**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Blind simulated samples** | | | |  | **Case work samples** | | | |
| **Class** | **1 (S2)** | **2 (S3)** | **3 (S18)** |  | **Class** | **1 (A2)** | **2 (B3)** | **3 (C1)** |
| **1 (S2)** | 0 | 2.92 | 2.34 |  | **1 (A2)** | 0 | 3.72 | 5.22 |
| **2 (S3)** | 2.92 | 0 | 3.63 |  | **2 (B3)** | 3.72 | 0 | 2.62 |
| **3 (S18)** | 2.34 | 3.63 | 0 |  | **3 (C1)** | 5.22 | 2.62 | 0 |



**Figure 3: Dendrograms of (A) blind simulated nyaope samples and (B) case work samples analysed by HCA using unweighted linkage and Euclidean distance** **for the time interval t = 72 hours.**

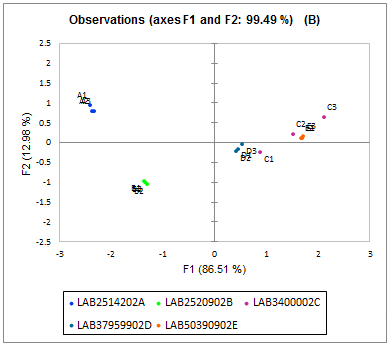
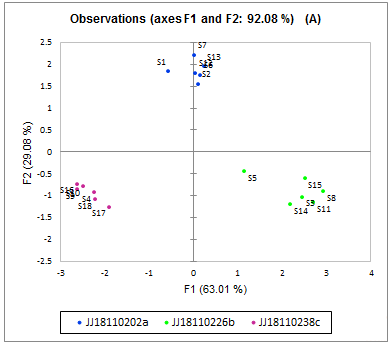
**3.8 Principal Component Analysis**

The correlation matrices for the PCA analysis of both simulated and case work nyaope samples (Table 7) indicate that all values are different from zero with a significant level α = 0.95 (two-tailed) which indicate that there is a linear correlation between the variables28. The transpose of these correlation matrices are identical to the matrices themselves. As a result, their product would yield identity matrices, which demonstrate that the PCA is orthogonal [ibid]. The principal component analysis indicates that there are three principal components, F1, F2 and F3 and one principal component F1 for the simulated and case work samples respectively, that explains the variability of the variables.

The eigenvalues of these principal components were greater than 1.00. The total variability (%) of the principal components (F1 and F2) for the simulated samples were more than the minimum 70%, while the principal component F1 alone accounted for more than 70% variability for the case work sample. This further indicates that the two principal components F1 and F2 for the simulated samples and the single component F1 for the case work sample are sufficient to explain the variability of the data set29,30. As indicated in Figure 4 the PCA has discriminated the simulated samples into three different batches and the case work samples into five different batches similar to the observation made using chromatographic profiles and HCA. The PCA further demonstrated that the simulated nyaope samples can still be discriminated even after 72 hours of autosampler storage confirming the stability of the samples once extracted into tertiary butyl alcohol for 72 hours.

**Table 7: Pearson correlation matrices for the simulated and case work samples.**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Simulated samples** | | |  |  | | | **Case work** | | | |
| Variables | CAFF | DAM | EFV | NVP | PNT | Δ9 -THC | Variables | CAFF | DAM | THC |
| CAFF | **1** | **0.884** | **0.595** | **-0.771** | **0.125** | **0.981** | CAFF | **1** | **0.984** | **-0.710** |
| DAM | **0.884** | **1** | **0.290** | **-0.471** | **0.556** | **0.858** | DAM | **0.984** | **1** | **-0.687** |
| EFV | **0.595** | **0.290** | **1** | **-0.645** | **-0.437** | **0.512** | THC | **-0.710** | **-0.687** | **1** |
| NVP | **-0.771** | **-0.471** | **-0.645** | **1** | **0.412** | **-0.801** |  |  |  |  |
| PNT | **0.125** | **0.556** | **-0.437** | **0.412** | **1** | **0.089** |  |  |  |  |
| Δ9 -THC | **0.981** | **0.858** | **0.512** | **-0.801** | **0.089** | **1** |  |  |  |  |



**Figure 4: Observation axis for (A) blind simulated nyaope and (B) case work samples analysed by PCA**

**Conclusions.**

This paper describes, for the first time, a method for the comparative analysis of nyaope. Provided that the samples are correctly seized and stored13, extracted into tertiary butyl alcohol12, and analysed within 72 hours12, this study demonstrates, for the first time, that quantitative comparisons of nyaope samples can be made. It also demonstrates, for the first time, that clustering techniques can be successfully applied to nyaope samples to identify different members of the same batch. This means that law enforcement agencies in the Southern African Development Community (SADC) and beyond have, for the first time, the ability to analyse nyaope and compare forensic science data. This will allow distribution and trafficking routes to be identified and will assist in the determination of the origins of this drug type. It does, however, require that decisions are made about how the samples will be treated prior to any investigative activity. It has been shown12 that the extraction of the drugs for analysis should be made as soon as possible after samples are seized and this requires planning before any police action.

It is also interesting that whilst antiretrovirals have been reported to be present in nyaope, in the five casework samples analysed here, the antiretrovirals efavirenz and nevirapine were not identified. It may be that they were below the detection limit of the instrument or that they were indeed absent. However, the method does provide for the determination of these antiretrovirals where they are present at concentrations above the detection threshold. It should however be noted that other antiretrovirals cannot be detected by GC – MS, but that LC-MS is a suitable method. However, LC-MS is not currently readily available to SAPS.

This method assists law enforcement and public health officials in a number of ways. It assists the law enforcement agencies in the identification and comparison of nyaope samples. It allows the establishment, for the first time, of a database on the composition of nyaope. It allows exchange of analytical data between jurisdictions provided that the necessary quality control protocols are in place. It also facilitates the prosecution of trafficking offences. In terms of public health it allows determination of the drugs present in nyaope and better public health information to be disseminated amongst the users of nyaope. In turn they may choose, having this information, to avoid using this drug cocktail.

**References**

[1] Mokwena K. “Consider our plight”: a cry for help from nyaope users, Health SA Gesondheid. 2016 Dec 2016, 21:137-142. URL: <https://doi.org/10.1016/j.hsag.2015.09.001>.

[2] Grelotti DJ, Closson EF, Smit JA, Mabude Z, Matthews LT, Safren SA, Bangsberg DR, Mimiaga MJ. Whoonga: Potential Recreational Use of HIV Antiretroviral Medication in South Africa. AIDS and Behav. 2014 Mar, 18: 511–518. URL

. <https://doi.org/10.1007/s10461-013-0575-0>.

[3] Mokwena KE. The Novel Psychoactive Substance ‘Nyaope’ Brings Unique

Challenges to Mental Health Services in South Africa. International Journal of Emergency Mental Health and Human Resilience. 2015, 17(1): 251-252. URL: <https://doi.org/10.4172/1522-4821.1000152>

[4] Monyakane M M-E M. A Rehabilitative South African Criminal Law Response to Nyaope, Drug Addiction: - A Recommendation for Health Oriented Nyaope Drug Weaning. Research in Pediatrics & Neonatology. 2018 Oct 5, 3(1): 206 – 214: URL <http://dx.doi.org/10.31031/rpn.2018.03.000554>

[5] J. Alegre, Portuguese Criminal Police, personal communication

[6] Masombuka J. Children’s Addiction to the Drug “NYAOPE” in Soshanguve

Township: Parents’ Experiences and Support Needs. Thesis 154 p, University of

South Africa, 2013. URL: <http://hdl.handle.net/10500/11903>.

[7] Thomas R, Velaphi S. Abuse of antiretroviral drugs combined with addictive drugs by pregnant women is associated with adverse effects in infants and risk of resistance, S. Afr. J. Child Health. 2014 May, 8 (2): 78–79. URL: <https://doi.org/10.7196/sajch.734>.

[8] Meel R, Essop MR. Striking increase in the incidence of infective endocarditis associated with recreational drug abuse in urban South Africa, *S Afri Med J.* 2018 June 26;108(7):585-589.URL: <https://doi.org/10.7196/samj.2018.v108i7.13007>

[9] Chambers D. 10 die after destroying their hearts by mainlining nyaope. TimesLive South Africa, 02 July 2018. Available at: <https://www.timeslive.co.za/news/south-africa/2018-07-02-10-die-after-destroying-their-hearts-by-mainlining-nyaope/> accessed 6th June 2019

[10] Recovery Direct, Cape Town's Top Whoonga / Nyaope Rehab Centre, <https://www.recoverydirect.co.za/drug/whoonga-addiction/> accessed 6th June 2019

[11] Haysom S. Hiding in plain sight: Heroin’s stealthy takeover of South Africa. ENACT Heroin Policy Brief 07, March 2019. Available at: <https://enactafrica.org/research/policy-briefs/hiding-in-plain-sight-heroins-stealthy-takeover-of> south-africa

[12] Mthembi PM, Mwenesongole EM, Cole MD. Chemical profiling of the street cocktail drug ‘nyaope’ in South Africa using GC–MS II: Stability studies of the cannabinoid, opiate and antiretroviral components during sample storage. Forensic Sci Int. 2019 Jul, 300: 187-192. URL: <https://doi.org/10.1016/j.forsciint.2019.04.040>.

[13] Mthembi PM, Mwenesongole EM, Cole MD. Chemical profiling of the street cocktail drug ‘nyaope’ in South Africa using GC–MS I: Stability studies of components of ‘nyaope’ in organic solvents. Forensic Sci Int. 2018 Nov, 292: 115-124. URL: <https://doi.org/10.1016/j.forsciint.2018.08.001>.

[14] ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text And Methodology, Q2 (R1), 2005, available at: <http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf> (Accessed 7 July 2019)

[15] UNODC in: S. Section (Ed.) Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens, United Nations, Vienna, 2009.

[16] Bonfilio R, Cazedey E C L, de Arańjo M B, Salgado H R N. Analytical Validation of Quantitative High-Performance Liquid Chromatographic Methods in Pharmaceutical Analysis: A Practical Approach. Crit Rev Anal Chem. 2012 Jan, 2: 87–100. URL: <https://doi.org/10.1080/10408347.2012.630926>.

[17] D.A. Skoog, D.M. West, F.J. Holler, S.R. Crouch, Fundamentals of Analytical Chemistry. 8th ed., Chapter 7 p. 163. Brooks/Cole – Thomson Learning Inc. Toronto, 2004.

[18] Ahmad U K, Muniandy Y, Hassan M S. Physical Analysis and Chemical Profiling of Illicit Herbal Cannabis using Multivariate Analysis. Malaysian J Forensic Sci. 2005, 5(1): 26 – 34. URL: <http://forensics.org.my/mjofs/pdf/fssmVol.5No.1/Article%2005.pdf>.

[19] United Nations Office on Drugs and Crime, 2009. *Recommended methods for the identification and analysis of cannabis and cannabis products*. United Nations Publications.

[20] Peters FT, Drummer O H, Musshoff F. Validation of analytical methods. Forensic Sci Int. 2007 Jan 17, 165 (2 -3): 216-224. URL: <https://doi.org/10.1016/j.forsciint.2006.05.021>.

[21] González O, Blanco M E, Iriarte G, Bartoloméd L, Maguregui M I, Alonso R M. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. J Chrom A. 2014 Aug 1, 1353: 10–27. <https://doi.org/10.1016/j.chroma.2014.03.077>.

[22] Kadiana N, Raju K S R, Rashid M, Malika M Y, Taneja I, Wahajuddin M. Comparative assessment of bioanalytical method validation guidelines for pharmaceutical industry. J Pharm Biomed Anal. 2016 Jul 15, 126: 83–97. URL: <https://doi.org/10.1016/j.jpba.2016.03.052>.

[23] TarcomnicuI I, van Nuijs A L, Simons W, Bervoets L, Blust R, Jorens PG, Neels H, Covaci A. Simultaneous determination of 15 top-prescribed pharmaceuticals and their metabolites in influent waste water by reversed-phase liquid chromatography coupled to tandem mass spectrometry. Talanta. 2011 Jan 15, 83: 795-803. URL: <https://doi.org/10.1016/j.talanta.2010.10.045>.

[24] Karolak S, Nefau T, Bailly E, Solgadi A, Levia Y. Estimation of illicit drug consumption by waste water analysis in Paris area (France). Forensic Sci Int. 2010 Jul 15, 200 (1-3): 153-160. URL: <https://doi.org/10.1016/j.forsciint.2010.04.007>.

[25] United Nations Office on Drugs, Crime. Laboratory and Scientific Section, 2005. *Methods for Impurity Profiling of Heroin and Cocaine: Manual for Use by National Drug Testing Laboratories*. United Nations Publications.

[26] Hennig C. Dissolution point and isolation robustness: Robustness criteria for general cluster analysis methods. Journal of Multivariate Analysis. 2008 Jul, 99 (6): 1154 – 1176. URL: <https://doi.org/10.1016/j.jmva.2007.07.002>.

[27] Shlens J. A Tutorial on Principal Component Analysis (Internet). 2005 [Cited 2020 March 22] Available at: <http://www.cs.cmu,edu/~elaw/papers/pca.pdf>.

[28] Kaiser H . A second generation Little Jiffy. Psychometrika. 1970, 35(4): 401 – 415. URL: <https://doi.org/10.1007/bf02291817>.

[29] Jolliffe IT, Cadima J..Principal component analysis: a review and recent developments. Philos Trans R Soc A. 2016 Apr 16, 374: 20150202. UR:L:<http://dx.doi.org/10.1098/rsta.2015.0202> .