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Applications of Sample Preparation Techniques in the Analysis of New Psychoactive Substances

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Abstract: The global rise of new psychoactive substances (NPSs) poses challenges for their analysis in biological matrices due to their complex chemistries and short market lifespan. A comparative study for the simultaneous extraction, separation, and detection of 19 NPSs was conducted. Six solid-phase extraction (SPE) methods and one supported liquid extraction method (SLE) were compared for the extraction of analytes from blood, serum, plasma, and urine. Comparisons of four derivatization agents were conducted, at four temperatures and two incubation times. Extraction methods were assessed by precision, sensitivity, and extraction efficiency. Derivatizing agents were assessed on their selectivity and sensitivity, and a three-way ANOVA was conducted to determine statistical significance. CSDAU SPE cartridges were shown to be the most efficient when extracting analytes from blood, serum, and plasma, whereas Xcel I cartridges performed the strongest when extracting analytes from urine. SPE extraction efficiencies, when utilizing the best-performing cartridges, ranged from 49 to 119%. SLE successfully extracted all analytes from all matrices (ranging from 22 to 120%). Pentafluoropropionic anhydride: ethyl acetate was the most successful derivatizing agent, allowing all analytes to be detected, with the highest peak area responses and more unique spectra. The optimum temperature for incubation was 37 °C, with no statistical difference found between the two incubation times.

Keywords: new psychoactive substance; solid-phase extraction; support liquid extraction; derivatization; extraction

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1. Introduction

New psychoactive substances (NPSs) have appeared on the recreational drug market at an unprecedented rate in the last decade, with the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) now monitoring over 800 NPSs [1]. These compounds have been designed to mimic existing illicit compounds, such as cannabis and 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), whilst evading current legislative control [2]. In order to limit the number of uncontrolled new compounds entering the recreational drug market, many countries, including the United Kingdom (UK) and the United States of America (USA), have introduced sweeping legislation that bans classes of compounds, rather than just those specifically named [3]. Despite this, recreational abuse of these compounds continues worldwide, with stimulants comprising 35% of all NPSs reported to the United Nations Office on Drugs and Crime Early Warning Advisory System [4]. For example, NPS Discovery, an open-access drug early-warning system based in the USA, continued to identify mephedrone in 2024, with this compound also continuing to be listed in drug-related deaths in the UK [5,6]. As the use of NPSs increases, so does knowledge in relation to the administration and dosing for each substance, reducing negative outcomes. This can falsely indicate that some NPSs are no longer in circulation

such as 6-(2-aminopropyl)benzofuran (6-APB) and ethylone, which were both detected by the Welsh Emerging Drugs and Identification of Novel Substances (WEDINOS) project in 2024 [7]. This changing market therefore poses a unique challenge to forensic toxicology laboratories attempting to keep pace with the changing drug landscape [8].

The term NPS encompasses a wide range of compounds with different physicochemical properties, including solubility, volatility, and stability, making the development of methods capable of extracting, separating, and analyzing NPSs challenging [9]. Many of these compounds are only in circulation for a limited time, and therefore full-method optimization and validation is not always possible or cost-effective. As a result, laboratories may seek to detect these compounds using existing methods, resulting in quicker method validation.

Sample preparation is a critical step in the analysis of compounds in biological matrices, and is a leading cause of error during analysis [10]. Forensic biological samples are typically complex matrices containing lipids, fatty acids, and proteins. The degree to which sample clean-up is required will be determined by the sample type, (post or ante-mortem), as well as the instrument being used for analysis. The efficiency of sample extraction methods will have a direct effect on the sensitivity and specificity of the detection method, impacting the methods' limit of detection and quantification. This is an important point to note as many NPSs are more potent than the drugs that they are designed to mimic, and so are detected at lower concentrations [11].

As well as optimizing the extraction technique to be used for the detection of NPSs, when compounds are to be analyzed by gas chromatography–mass spectrometry (GC-MS), derivatization may be necessary due to the high polarity of many of these compounds [12]. Although many laboratories have transitioned to the use of liquid chromatography–tandem mass spectrometry (LC-MS/MS), due to the increased potency of NPSs, GC-MS continues to be widely used for both screening and confirmation analysis in clinical and postmortem toxicology [13]. This is especially true in developing countries where significant cost barriers can prohibit the purchase and maintenance of LC-MS/MS instrumentation [14]. As NPSs are structurally diverse, their physicochemical properties can vary widely, making them difficult to detect and quantify. The close structural similarities between compounds can also result in mass spectra with similar ion fragmentation patterns, making the differentiation of these compounds more difficult. Derivatization can overcome these challenges by modifying the target compounds to produce derivatives that have improved chromatographic properties, such as increased volatility, polarity, or ionizability, which can enhance their separation and detection, thus increasing method sensitivity and selectivity. Two of the most commonly used derivatization methods for the analysis of drugs are silylation and acylation. In silylation reactions, the reagent reacts with active hydrogens, whereas in acylation reactions, the reagent reacts with polar functional groups.

Although there has been a wide range of published methods for the detection of NPSs, these involve several different extraction methods, derivatization solvents, and incubation conditions, making it more challenging for analysts to identify optimum parameters [15–19]. This research evaluated four commonly encountered derivatization agents, along with varying incubation times and temperatures, to identify the optimum derivatization conditions that allow for the simultaneous detection of a wide range of NPSs (shown in Table 1). The work also evaluated six different solid-phase extraction (SPE) cartridges as well as a supported liquid extraction (SLE) method for the extraction of these compounds from blood, urine, plasma, and serum.

Table 1. Compound names, CAS numbers, and chemical structures of the NPS and derivatizing agents used in this study.

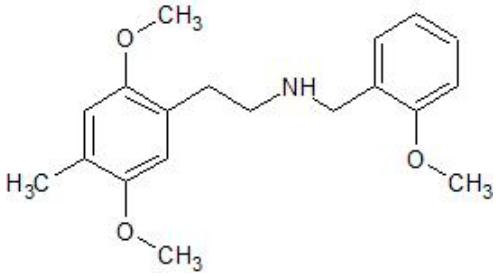
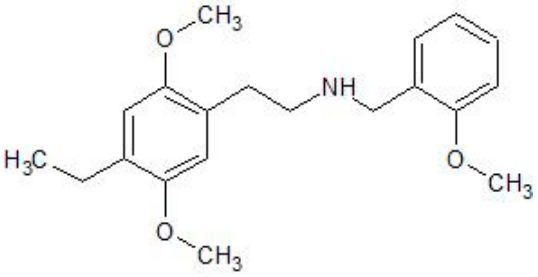
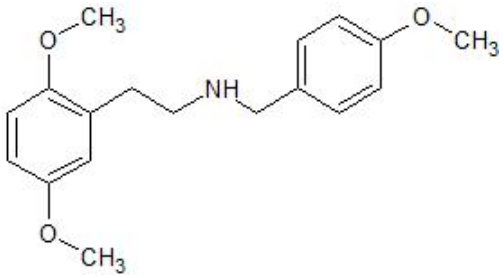
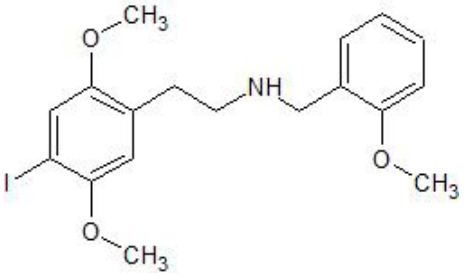
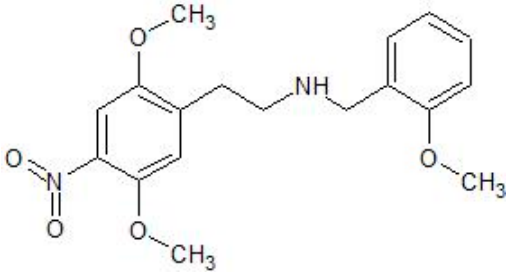
Drug Name	CAS No	Chemical Class	Chemical Structure
25D-NBOME	1539266-35-7	Phenethylamine	
25E-NBOMe	1539266-39-1	Phenethylamine	
25H-NBOME	1566571-52-5	Phenethylamine	
25I-NBOMe	1043868-97-8	Phenethylamine	
25N-NBOMe	1566571-65-0	Phenethylamine	

Table 1. *Cont.*

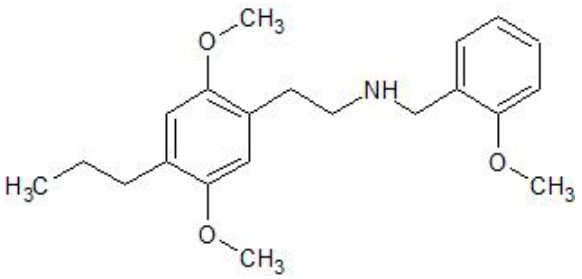
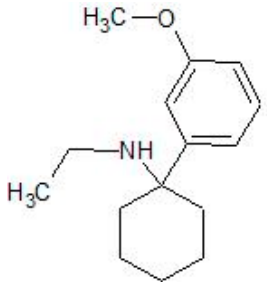
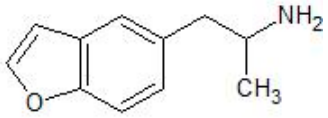
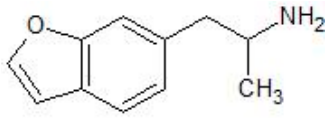
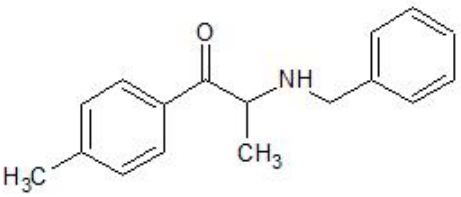
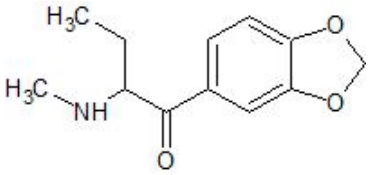
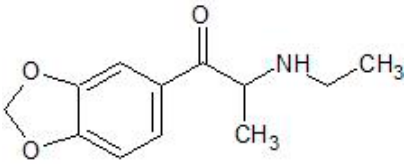
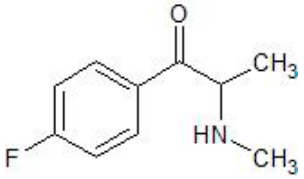
Drug Name	CAS No	Chemical Class	Chemical Structure
25P-NBOMe	1539266-43-7	Phenethylamine	
3-MEO-PCE	1797121-52-8	Arylcyclohexylamine	
5-APB	286834-80-8	Phenethylamine	
6-APB	286834-84-2	Phenethylamine	
Benzedrone	1797979-43-1	Phenethylamine	
Butylone	17762-90-2	Phenethylamine	
Ethylone	1454266-19-3	Phenethylamine	
Flephedrone	7589-35-7	Phenethylamine	

Table 1. *Cont.*

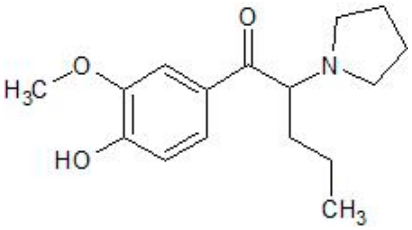
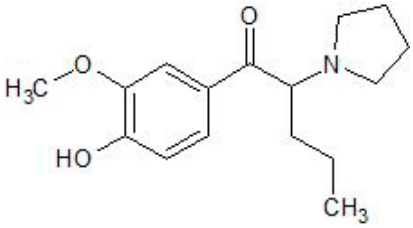
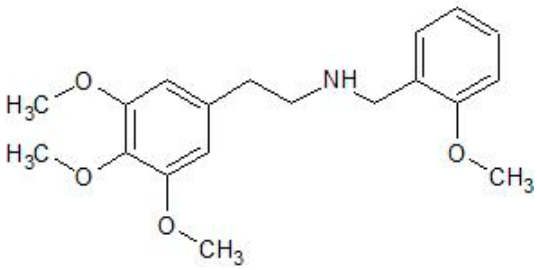
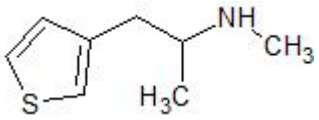
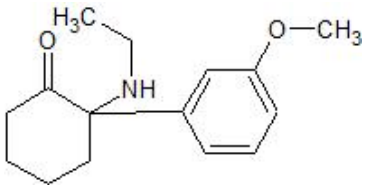
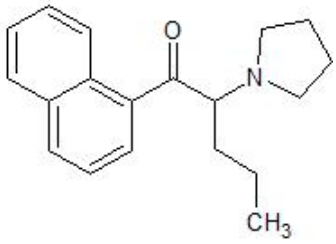
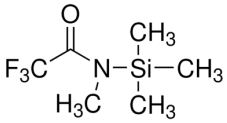
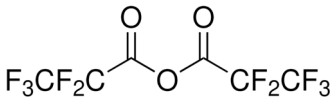
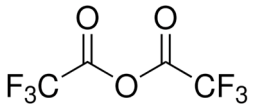
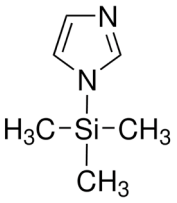
Drug Name	CAS No	Chemical Class	Chemical Structure
MDPV	2748590-34-1	Phenethylamine	
Mephedrone	1189726-22-4	Phenethylamine	
Mescaline-NBOMe	1354632-01-1	Phenethylamine	
Methiopropamine	7464-94-0	Thiophene analogue of methamphetamine	
Methoxetamine	1239908-48-5	Arylcyclohexylamine	
Naphyrone	850352-11-3	Phenethylamine	
N-Methyl-N-(trimethylsilyl)trifluoroacetamide	24589-78-4	Silylating reagent	
Pentafluoropropionic anhydride	356-42-3	Acylation reagent	

Table 1. *Cont.*

Drug Name	CAS No	Chemical Class	Chemical Structure
Trifluoroacetic anhydride	407-25-0	Acylation reagents	
N-Trimethylsilylimidazole	18156-74-6	Silylating reagent	

2. Materials and Methods

2.1. Chemicals and Reagents

Butylone, ethylone, Methylenedioxypyrovalerone (MDPV), naphyrone, 3,4,5-trimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (mescaline-NBOMe), 2-(2,5-Dimethoxy-4-nitrophenyl)-*N*-[(2-methoxyphenyl)methyl]ethan-1-amine (25N-NBOMe), and 2-(4-Propyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl] (25P-NBOMe) were purchased from LIPOMED (MA, USA). 2-(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine (25D-NBOMe), 2-(4-ethyl-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethan-1-amine (25E-NBOMe), 2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25H-NBOMe), 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe), and 25I-NBOMe-D_3 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Flephedrone, mephedrone, methoxetamine (MXE), MDPV-D_8, methylone-D_3, and mephedrone-D_3 were purchased from Cerilliant (Round Rock, TX, USA). 3-methoxyeticyclidine (3-MeO-PCE), 5-(2-aminoproprly)benzofuran (5-APB) and 6-APB, benzedrone, methiopropamine (MPA), and ethylone-D_5 were purchased from Logical (Milford, MA, USA). All reference material was purchased as 1 mg/mL ampules.

Pentafluoropropionic anhydride (PFPA) and trifluoroacetic anhydride (TFAA) were purchased from Sigma Aldrich (St Louis, MO, USA). N-Methyl-N-(trimethylsilyl)trifluoro acetamide (MSTFA) was purchased from Pierce, (Rockford, IL, USA), and N-Trimethylsily limidazole (TMSI):pyridine (1:4) was purchased from Supelco (Bellefonte, PA, USA). All other chemicals such as ethyl acetate (EtOAc) and methanol (MeOH) were of analytical grade and purchased from Grainger (Ann Arbor, MI, USA).

2.2. Preparation of Standards

Stock solutions (100 µg/mL) of each analyte were prepared via a 1:10 dilution with MeOH. These were then used to prepare mixed methanolic working solutions (10 µg/mL) by further 1:10 dilution. An internal standard (I.S.) mixed methanolic solution (10 µg/mL) containing all 5 internal standards was prepared in the same manner.

2.3. Biological Specimens

Blank human whole blood, plasma, and serum were purchased from Golden West Biologicals Inc.® (Temecula, CA, USA). Blank drug-free human urine was collected in-house from a willing donor. A 1 mL aliquot of each specimen was used during analysis.

2.4. Extraction Cartridges

SPE cartridges (ZSDAU020, CSDAU133, XRDAH206, XRPCH50z, and XCEL I) were supplied by United Chemical Technologies (Bristol, PA, USA). Oasis® cartridges were supplied by Waters (Milford, MA, USA). SLE ISOLUTE® cartridges were supplied by Biotage (Charlotte, NC, USA).

2.5. Derivatization Method Optimization

To 96 test tubes, 100 µL of each methanolic working solution (10 µg/mL) was added. The solvent was then evaporated to dryness using a TurboVap® LV (Biotage, Charlotte, NC, USA) sample concentrator at room temperature. To a set of test tubes (n = 24), 50 µL of PFPA:EtOAc (2:1), 100 µL of MSTFA:toluene (1:3), 100 µL of TMSI:pyridine (1:4), or 100 µL of TFAA:EtOAc (2:1) was added. Each set of samples was then derivatized at room temperature 24°, 37 °C, 50 °C, or 70 °C for 20 or 40 min. Each temperature and incubation time combination was prepared in triplicate. After derivatization, samples were evaporated to dryness before being reconstituted in 100 µL of EtOAc and analyzed by GC-MS. The GC-MS was operated in full scan mode (*m*/*z* 40–450 amu). Peak areas were compared, and three-way ANOVA was undertaken to determine significant statistical differences between the derivatizing agent, incubation temperature, and incubation time for each NPS using SPSS (IBM, Statistics 26). The quality of fragmentation ions was also assessed, with derivatizing agents providing more unique spectra and higher *m*/*z* fragments being favored. A graphical representation of the derivatization study design can be found in Figure 1.

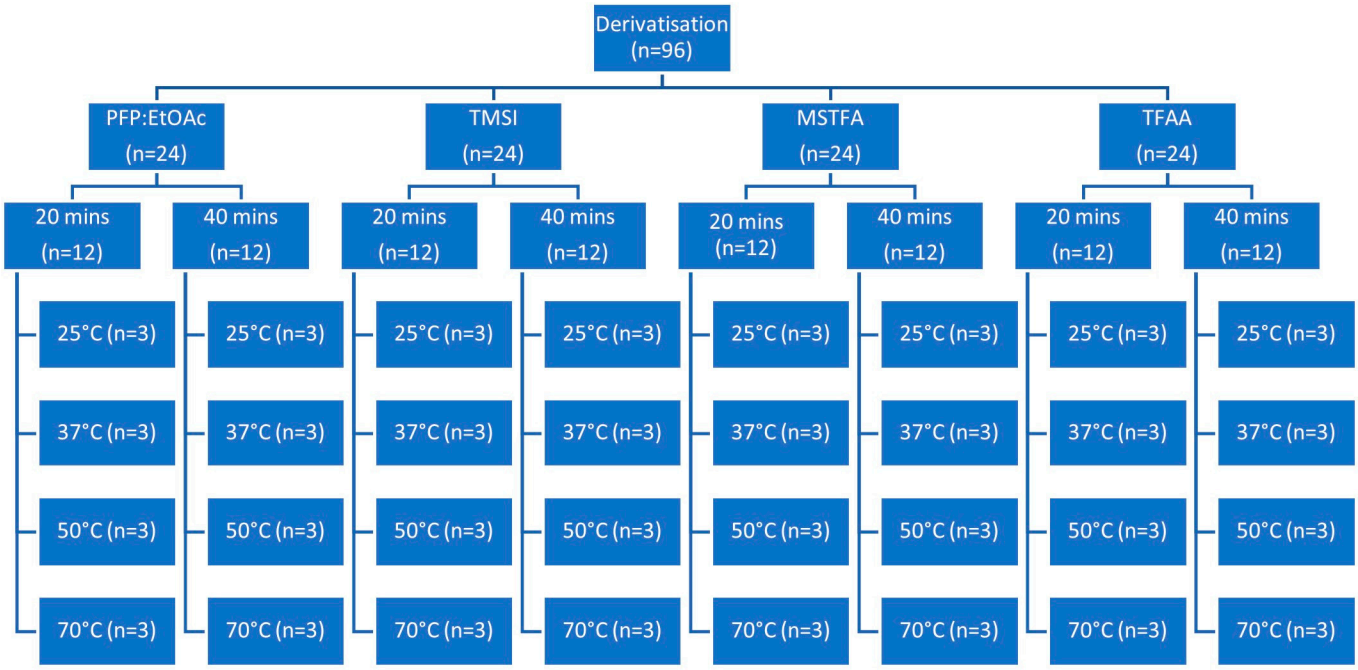


Figure 1. Derivatization experimental design.

2.6. Sample Extraction Evaluation

2.6.1. Unextracted Methanolic Sample Preparation

To assess the extraction efficiency for each extraction method, unextracted methanolic standards were prepared in triplicate for each extraction procedure to account for inter-day instrumentation differences. Methanolic standards were made by pipetting 100 µL of each working solution mixture (10 µg/mL) and 100 µL of the I.S. mixture (10 µg/mL) into glass test tubes before evaporating to dryness under nitrogen gas at room temperature. The methanolic standards were then derivatized using 50 µL of PFPA:EtOAc (2:1) for 20 min at 37 °C before being evaporated to dryness once again. The methanolic standards were then reconstituted in 100 µL of EtOAc before being analyzed by GC-MS.

2.6.2. Solid-Phase Extraction Method-ZSDAU020, CSDAU133, XRDAH206, XRPCH50z

To 1 mL of each biological matrix, 1 mL of 0.1 M phosphate buffer (pH 6.0) was added. Samples were then vortex-mixed for 30 s and centrifuged for 5 min at 3500 rpm using a Combo V24 centrifuge (Global Industrial, Buford, GA, USA). The cartridges were

conditioned using 3 mL MeOH, 3 mL deionized water (dH_2O), and 1 mL phosphate buffer (0.1 M, pH 6.0). Samples were then loaded to the cartridges at a rate of 1 mL/min. Cartridges were then washed using dH_2O (3 mL), 0.1 M acetic acid (1 mL), and MeOH (3 mL), before being left to dry under vacuum for 5 min. Samples were eluted using dichloromethane (DCM); iso-propanol (IPA); ammonium hydroxide (NH_4OH) at a ratio of 78:20:2. The elution mixture was prepared fresh each day.

2.6.3. Solid-Phase Extraction Method—XCEL I

To 1 mL of each biological matrix, 1 mL of 0.1 M phosphate buffer (pH 6.0) was added. Samples were then vortex-mixed for 30 s and centrifuged for 5 min at 3500 rpm. Samples were loaded directly to the XCEL I cartridges and washed with 2% acetic acid/98% methanol (1 mL) before elution with 1 mL DCM/IPA/NH_4OH (78:20:2).

2.6.4. Solid-Phase Extraction Method—OASIS®

Prior to extraction, 1 mL of each biological matrix was vortexed for 30 s and centrifuged for 5 min at 3500 rpm. Cartridges were conditioned with 1 mL of MeOH and 1 mL dH_2O prior to loading samples. These were then washed using 2% acetic acid (2 mL) and MeOH (1 mL) prior to elution with 95% methanol with 5% ammonium hydroxide (2 mL).

2.6.5. Post Solid-Phase Extraction Method

After extraction, I.S. (mephedrone-D_3, methylone-D_3, ethylone-D_5, MDPV-D_8, and 25I-NBOMe-D_3) was added to the collection tubes prior to elution. Samples were then evaporated to dryness with nitrogen gas using a Pierce 18830 Reacti-Therm III heating module with Pierce Reacti-Vap (American Instrument Exchange, Haverhill, MA, USA), before derivatization using 50 µL of PFPA:EtOAc (2:1) for 20 min at 37 °C. Samples were then evaporated to dryness once again and reconstituted in 100 µL of EtOAc before being analyzed by GC-MS.

Each extraction was performed in triplicate, and each SPE cartridge's composition is shown in Table 2.

Table 2. Solid-phase extraction sorbent type, sorbent size, and cartridge size for each cartridge used.

Cartridge Type	Sorbent Type	Particle Size	Cartridge Size
ZSDAU020	Octyl + benzyl sulfonic acid	200 mg	10 mL
CSDAU133	Octyl + benzyl sulfonic acid	130 mg	3 mL
XRDAH206	H.F. C8 + benzyl sulfonic acid	200 mg	6 mL
XRPCH50z	H.F. propylsulfonic acid	500 mg	10 mL
XCEL 1	Proprietary	130 mg	3 mL
Oasis	Oasis MCX	150 mg	6 mL
SLE ISOLUTE®	Diatomaceous Earth	-	1 mL

2.6.6. Supported Liquid Extraction (SLE) Method

Urine, blood, plasma, and serum samples (1 mL) were spiked with 100 µL of each working solution (10 µg/mL). The pH of each sample was then adjusted to pH 10 using 1% NH_4OH. Samples were centrifuged for 10 min at 4500 rpm and loaded directly to the SLE ISOLUTE® columns. The samples were left on the columns for 5 min before being eluted twice using 4 mL of EtOAc. To the eluant 100 µL of I.S. working solution (10 µg/mL) was then added before samples were vortexed for 30 s and evaporated under nitrogen gas at room temperature. The samples were then derivatized using 50 µL of PFPA:EtOAc (2:1) at 37 °C for 20 min before being evaporated again and reconstituted in 100 µL of EtOAc for analysis via GC-MS.

2.6.7. Extraction Efficiency Determination for Both SPE and SLE

Extraction efficiency was determined by calculating the difference in peak area ratio (PAR) between those obtained from samples that underwent extraction to those from the methanolic standards as per Equation (1). The extraction efficiencies obtained were compared to those of the best-performing SPE cartridge (CSDAU133). A paired *t*-test was conducted to determine any statistical significance between extraction methods, using SPSS (IBM, Statistics 26).

I.S. was added after SPE or SLE extraction in all cases. This meant that only the analytes were impacted by the extraction process, and therefore changes in PAR could be attributed to this step. The use of I.S. also ensured that any changes in analyte concentration post-extraction, via the evaporation or derivatization process, were accounted for as this should happen to both analyte and I.S.

$$\%\text{Extraction Efficiency} = \frac{\text{PAR Extracted Sample}}{\text{PAR Methanolic Standard}} \times 100 \quad (1)$$

Equation (1): % extraction efficiency.

2.7. Instrumentation

Analysis was carried out using an Agilent GC-MSD 5975C series instrument (Agilent Technologies, Santa Clara, CA, USA) fitted with a J&W DB-5ms low bleed column (30 m × 0.32 mm i.d.; film thickness 0.25 µm). The GC-MS was operated in full scan mode with an EI source and a splitless injection. The injection port temperature was 225 °C; the transfer line temperature was 250 °C with an MS source temperature of 200 °C. The carrier gas was helium at a continuous flow of 1.5 mL/min. The initial oven temperature was 80 °C, which was held for 2 min, before ramping to 170 °C at a rate of 25 °C/min and held for 1 min. The temperature was then further increased to 200 °C at 5 °C/min and held for 1 min before being increased to 250 °C at a rate of 15 °C/min. Finally, the oven temperature was increased to 300 °C at a rate of 5 °C and held for 3 min. The total run time was 30 min. Data were analyzed using Agilent's ChemStation software (version 02.02.1431). The method was previously validated to SWGTOX guidelines [18].

3. Results and Discussion

3.1. Derivatization

The ions used for the detection of each NPS when incubated with each derivatization agent are shown in Table 3. The target ions (highlighted in bold) were used to determine peak areas for each analyte after derivatization with each agent. From this table, it is evident that only PFPA:EtOAc and TFAA:EtOAc resulted in the detection of all 19 NPSs. Using MSTFA:toluene, MPA and flephedrone were unable to be detected; however, it is possible that this is a result of these compounds eluting during the GC-MS method solvent delay. Using TMSI:pyridine, 5-APB, 6-APB, benzedrone, butylone, ethylone, flephedrone, mephedrone, and MPA were unable to be detected. This is because these compounds do not contain hydroxyl groups but instead contain ketones and amines. Although TMSI can react with some carbonyl groups, these reactions are often slow and result in poor yields. Furthermore, TMSI cannot react with amine groups, present in many of the compounds used in this study [20].

The optimum derivatization temperature and time for each compound using each reagent is shown in Table 4. The derivatizing agent, incubation temperature, and incubation time that provided the highest peak area overall have been highlighted in bold. Although both PFPA:EtOAc and TFAA:EtOAc allowed for all compounds to be detected, PFPA:EtOAc was the best derivatizing agent overall, providing the greater peak areas for 17 out of the 19 compounds examined. The average peak area of analytes derivatized by PFPA:EtOAc was 4,781,426 in comparison with MSTFA:toluene (1,223,928), TFFA:EtOAc (1,324,905), and TMSI:pyridine (1,703,460).

Table 3. Ions used for identification of each compound using each derivatization agent. Qualifier ions are marked in bold.

DRUG NAME	PFPA:EtOAc	MSTFA:Toluene	TFAA:EtOAc	TMSI:Pyridine
25D-NBOME	**178**, 121, 461	**166**, 150, 121	**178**, 165, 135	**150**, 149, 121
25E-NBOMe	**192**, 475, 121	**180**, 150, 121	**192**, 179, 121	**167**, 149, 121
25H-NBOME	**164**, 121, 447	**150**, 121, 91	**164**, 151, 121	**150**, 121, 91
25I-NBOMe	**121**, 573, 185	**150**, 121, 91	**290**, 277, 121	**150**, 121, 91
25N-NBOMe	**209**, 166, 121	**167**, 150, 121	**209**, 166, 121	**150**, 121, 91
25P-NBOMe	**206**, 193, 489	**194**, 150, 121	**206**, 198, 193	**194**, 150, 121
3-MEO-PCE	**190**, 233, 176	**233**, 190, 176	**190**, 191, 176	**190**, 233, 72
5-APB	**131**, 158,190	**132**,131,77	**158**, 140, 131	Undetected
6-APB	**131**, 158, 190	**132**,131,77	**158**, 140, 131	Undetected
Benzedrone	**91**, 119, 148	**135**, 134, 91	**230**, 148, 119	Undetected
Butylone	**149**, 218, 121	**149**, 121,72	**168**, 149, 121	Undetected
Ethylone	**218**, 190, 367	**149**,121,72	**168**, 149, 121	Undetected
Flephedrone	**123**, 204, 160	Undetected	**154**, 123, 110	Undetected
MDPV	**126**, 149, 110	**126**, 149, 110	**126**, 149, 110	**126**, 149, 110
Mephedrone	**204**, 160, 323	**119**, 91, 58	**154**, 119, 110	Undetected
Mescaline-NBOMe	**194**, 181, 477	**247**, 73,149	**194**, 181, 121	**182**, 150, 121
MPA	**124**, 204, 160	Undetected	**154**, 124, 110	Undetected
MXE	**190**, 219, 134	**219**, 190, 176	**219**, 218, 190	**219**, 190, 176
Naphyrone	**126**, 155, 127	**126**, 155, 127	**126**, 155, 127	**126**, 149, 127

The ability of PFPA to react with a wide range of functional groups including primary and secondary amines, phenols, and carboxylic acids makes it particularly useful when analyzing unknown compounds of different chemistries [21]. It is not surprising, therefore, that PFPA has been previously used for the detection of NPSs including synthetic cathinones and NBOMes [18,22,23]. Although NBOMes are typically analyzed using LC-MS/MS, in the case of fatal overdose, concentrations may be high enough to be detected via GC-MS [18,24–26]. The use of PFPA:EtOAc allows for easier differentiation between NBOMe compounds with the derivatized parent molecule frequently detected. Figure 2 illustrates the derivatized 25D-NBOMe with a molecular weight of 461, which is detected when analyzed by GC-MS, unlike the non-derivatized molecule, which has a molecular weight of 315. Due to their structural similarity, these compounds can be difficult to separate chromatographically. Derivatization, which modifies the compounds, aids in their separation by producing unique ions that improve differentiation. This is particularly important as these compounds have been shown to have differing potencies [27].

Table 4. Optimum incubation temperature (°C) and time (minutes) for each derivatization reagent with the average peak area of each new psychoactive substance analyzed. Bolded compounds show the overall optimum conditions for each compound.

Analyte	PFPA:EtOAc		MSTFA:Toluene		TFAA:EtOAc		TMSI:Pyridine		Statistically Significant Factor
	Incubation Temperature (°C)	Incubation Time (min)	Incubation Temperature (°C)	Incubation Time (min)	Incubation Temperature (°C)	Incubation Time (min)	Incubation Temperature (°C)	Incubation Time (min)	
25D-NBOME	37	20	50	20	50	40	37	20	Agent (p = 0.000)
25E-NBOMe	37	20	50	20	50	40	37	20	Agent (p = 0.000)
25H-NBOME	24	40	50	20	50	40	37	20	Agent (p = 0.000)
25I-NBOMe	37	20	50	20	50	40	37	20	Agent (p = 0.000) Time (p = 0.002)
25N-NBOMe	70	20	50	20	50	40	37	20	Agent (p = 0.000)
25P-NBOMe	37	40	50	20	50	40	37	20	Agent (p = 0.000)
3-MEO-PCE	24	40	24	40	37	40	37	20	Agent (p = 0.000)
5-APB	24	40	24	40	24	40	Not Detected		Agent (p = 0.000)
6-APB	24	40	24	40	24	40	Not Detected		Agent (p = 0.000)
Benzedrone	70	20	24	40	37	40	Not Detected		Agent (p = 0.000)
Butylone	24	40	24	40	37	40	Not Detected		Agent (p = 0.000)
Ethylone	24	40	24	40	37	40	Not Detected		Agent (p = 0.000)
Flephedrone	70	20	Not Detected		37	40	Not Detected		Agent (p = 0.000)
MDPV	24	40	24	40	37	40	Agent (p = 0.000)	20	Agent (p = 0.000)
Mephedrone	70	20	24	40	37	40	Agent (p = 0.000)		Agent (p = 0.000)
Mescaline-NBOMe	37	40	50	20	50	40	Agent (p = 0.000)	20	Agent (p = 0.000)
MPA	70	20	Not Detected		37	40	Not Detected		Agent (p = 0.000)
MXE	24	40	24	40	37	40	37	20	Agent (p = 0.000)
Naphyrone	70	20	24	40	37	40	37	20	Agent (p = 0.000)

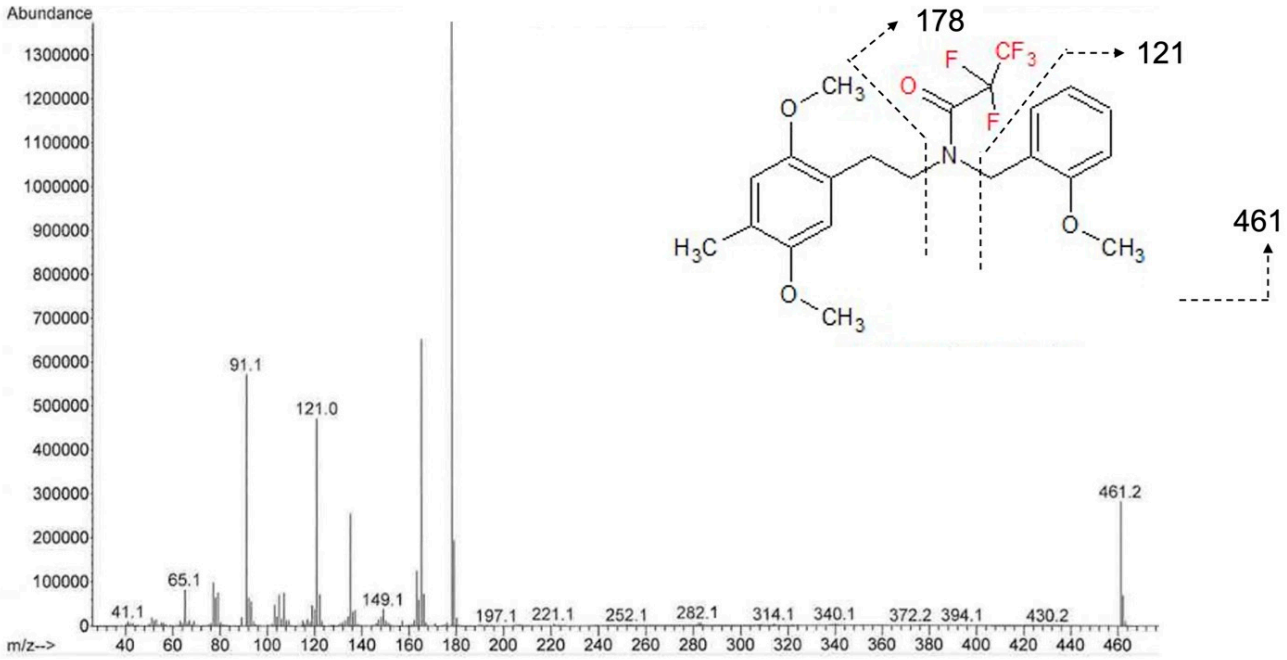


Figure 2. *m*/*z* fragmentation pattern of PFPA derivatization of 25D-NBOMe.

As shown in Table 4, incubation temperature was deemed a statistically significant variable for 25I-NBOMe only ($p = 0.002$). The optimum incubation temperature varied for each derivatizing agent, with compounds derivatized using PFPA:EtOAc and MSTFA:toluene performing best at 24 °C, in comparison to TFAA:EtOAc, which performed better at 37 °C and TMSI:pyridine, which performed better at 37 °C. There was a clear difference between synthetic cathinones and NBOMes with regards to optimum incubation temperatures when derivatized using MSTFA:toluene and TFAA:EtOAc, with NBOMes preferring 50 °C, and synthetic cathinones 24 °C and 37 °C, respectively. When derivatized using PFPA:EtOAc, there was more variation between optimum temperatures, with five of the seven NBOMes analyzed preferring 37 °C, and four of the seven synthetic cathinones preferring 70 °C. Previous work examining optimum derivatization temperatures for synthetic cathinones also found 70 °C to be the optimum temperature for these compounds using PFPA:EtOAc [28]. However, the use of lower temperatures for the analysis of NBOMes contradicts previously published methods, which used higher temperatures [28]. Previously published literature incubated these analytes at 70 °C for 40 min, which this study found not to be necessary [29]. This previous research focused on the detection of NBOMes in blotter papers where concentrations are much higher than those found in biological matrices, and therefore analyte loss in these instances would not be as important to avoid. In toxicological cases, NBOMe doses are much lower than those of cathinones (typically in the µg range), and thus their identification should be prioritized over compounds where the concentrations detected in biological matrices will be higher.

Incubation time did not have any statistically significant effect on the peak areas detected for each derivatizing agent at each incubation temperature; however, 10 analytes achieved optimum peak areas when incubated for 40 min compared to 9 preferring 20 min. As there was no statistical significance between the two times; however, the shorter 20 min time is recommended, especially for high-throughput laboratories, or instances where GC-MS is used for screening.

Not all NPSs used within this study underwent derivatization in the presence of the agents used such as MPDV and naphyrone. These compounds contain nitrogen atoms capable of forming a protonated ion during analysis by mass spectrometry, allowing detection without derivatization. It is important however to include these compounds, as although they do not require derivatization, in the case of naphyrone, their metabolites

do [30]. Polydrug use is also common in cases involving NPSs and therefore it is likely that compounds that do not require derivatization, such as these, will undergo this process during analysis to allow for the detection of other compounds within the sample such as amphetamines [31].

This study highlights the importance of choosing the correct derivatization agent to allow for the detection of these analytes using GC-MS. Although LC-MS/MS is now viewed as the gold standard for the detection of analytes within biological samples, there are laboratories that continue to rely on GC-MS for both screening and quantification, particularly within the global south where NPS detection is thought to be under-reported [32]. Optimizing derivatization reagent choice and incubation parameters is important to ensure these laboratories can still detect these substances.

3.2. Solid-Phase Extraction

All cartridges were able to extract each NPS from each matrix except for the XRPCH50Z cartridge, which failed to extract any of the NPSs. As the compounds used in this study were predominantly synthetic cathinones, which are often ionized in aqueous solutions, they are less likely to interact with hydrophobic sorbents such as that found within the XRPCH50Z cartridge [33]. The type of cartridge that provided the highest extraction efficiency, along with the extraction efficiency for each drug, is shown in Table 5.

Table 5. The cartridge type that achieved the highest extraction efficiency (%) for each analyte in each matrix type.

Analyte	Blood		Urine		Plasma		Serum	
	Cartridge Type	**% Extraction Efficiency**	**Cartridge Type**	**% Extraction Efficiency**	**Cartridge Type**	**% Extraction Efficiency**	**Cartridge Type**	**% Extraction Efficiency**
25D-NBOME	CSDAU	102	XRDAH 206	109	ZSDAU	117	ZSDAU	111
25E-NBOMe	CSDAU	110	CSDAU	110	ZSDAU	111	ZSDAU	108
25H-NBOME	CSDAU	99	CSDAU	81	CSDAU	93	CSDAU	81
25I-NBOMe	CSDAU	90	ZSDAU	90	CSDAU	98	ZSDAU	118
25N-NBOMe	ZSDAU	81	ZSDAU	98	ZSDAU	105	ZSDAU	118
25P-NBOMe	XRDAH 206	69	ZSDAU	76	CSDAU	70	CSDAU	95
3-MEO-PCE	CSDAU	58	XCEL 1	96	XRDAH 206	60	ZSDAU	50
5-APB	CSDAU	49	XCEL 1	95	CSDAU	63	CSDAU	71
6-APB	CSDAU	65	XCEL 1	94	CSDAU	98	CSDAU	51
Benzedrone	CSDAU	104	OASIS®	114	CSDAU	105	XRDAH 206	32
Butylone	XCEL 1	80	OASIS®	119	XCEL 1	101	XCEL 1	87
Ethylone	ZSDAU	115	OASIS®	87	XCEL 1	79	XCEL 1	79
Flephedrone	CSDAU	65	CSDAU	81	CSDAU	67	CSDAU	63
MDPV	CSDAU	106	XCEL 1	105	XCEL 1	109	XCEL 1	100
Mephedrone	CSDAU	112	CSDAU	101	CSDAU	88	CSDAU	106
Mescaline-NBOMe	CSDAU	71	CSDAU	77	CSDAU	91	ZSDAU	63
MPA	CSDAU	90	XCEL 1	80	CSDAU	49	CSDAU	88
MXE	CSDAU	57	XCEL 1	70	CSDAU	100	CSDAU	60
Naphyrone	CSDAU	74	CSDAU	116	CSDAU	105	CSDAU	66
Average Extraction Efficiency	-	84	-	95	-	90	-	81

The average optimum extraction efficiency for analytes from blood was 84% (ranging 49–115%). The cartridge that proved to be the most effective at recovering each of the 19 NPSs in blood was the CSDAU133, (n = 15), followed by ZSDAU020 (n = 2) and XRDAH

206 (n = 1) and XCEL 1 (n = 1). The optimum extraction efficiency for urine samples was the highest of all matrices tested at 95%, (ranging 70–119%). This is not surprising as urine was the simplest matrix tested. Both CSDAU133 and XCEL 1 cartridges had the best extraction efficiency for six analytes from urine, followed by ZSDAU020 (n = 3). XCEL 1 cartridges do not require conditioning prior to sample loading, and therefore are particularly suited for urine, especially for clinical high throughput laboratories. The average extraction efficiency of the 19 analytes extracted from plasma was 90% (ranging 49–117%). Again, the CSDAU133 cartridges performed well, achieving the highest extraction efficiency for 12 analytes, followed by the ZSDAU020 cartridges (n = 3). The average extraction efficiency of the 19 analytes extracted from serum was 81% (ranging 32–118%). Again, the CSDAU133 cartridge performed the strongest, achieving the highest extraction efficiency for 9 of the 19 analytes extracted.

Overall, the CSDAU133 cartridge performed strongest, achieving the highest extraction efficiency for 55% of analytes across all matrices. This was followed by the ZSDAU020 and XCEL 1 cartridges, which allowed the highest extraction efficiency for 18% and 17% of the analytes across all matrices. CSDSAU133 and ZSDAU020 both use the mixed mode (reverse phase and an ion exchange co-polymeric bonded phase (octyl and benzyl sulfonic acid)) sorbents, which are highly selective for basic compounds, including illicit drugs containing amine groups, such as those used in this research, and therefore their strong performance is to be expected. Previous studies using cation exchange SPE cartridges have been published, with extraction efficiencies ranging from 92 to 115%. Extraction efficiency results using CSDAU133 cartridges for the extraction of NBOMes from whole blood compare favorably to those already published in the literature. Johnson et al. used DAU SPE cartridges for the extraction of NBOMes from whole blood, reporting extraction efficiencies of 82, 85, and 54% for 25D-, 25H-, and 25I-NBOMe, respectively, which are all lower than those reported here [34]. The OASIS® cartridges were not suitable for the extraction of the NPSs tested from blood, plasma, and serum. Sample breakthrough was noticed on several occasions when utilizing this cartridge, suggesting that the cartridge and sample were not properly equilibrated prior to sample loading. Further optimization of this step would be required should these cartridges be used for the detection of NPSs.

The difference in performance between CSDAU133 and ZSDAU020 cartridges is surprising as these only differ in sorbent size, 130 mg and 200 mg, respectively. Both have been used for the extraction of a wide range of NPSs from biological matrices in the literature; however, it has been shown previously that the amount of sorbent present can impact extraction efficiency [18,28,35,36]. We propose that the increased sorbent amount within the ZSDAU020 cartridge leads to an increase in the non-specific binding of other sample components, reducing the recovery of target analytes in comparison to the CSDAU133 cartridge. The reduced cartridge size (3 mL versus 10 mL) uses less solvent, and this should also be considered when selecting SPE cartridges.

Although the performance of the OASIS® cartridges in this work was poor with the exception of benzedrone, butylone, and ethylone from urine (114%, 119%, and 87%, respectively), these cartridges have been used successfully in other studies, in particular in the detection of illicit substances from wastewater [37–39].

3.3. Supported Liquid Extraction

Using CSDAU133 cartridges, a further comparison was made between the use of SPE and SLE for the extraction of a wide range of NPSs from blood, urine, plasma, and serum. Both SLE and SPE methods extracted all 19 NPSs from each of the matrices tested. The extraction efficiency of each NPS from each matrix using SLE and CSDAU133 SPE cartridges is shown in Table 6. The average extraction efficiency achieved using SLE for the 19 NPSs in blood was 76% (ranging 32–117%) in comparison to SPE, which achieved an average of 78% (ranging 49–112%). SPE achieved the best extraction for 10 NPSs versus SLE, which extracted 9 NPSs more efficiently than SPE.

Table 6. Comparison of extraction efficiency (%) for each analyte in each matrix using solid-phase extraction (SPE) and supported liquid extraction (SLE), including any difference.

	Blood % Extraction Efficiency			Urine % Extraction Efficiency			Plasma % Extraction Efficiency			Serum % Extraction Efficiency		
Analyte	**SLE**	**SPE**	**% Difference**	**SLE**	**SPE**	**% Difference**	**SLE**	**SPE**	**% Difference**	**SLE**	**SPE**	**% Difference**
25D-NBOMe	71	102	−31	108	63	45	97	59	38	120	85	35
25E-NBOMe	82	110	−28	75	110	−35	22	65	−43	43	56	−13
25H-NBOMe	77	99	−22	49	81	−32	41	93	−52	40	81	−41
25I-NBOMe	81	90	−9	48	83	−35	34	98	−64	33	78	−45
25N-NBOMe	105	56	49	65	76	−11	52	67	−15	55	54	1
25P-NBOMe	74	51	23	61	74	−13	43	70	−27	32	95	−63
3-MEO-PCE	88	58	30	108	86	22	77	50	27	100	36	64
5-APB	75	49	26	59	74	−15	64	63	1	70	71	−1
6-APB	61	65	−4	69	93	−24	56	98	−42	34	51	−17
Benzedrone	32	104	−72	71	88	−17	72	105	−33	70	22	48
Butylone	61	65	−4	72	83	−11	69	65	4	69	29	40
Ethylone	90	70	20	73	79	−6	69	74	−5	70	31	39
Flephedrone	79	65	14	73	81	−8	74	67	7	69	63	6
MDPV	117	106	11	75	74	1	64	75	−11	68	75	−7
Mephedrone	77	112	−35	73	101	−28	71	88	−17	71	106	−35
Mescaline-NBOMe	78	71	7	72	77	−5	66	91	−25	67	63	4
Methiopropamine	43	90	−47	73	55	18	63	49	14	63	88	−25
Methoxetamine	87	57	30	73	53	20	66	100	−34	66	60	6
Naphyrone	72	74	−2	94	116	−22	104	105	−1	87	66	21
Average	76	79	−2	73	81	−8	63	78	−15	65	64	1
Statistical Difference (p)		0.729			0.113			0.029			0.911	

The average extraction efficiency of the 19 NPSs extracted from urine using SLE was 73%, (ranging 48–108%) in comparison to SPE, which had an average extraction efficiency of 81% (ranging 53–116%). Although the overall extraction efficiency between SPE and SLE only differed by 8%, SPE outperformed SLE for the extraction of 14 of the analytes.

The average extraction efficiency of the 19 NPSs extracted from plasma using SLE was 63%, (ranging 22–104%), in comparison to SPE, which had an average extraction efficiency of 78% (ranging 49–105%). Again, although similar in overall extraction efficiency, SPE outperformed SLE for 13 of the 19 NPSs.

The average extraction efficiency of the 19 NPSs extracted from serum using SLE was 65%, (ranging 32–120%), in comparison to SPE, which had an average extraction efficiency of 64% (ranging 22–106%). Serum was the only matrix where SLE outperformed SPE, having the best extraction efficiency for 10 of the 19 NPSs extracted.

Although differences were observed between each analyte, assessing overall performance using a paired *t*-test showed that the slight differences in extraction efficiency between both SPE and SLE were not significant, except for those from plasma ($p = 0.023$). SLE is a much faster process as the majority of SPE cartridges still require conditioning and wash steps, which are omitted when using SLE; therefore, although there was not a statistically significant difference between techniques for blood, urine, and serum samples, the ability to process more samples faster should be taken into consideration when determining which techniques to implement within the laboratory. The omission of conditioning and wash steps also reduces the volume of solvents used during analysis and the amount of solvent waste produced by the laboratory, which should also be considered.

4. Conclusions

Of all the derivatization agents tested in this work, PFPA:EtOAc at 37 °C achieved the highest peak area responses and the most unique spectra. Incubation time did not have a significant effect on peak area, and therefore it is suggested that incubation times should be based upon the needs of the laboratory and which analytes they most commonly encounter. This SPE study showed that when analyzing biological matrices, the smaller bed size of the CSDAU133 cartridges is preferable. XRPCH50z cartridges, using the method tested, are not recommended for sample extraction of NPSs. SPE using CSDAU133 cartridges performed slightly better than SLE; however, this difference was not statistically significant. Additional factors such as time and solvent requirements should be considered when implementing extraction protocols within the laboratory. This work is particularly relevant to laboratories still reliant upon GC-MS for the analysis of biological materials.

Laboratories should continue to track which analytes are most commonly encountered within their location to prioritize which analytes are of high importance when determining which cartridge type to use. Extraction efficiencies should continue to be monitored as more NPSs are detected within the recreational drug market to ensure that methods remain current and fit for purpose. This research shows that existing methods routinely used by laboratories, such as the use of CSDAU133 and ZDSAU020 cartridges, can extract a wide range of analytes, and therefore it should be possible to detect new and emerging compounds using existing laboratory methods.

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