Tatu Köli

Testing of the Recommended Operating Procedures (ROPs) for central nervous system acting chemicals

Metropolia University of Applied Sciences

Bachelor of Laboratory Services

Laboratory Sciences

Bachelor's thesis

4.12.2015

Metropolia

Haluan kiittää koko VERIFINin henkilökuntaa saamastani asiantuntevasta avusta opinnäytetyöni kanssa. Erityiskiitos professori Paula Vanniselle mahdollisuudesta päästä suorittamaan opinnäytetyöni VERIFINillä sekä työni ohjauksesta. Iso kiitos kuuluu yliopettaja Jukka Niiraselle ohjauksesta sekä asiantuntevasta opetuksesta.

Metropolia

Tekijä Otsikko Sivumäärä Aika	Tatu Köli Testing of the Recommended Operating Procedures (ROPs) for central nervous system acting chemicals 56 sivua + 6 liitettä 4.12.2015
Tutkinto	Laboratorioanalyytikko
Koulutusohjelma	Laboratorioala
Ohjaajat	Professori, FT, Paula Vanninen Yliopettaja Jukka Niiranen

Tässä opinnäytetyössä testattiin kemiallisten aseiden analysointiin, seulontaan ja tunnistamiseen tarkoitettuja menetelmiä (Recommended Operating Procedure, ROP) keskushermostoon vaikuttaville yhdisteille, jotka kuuluvat inkapasitoivien kemiallisten aineiden piiriin. Kokeissa käytettäväksi näytematriisiksi valittiin pyyhintänäytteet ja tutkittaviksi aineiksi keskushermostoon vaikuttavia yhdisteitä: fentanyyli, naloksoni sekä amfetamiini. Näytteenkäsittely perustui pyyhintänäytteen uuttamiseen ensin orgaanisella liuottimella ja tämän jälkeen vedellä. Vertailtaviksi orgaanisiksi uuttoliuottimiksi valittiin dikloorimetaani sekä asetoni ja kokeita suoritettiin kolmelle eri pyyhintänäytemateriaalille: Whatman-suodatinpaperille, vanupuikolle sekä puuvillaliinalle. Asetonilla uutettaessa saavutettiin huomattavasti suuremmat saannot valituille analyyteille kuin dikloorimetaanilla. Saannot olivat myös suhteellisen korkeita vesifraktioissa, jotka olivat uutettu dikloorimetaaniuuton jälkeen.

Valittuja yhdisteitä seulottiin ja tunnistettiin pyyhintänäyteuutteista käyttäen sekä nestekromatografia-sähkösumutusionisaatio-tandem-massaspektrometriaa (LC–ESI–MS/MS) että kaasukromatografia-massaspektrometriaa (GC–MS). GC–MS-seulonnassa ja -tunnistuksessa hyödynnettiin AMDIS-tietokoneohjelmaa. LC–MS/MS -seulonta perustui tunnettujen yhdisteiden etsimiseen käyttäen full scan- ja MRM-menetelmää. GC–MS- ja LC–MS/MS-tekniikoiden sekä ROP-menetelmien todettiin olevan soveltuvia valittujen yhdisteiden seulontaan ja tunnistamiseen miljoonasosa-konsentraatiotasolla (ppm, µg/g). Analyysitekniikoista LC–MS/MS osoittautui soveltuvammaksi valittujen yhdisteiden analysointiin.

Pyyhintänäytekokeiden lisäksi tässä työssä validoitiin menetelmä fentanyylin määrittämiseksi virtsasta LC–ESI–MS/MS-tekniikalla. Menetelmän näytteenkäsittely perustui analyyttien uuttamiseen virtsasta kiinteäfaasiuutolla isotooppileimatun fentanyyli-d_5:n toimiessa sisäisenä standardina kvantitoinnissa. Validointi suoritettiin pitoisuusalueella 0,5–50 ng/ml fentanyyliä virtsassa. Validointia varten valmistettiin ja analysoitiin kolme rinnakkaista näytettä seitsemällä eri pitoisuustasolla päivittäin kolmen päivän ajan. Havaitsemis- ja määritysrajan (LOD ja LOQ) todettiin olevan 0,5 ng/ml fentanyyliä virtsassa. Mittausten tarkkuus vaihteli -1,2 ja 14,3 %:n välillä ja täsmällisyys 2,7 ja 6,1 %:n välillä. Fentanyylin saannoksi kiinteäfaasiuutosta määritettiin 83,8 % pitoisuustasolla 1 ng/ml ja 90,1 % pitoisuustasolla 25 ng/ml.

Avainsanat	keskushermostoon vaikuttavat aineet, inkapasitoivat taisteluaineet, fentanyyli, amfetamiini, naloksoni, LC–MS/MS, GC–MS, validointi, virtsa-analyysi, kiinteäfaasiuutto

Author Title Number of Pages Date	Tatu Köli Testing of the Recommended Operating Procedures (ROPs) for central nervous system acting chemicals 56 pages + 6 appendices 4 December 2015
Degree	Bachelor of Laboratory Services
Degree Programme	Laboratory Sciences
Instructors	Paula Vanninen, PhD, Professor Jukka Niiranen, Principal Lecturer

In this thesis Recommended Operating Procedures (ROPs) were tested for sample preparation, analysis, screening and identification of central nervous system (CNS) acting chemicals, a class of incapacitating chemical agents (ICAs), in wipe samples. The selected candidate chemicals were CNS acting drugs: fentanyl, naloxone and amphetamine. The sample preparation was based on extraction of analytes from spiked wipe samples successively with organic solvent and aqueous solvent. Two different organic solvents, dichloromethane and acetone, and three different wipe materials, cotton swab, Whatman filter paper and cotton wipe, were used in the ROP testing experiments. Acetone provided high recoveries for the candidate chemicals whereas dichloromethane extracted the analytes poorly. Relatively high recoveries were achieved with water extraction performed after the extraction with dichloromethane.

Screening and identification of the candidate chemicals in cotton wipe were performed by using both liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) and gas chromatography-mass spectrometry (GC–MS). AMDIS software was utilized in GC–MS screening. The LC–MS/MS screening was targeted screening using full scan mode and multiple reaction monitoring (MRM). Both GC–MS and LC–MS/MS techniques and the tested ROPs were evaluated to be valid for screening and identification of the chemicals in question at parts-per-million (ppm, µg/g) concentration levels. From these two analysis techniques, the LC–MS/MS was found to be more appropriate technique for analysis of the candidate chemicals.

In addition to the wipe sample study, a quantitative method for determining fentanyl in urine by LC–ESI–MS/MS was validated. The assay was based on extraction of fentanyl from human urine using solid phase extraction (SPE). Deuterium labeled fentanyl-d_5 was used as internal standard in quantitation. Validation of the method was studied in the concentration range of 0.5–50 ng/ml. The validation experiments were carried out by preparing and analyzing three replicate calibration standards at seven different concentration levels each day during three days. Limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 0.5 ng/ml. The accuracy ranged from -1.2 to 14.3 % and the intermediate precision from 2.7 to 6.1 %. The extraction recovery of fentanyl was determined to be 83.8 and 90.1 % at concentration levels of 1 and 25 ng/ml, respectively.

Keywords	central nervous system acting chemical, incapacitating chemical agent, recommended operating procedure, wipe sample, amphetamine, naloxone, fentanyl, validation, LC–ESI–MS/MS, GC–MS, urine analysis, solid phase extraction

Contents

Appendices

Abbreviations

ADHD	attention deficit-hyperactivity disorder
AMDIS	the Automated Mass Spectral Deconvolution and Identification System
ANOVA	analysis of variance
BSTFA	N,O-bis(trimethylsilyl)-trifluoroacetamide
CAS	Chemical Abstracts Service
CNS	central nervous system
CW	chemical weapon
CWA	chemical warfare agent
CWC	the Chemical Weapons Convention
DC	direct current
DCM	dichloromethane
EI	electron ionization
ESI	electrospray ionization
FDA	US Food and Drug Administration
Fimea	Finnish Medicines Agency
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
HPLC	high-performance liquid chromatography

ICA	incapacitating chemical agent
IS	internal standard
LC	liquid chromatography
LC–MS/MS	liquid chromatography–tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
MF	match factor
MTBSTFA	N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide
MW	molecular weight
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NIST	National Institute of Standards and Technology
NMF	net match factor
OCAD	OPCW Central Analytical Database
OPCW	the Organization for the Prohibition of Chemical Weapons
Q	quantifier ion
q	qualifier ion

QC	quality control
R^2	correlation coefficient
RCA	riot control agent
RF	radio frequency
RMF	reverse match factor
ROP	Recommended Operating Procedure
RP	reversed phase
RSD	relative standard deviation
RT	retention time
SD	standard deviation
SIM	selected ion monitoring
S/N	signal-to-noise ratio
SPE	solid phase extraction
TBDMS	tert-butyldimethylsilyl
TIC	total ion chromatogram
TMS	trimethylsilyl
WADA	World Anti-Doping Agency
WCOT	wall coated open tubular

1 Introduction

This thesis was conducted at Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN) which operates under the Department of Chemistry at the University of Helsinki. The institute was established in 1994 to continue the Chemical Weapon research project (CW Project) started in 1973. VERIFIN supports the verification of the Chemical Weapons Convention (CWC) in the fields of research and training. The research in VERIFIN focuses on developing analytical methods for screening and identification of chemical warfare agents (CWAs), including their degradation products and starting materials. The institute trains and organizes courses for chemists from the developing countries. VERIFIN also acts as the National Authority of Finland for the CWC. [1]

This thesis focuses on analytical methods for detecting central nervous system (CNS) acting chemicals, a class of incapacitating chemical agents (ICAs). There has been an interest in the possibility of using chemicals that can cause incapacitation in humans for military, law enforcement or counter-terrorist purposes. These agents include a large variety of different chemicals with separate actions and effects. Especially drug-related compounds have been investigated for use as incapacitans [2, pp. 23–25]. Development of ICAs and their delivery system has been continued for over 50 years [3, p. 2]. A number of programs on research and development of ICAs have been reported, including programs taking place during the Cold War and contemporary programs [3]. The use of CNS acting chemicals against Chechen terrorist at the Dubrovka Theatre in Moscow 2002 focused the attention on these agents and their possible application as a counter-terrorist tool. In addition, today's advancement in drug research and development together with growing knowledge and understanding of human physiology and how human mind works have increased the interest in ICAs. [2, pp. 5–6]

The purpose of this thesis was to test the existing recommended operating procedures (ROPs, introduced in The Blue Book, Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament) for sample preparation, screening and identification of CNS acting chemical agents in environmental samples and to validate a method for determining fentanyl, a CNS acting drug with possible application as an incapacitant, in urine [4]. The candidate chemicals were selected for

the study and the availability of the reference chemicals was investigated. A review of literature on the candidate chemicals and methods for analyzing the substances was carried out. The analysis of fentanyl in urine was based on the review of existing literature. The sample preparation and analysis of the environmental samples were conducted according to the ROPs. In addition, electron ionization (EI) mass spectra for the candidate chemicals were produced and the spectral data will be submitted to be evaluated and included in the OPCW Central Analytical Database (OCAD) in future.

From different environmental sample matrices, wipe samples were selected for the ROP testing experiments. A wipe is an appropriate tool for collecting samples from various surfaces, such as, the inside of reactor vessels, containers and fume hoods. In addition, wipes can be used for sample collection when no apparent liquid or solid samples are available. For example, in United States wipe sampling of household surfaces is used for revealing methamphetamine contamination caused by clandestine drug laboratories [5, p. 23]. [6, pp. 39–40]

The ROPs that are applied in the analysis of wipe samples are introduced in The Blue Book, Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament, published by University of Helsinki. These ROPs introduce the methods and guidelines to be followed in designated laboratories of the Organization for the Prohibition of Chemical Weapons (OPCW) or in laboratories applying for designation. The ROPs include guidelines, for instance, for preparation of different environmental sample matrices and for screening and identification of CWC-related chemicals. The Blue Book is edited by VERIFIN and the ROPs are regularly updated by the collaborating expert laboratories working with CWC-related chemicals. [4]

2 Incapacitating chemical agents

Incapacitating chemical agents can be defined as toxic chemicals that cause temporary incapacitation to humans or animals, but not usually death or permanent harm, differing from riot control agents (RCA) in longer duration of action [7, p. 5]. The definitions may vary depending on the context they are used in. In some contexts the ICAs have been described as “non-lethal” or “less-than-lethal” agents. According to some experts, ICAs should not be considered as non-lethal due to the fact that they can cause death in actual use. The lethality of ICAs is dependent on several factors, such as the actual

dose of the agent, the physiology of the victim and the availability of medical care and antidote. [2, p. 5]

The CWC does not define the term incapacitating chemical agent. However, in the CWC ICAs are covered under the definition of “toxic chemicals”. The CWC Article II.2 defines toxic chemicals as follows:

> Any chemical which through its chemical action on life processes can cause death, temporary incapacitation or permanent harm to humans or animals. This includes all such chemicals, regardless of their origin or of their method of production, and regardless of whether they are produced in facilities, in munitions or elsewhere.

This is a so-called general purpose criterion.

There is a large variety of substances that can potentially be used as ICAs. Recently the research has focused on chemicals such as anesthetic agents, skeletal muscle relaxants, opioid analgesics, anxioltytics, antipsychotics, antidepressants and sedative-hypnotic agents [8, p. 58]. Many of these chemicals are used in human or veterinary medicine as tranquilizing or anesthetic agents [8, p. 58]. The chemicals selected for this research were CNS acting drugs: fentanyl, amphetamine and naloxone. Diazepam was also among the selected candidate agents but due to delivery problems of the reference chemical it was left outside of the ROP testing study. Fentanyl and diazepam are categorized as calmatives and amphetamine as a CNS stimulant [9, pp. 15–16][10, p. 612]. Naloxone works as an opioid antagonist [10, p. 605].

2.1 Moscow hostage crisis

The most prominent use of CNS acting drugs for counter-terrorist purposes was at the Dubrovka Theater in Moscow during the hostage crisis in 2002. The crisis began when Chechen militants, equipped with explosives, seized the Dubrovka Theater in Moscow taking over 800 hostages during a sold-out performance. The Russian Special Forces surrounded the theater. After a two-and-a-half day’s siege the Special Forces pumped a chemical aerosol into the building’s ventilation system and raided the theater. The chemical deployed to the theater hall rendered most of the hostages and terrorists

unconscious. Over 120 hostages and all of the terrorists died during the hostage crisis. [11]

Most of the hostages killed in the raid died from the effects of the chemical. The medical personnel were not informed about the composition of the aerosol deployed to the concert hall and they were not able to offer adequate treatment for the victims. It is possible that by preparing the medical personnel and reserving enough antidote, such as naloxone, the casualties could have been minimized. Afterwards the Russian Health Minister stated that the chemical used in the raid was a fentanyl-based substance [12]. Traces of remifentanil and carfentanil (derivatives of fentanyl) were found in the clothing of British hostages [11]. [13, p. 20]

2.2 Fentanyl

Fentanyl (Figure 1) N-phenyl-N-(1-(2-phenylethyl)-4-piperidinyl)propanamide, is a synthetic opioid, related to meperidine. The chemical and physical properties of fentanyl are listed in Table 1. Fentanyl was first synthesized by Paul Janssen in 1960 [14]. It is a highly potent narcotic that is commonly used as a surgical anesthetic and for pain treatment (a therapeutic plasma concentration for analgesia is usually 1–2 ng/ml and for anesthesia it is 10–20 ng/ml) [15]. Fentanyl has been reported to be approximately 250 times more potent than morphine [14].

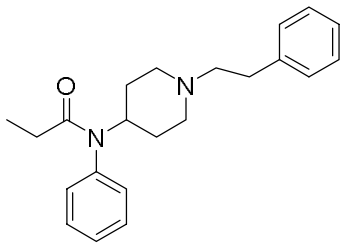


Figure 1. Molecular structure of fentanyl.

Table 1. Physical and chemical properties of fentanyl [16].

Properties	Fentanyl	Fentanyl-d_5	Fentanyl citrate
CAS Number	437-38-7	118357-29-2	990-73-8
MW	336.47	341.50	528.59
pKa	8.4	-	-
Melting point (^{o}C)	83–84	-	149–151
Partition coefficient (*n*-octanol/water)	860	-	-

Fentanyl is a strong μ-opioid receptor agonist that has very rapid onset of action and a short duration of action [17]. In human body it has a half-life of 1-2 hours [10, p. 606]. Fentanyl has a wide range of side effects including respiratory depression, nausea, dizziness, vomiting, fatigue, headache, constipation, anemia and peripheral edema [15]. Due to the pharmacological properties of fentanyl and its derivatives (e.g. carfentanil), they have potential applications as incapacitating agents [9, p. 49].

There is a variety of methods developed for the determination and quantitation of fentanyl and its derivatives in human urine and blood by liquid chromatography-tandem mass spectrometry (LC–MS/MS) and gas chromatography-mass spectrometry (GC–MS) [18][19][20][21]. A method for detection of fentanyls in wipe samples have also been reported [22]. A notable research, closely related to this study, is the analysis of fentanyls and their metabolites in clothing, urine and plasma of the survivors of the Moscow hostage crisis [11]. Riches et al. managed to find residues of carfentanil and remifentanil in the clothing samples taken from British hostages analyzed by LC–MS/MS. The concentrations detected were lower than 0.5 ng/ml.

2.3 Amphetamine

Amphetamine (Figure 2), 1-phenylpropan-2-amine, is a synthetic substance that acts as central nervous stimulant. Chemical and physical properties of amphetamine are given in Table 2. In human body amphetamine mainly acts by releasing noradrenaline and dopamine. The effects of the drug are increased heart rate and blood pressure, locomotion stimulation and euphoria. With large doses stereotyped behavior occurs. The duration of action is approximately a few hours. [10, pp. 612–613]

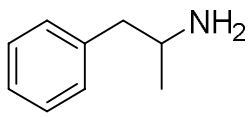


Figure 2. Molecular structure of amphetamine

Table 2. Physical and chemical properties of amphetamine [16]. RT refers to room temperature.

Properties	Amphetamine	Amphetamine sulphate	Amphetamine phosphate
CAS Number	300-62-9	60-13-9	139-10-6
MW	135.21	368.49	233.20
Melting point (°C)	Mobile liquid at RT	above 300	-
Boiling point (°C)	200–203	-	-

Amphetamine and its derivatives have therapeutic use in treatment of narcolepsy and attention deficit-hyperactivity disorder (ADHD) in children. Clinical use of amphetamines is very limited due to its many unwanted effects. The main importance of amphetamines is in drug abuse. US Army Chemical Corps has reported that psychochemicals, such as phenethylamines, could potentially be used as incapacitating agents [3, p. 4]. [10, p. 614]

The analysis of amphetamines by GC usually includes derivatization prior to analysis. This is not mandatory but it improves chromatographic properties and detectability of amphetamines. For example, analyses that include silylation of amphetamine with BSTFA and MTBSTFA have been described [23, p. 76][24]. Several methods for detecting amphetamine and related compounds by LC–MS/MS have also been developed, including a method for detecting amphetamine in wipe samples [25][26]. Free base of amphetamine is a liquid and volatile compound at room temperature. This should be taken into account in sample preparation because evaporating the sample to dryness can cause significant loss of the analyte. [23, p. 75]

2.4 Naloxone

Naloxone (Figure 3), (5α)-4,5-epoxy-3,14-dihydroxy-17-(2-propen-1-yl)morphinan-6-one, is a pure opioid antagonist that has affinity for all of the three opioid receptors: μ-, κ- and δ-receptors. The physical and chemical properties of naloxone are listed in Table 3. Naloxone blocks the action of both endogenous opioid peptides and morphine-related drugs. It is rapidly metabolized by liver and the duration of action is from 2 to 4 hours. It is clinically used in treatment of respiratory depression caused by opioid (e.g. morphine) overdose. Naloxone has little effects when given on its own, whereas it produces a rapid reversal of the effects of opioids. Naloxone does not have applications as an incapacitating agent as such, but it can be used as an antidote for opioid incapacitating agents [3, p. 15]. [10, p. 605]

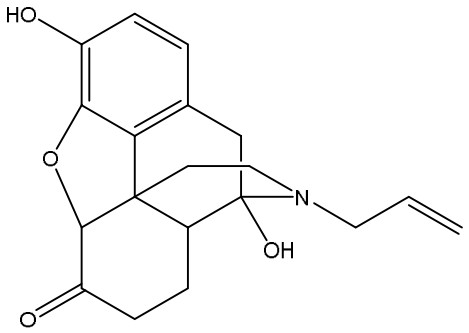


Figure 3. Molecular structure of naloxone.

Table 3. Physical and chemical properties of naloxone [16].

Properties	Naloxone	Naloxone hydrochloride
CAS Number	465-65-6	357-08-4
MW	327.27	363.84
Melting point (^{o}C)	184	200–205

Several methods for detecting naloxone from different biological matrices by LC–MS/MS have been reported [27][28]. Methods for detecting naloxone by GC–MS were not found in the review of literature. However, a method for analyzing naltrexone (a very similar compound to naloxone) by GC–MS using naloxone as internal standard was found. This method included silylation of naloxone and naltrexone prior to analysis. Due to keto-enol tautomerism that occurs on naloxone and naltrexone, these compounds have three potential hydroxyl groups where the silyl group can attach. Because of the keto-enol tautomerism and incomplete silylation, it is possible that naloxone and naltrexone form several different products when silylated. [29]

3 Sample preparation techniques

3.1 Silylation

Silylation is a derivatization technique where an active hydrogen bound to a heteroatom is replaced with a silyl group. Usually the compound is converted to a trimethylsilyl (TMS) derivative but also other derivatives can be used. For instance, a tert-butyldimethylsilyl (TBDMS) group is often used for substituting the hydrogen. These silylated compounds are generally less polar, more stable and more suitable for GC. The formation of a TMS derivative can be written as in Figure 4. [30, p.545]

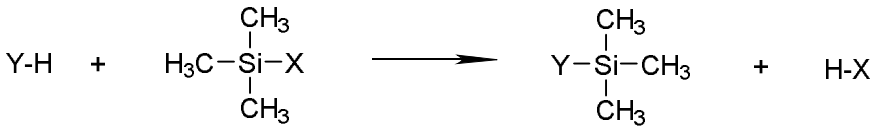


Figure 4. The formation of a TMS derivative. The active hydrogen of the compound Y-H is replaced with the TMS group of the silylation reagent TMS-X [30, p. 546].

There is a variety of different reagents for silylation and aprotic solvents that can be used as medium. In this thesis, BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide, Figure 5) was used as silylating reagent and high purity acetonitrile was used as solvent. BSTFA can silylate several different functional groups, including primary amines and hydroxyl groups [30, p. 553]. Therefore, it was presumed to be valid reagent for silylating amphetamine and naloxone. Fentanyl does not contain any functional groups that would react with the silylation reagent.

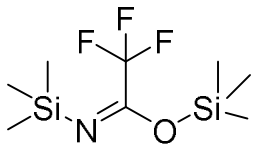


Figure 5. Molecular structure of BSTFA.

3.2 Solid phase extraction

Solid phase extraction (SPE) is a common sample preparation procedure used for cleanup and concentration of liquid samples. A certain amount of finely divided porous solid material is used for retaining the analytes of interest or the interfering compounds from a sample solution. The solid phase (from 50 mg to 10 g) is usually packed into a small column, cartridge or disc. In this thesis, SPE was employed to clean up the urine sample. The cleanup was based on the retention of the analyte and elution of the interfering compounds. Figure 6 shows the extraction procedure schematically. The diluted urine sample was introduced into the cartridge (A). The analytes retain on the solid phase while the solvent and some of the interferences drain from the cartridge. The cartridge is washed with wash solution in order to remove interferences from the solid phase (B). Finally the analytes are eluted from the cartridge using appropriate solvent (C). The eluate is collected for an analysis. [30, p. 341]

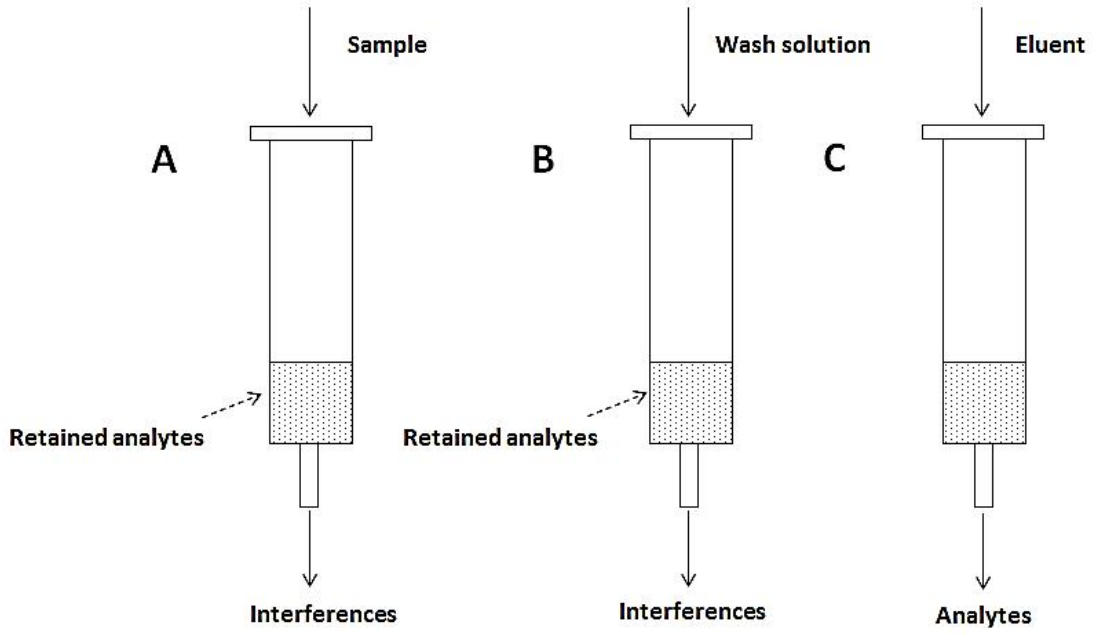


Figure 6. SPE cleanup with analyte retention (A) followed by washing (B) and elution (C) [30 p. 341].

4 Analytical methods

4.1 Gas chromatography

Gas chromatography (GC) is an analytical separation technique used to analyze volatile organic compounds in gaseous phase. The separation of the substances relies on different partition behavior of components between two phases: a gaseous mobile phase and a stationary phase. The mobile phase is an inert gas (often He) that carries the analytes into the column. The stationary phase is usually a thin film of liquid or polymer coated inside the tubular column. The carrier gas transports the gaseous sample inside the column where separation occurs. The separated compounds are detected when exiting the column. [31, pp. 565–566, 574, 579]

4.1.1 Injector

A volatile liquid or gaseous sample is introduced to the high-temperature injector where it vaporizes. The analyzed molecules have to be volatile and inert enough not to decompose in high temperature. The injection can be operated in split or splitless mode. Split injection is preferred when analytes constitute more than 0.1 % of the sample. For trace analysis, splitless injection is more appropriate. [31, pp. 577–578]

In this thesis, splitless injection was applied due to small concentration of the analytes of interest. In splitless injection (Figure 7) a relatively large volume (~2 µl) of liquid sample is injected with syringe through a rubber septum into a heated glass liner. The split vent is held closed. The liquid sample evaporates in the liner and the sample vapors are transported into the column by carrier gas. The sample spends relatively long time (~1 min) in the injection port. Slow flow of the septum purge cleans the septum and removes any vapors that escape the glass liner. After the injection the split vent is opened and the injector is quickly flushed. [31, p. 578]

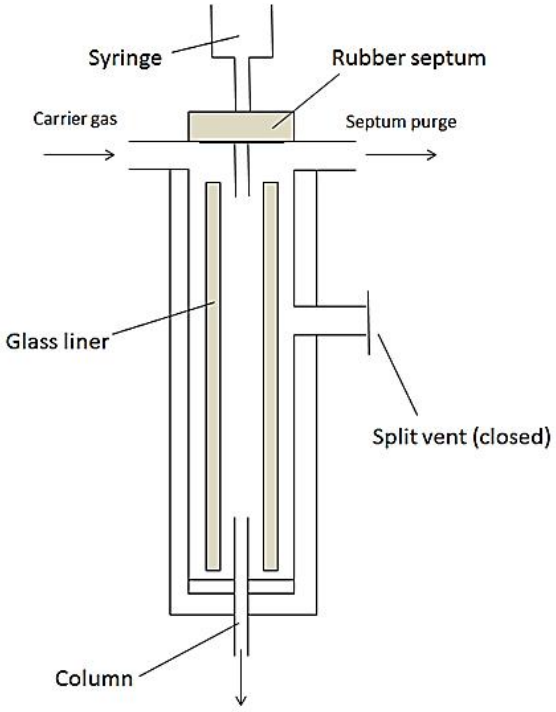


Figure 7. Schematic representation of splitless injection into an open tubular column.

The sample molecules are introduced into the column during the entire splitless time. This could cause an unacceptable broadening of the peaks in the chromatogram. The

broadening is avoided by using suitable initial column temperatures. When setting the temperature below the solvent's boiling point, the solvent and other solutes condense at the beginning of the column and the solvent slowly evaporates. This technique is called solvent trapping. A focusing mechanism called cold trapping can be applied to solutes with high boiling point. These solutes condense at the beginning of the column while solvent and other lower boiling components are eluted by the carrier gas. Chromatography is then initiated by raising the column temperature. [31, p. 578]

4.1.2 GC Column

The majority of the columns used in GC are long, narrow wall coated open tubular (WCOT) columns. The columns are usually made of fused silica coated with polyimide. Typical column dimensions are 30 m length and 0.1 to 0.5 mm internal diameter. The stationary phase is often a 0.25 µm thick film bonded to the inner wall of the column. Polysiloxanes are common materials used as stationary phases. [31, pp. 566-567, 569]

The column is located in a column oven. The oven can be operated in temperature programming in which the temperature is usually set to increase during the run. High temperature increases the vapor pressure of analytes and decreases retention time of late-eluting analytes making shorter run times possible. Temperature programming can also be utilized to achieve sharper peaks for late-eluting compounds. [31, pp. 573-574]

4.1.3 Electron ionization

Only charged molecules can be detected with mass spectrometer. In order to detect neutral molecules exiting the GC column, the gas phase molecules are converted into charged ions and fragments by electron ionization (EI). Molecules from the GC column, connected directly to the mass spectrometer, enter the ion source. A beam of electrons emitted from a hot filament is accelerated through 70 V. These energetic electrons collide with neutral molecules (M) in gas phase dislodging an electron from the molecules and forming molecular ion $M^{+\cdot}$. Usually the $M^{+\cdot}$ has enough internal energy to decompose into smaller fragments. In the ion source, the charged fragment ions are directed to the mass separator by a charged repeller. [31, pp. 503-504]

The ionization energy has a major effect on the fragmentation of the molecule and therefore on the mass spectrum. The reference EI mass spectra that exist in mass spectral libraries, such as OCAD, Wiley and NIST (National Institute of Standards and Technology), are recorded using the electron energy of 70 eV [32, p. 142]. In this thesis, ionization energy of 70 eV is used in order to obtain reproducible fragmentation pattern for compounds and to produce mass spectra that are comparable with the library spectra. [31, p. 505]

4.2 High-Performance Liquid chromatography

High-performance liquid chromatography (HPLC) is an analytical technique that uses high pressure to force solvent containing sample mixture through a closed column packed with fine particles. The chromatographic separation of the compounds relies on different partition behavior of components between two phases: a liquid mobile phase and a stationary phase. The mobile phase is liquid solvent (e.g. water, methanol, acetonitrile) and the stationary phase is either solid or liquid. [31, p. 596]

The basic instrumentation of the HPLC includes mobile phase reservoirs, pumps, an injector, a column and a detector. Liquid eluent is pumped from the eluent inlet at stable flow up to 2 ml/min. A loop injector is very commonly used injector type for HPLC. The liquid sample is introduced into a loop with a nominal volume. The pumps maintain constant flow rate through the HPLC system and the injection is completed by moving a rotating switch. This directs the flow through the loop and the liquid sample is flushed among the mobile phase into the column. The separation of the compounds occurs in the column and the separated compounds are detected after exiting the column. A method that uses a mobile phase of constant composition during entire elution is termed isocratic elution. In gradient elution the composition changes during the analysis. The rate at which the composition is changed has a major impact on the separation of the compounds. [33, pp. 10–12]

4.2.1 HPLC Column

A typical HPLC column consists of fine particles packed tightly into a steel reinforced tube. These particles are employed as the stationary phase. The stationary phase can be either solid, such as silica particles, or immiscible liquid bonded to a solid support.

HPLC column dimensions are typically 5–30 cm length and 1–5 mm inner diameter. The particle size of the stationary phase column is usually from 1.7 to 5 µm. The efficiency of a column can be increased by decreasing the stationary phase particle size. When using smaller particle size, higher pressure is required but improvement in resolution is achieved. Alternatively, the use of smaller particles shortens the run time while the same resolution is maintained. [31, pp. 596–602]

In this thesis, the HPLC analyses were carried out with hydrophobic reversed-phase (RP) column. The RP column stationary phase consists of nonpolar hydrocarbon chains bonded covalently to the silica surface. The very commonly used stationary phase is an octadecyl (C_{18}) alkyl group bonded to the silica surface (Figure 8). Interactions with nonpolar stationary phase cause longer retention times for nonpolar molecules while polar molecules elute more readily with the mobile phase. In RP chromatography less peak tailing occurs compared to normal-phase chromatography because the RP stationary phase has fewer sites that can strongly adsorb molecules and cause peak tailing. [31, p. 603]

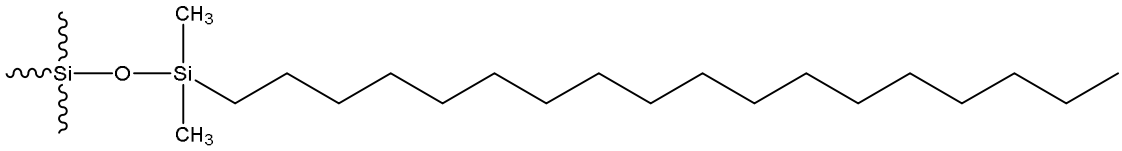


Figure 8. An octadecyl alkyl group attached to the silica surface [31, p. 600].

4.2.2 Electrospray ionization

Solvent in LC creates a significant volume of gas when vaporizing. Most of this gas must be removed before the analytes are introduced into the mass spectrometer. Electrospray ionization (ESI) is a technique in which protonated or deprotonated molecules are transferred from liquid solvent into gaseous phase and the excess gaseous eluent is disposed. ESI is a soft ionization technique that causes little fragmentation of analytes. It can be operated in both positive and negative mode. In positive mode, protonated molecules ($[M+H]^+$) or other adduct ions (e.g. $[M+Na]^+$) are formed and in negative mode, deprotonated ($[M-H]^-$) molecules are formed. [31, pp. 519–521]

Eluent from the HPLC enters a metal capillary needle at relatively low flow rate. A high voltage (2–6 kV) is applied to the capillary needle relative to the spray chamber. Liquid enters the spray chamber where a strong electric field and a coaxial flow of N_2 sheath gas disperses the solvent into a fine aerosol of highly charged droplets. Nitrogen drying gas evaporates the solvent diminishing the droplets until the repulsive force of charged molecules equals the cohesive force of surface tension. The droplets break up into smaller droplets which evaporate and release the charged molecules into the gaseous phase. The analytes pass through a sampling cone or a heated capillary and enter the mass analyzer. A schematic representation of ESI is shown in Figure 9. [34]

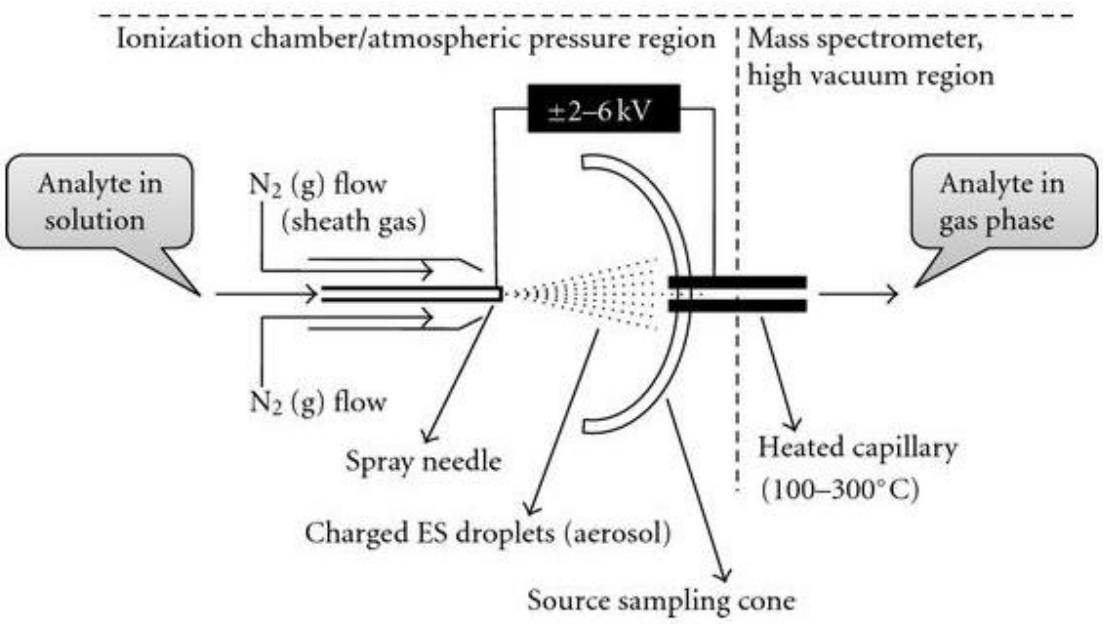


Figure 9. Schematic representation of ESI [34.].

4.3 Mass spectrometry

Mass spectrometer (MS) is a commonly used detector in chromatography that provides both quantitative and qualitative information on molecules [31, p. 519]. Mass spectrometry makes identification of compounds possible with a high degree of confidence. Also, compounds that have similar retention characteristics and are not fully resolved chromatographically can be differentiated by their mass spectra [33, p.3]. In mass spectrometry charged molecules or fragments of molecules are accelerated in vacuum by an electric field and separated according to their mass and charge (mass-to-charge ratio, *m/z*). Charged fragments of a compound with different *m/z* are

analyzed in the detector and a mass spectrum representing the detector response vs. *m/z* is obtained [31, p. 502].

4.3.1 Quadrupole mass spectrometer

In this thesis, quadrupole mass spectrometer was used. It is a common mass separator with ability to scan ions fast using low voltages which makes it suitable detector for chromatography. Figure 10 shows the structure of a quadrupole mass separator. Four parallel metal rods are applied with a constant (DC, direct current) voltage and a radio-frequency (RF) alternating voltage. Ions arriving from the ionization chamber migrate between the rods towards the detector. The applied voltages affect the trajectory of the charged ion. For given voltages, only ions of a particular *m/z* (resonant) reach the detector while others (nonresonant) collide with the rods. By rapidly and systematically varying the voltages, ions of different *m/z* reach the detector and a mass spectrum is produced. The size of the ions detected can be as high as 4000 *m/z* units [31, p. 514]. [33., p.41]

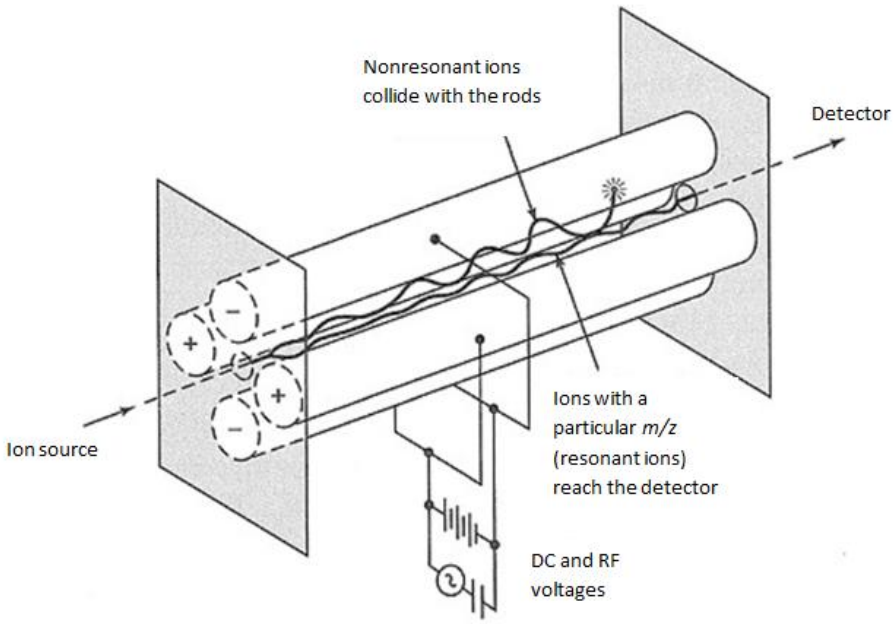


Figure 10. Structure of a quadrupole mass separator [31, p. 513].

4.3.2 Triple quadrupole mass spectrometer

Triple quadrupole mass spectrometer is a tandem mass spectrometer (MS/MS) where two mass separators are connected in series. The triple quadrupole mass spectrometer consists of two quadrupole mass separators and a quadrupole employed as a collision cell between them. The mass selective detection system can be operated in multiple reaction monitoring (MRM) that provides very sensitive and selective method for detecting targeted molecules: as low as parts per trillion levels can be achieved [31, p. 524]. The schematic representation of the triple quadrupole mass spectrometer and the principle of MRM are shown in Figure 11.

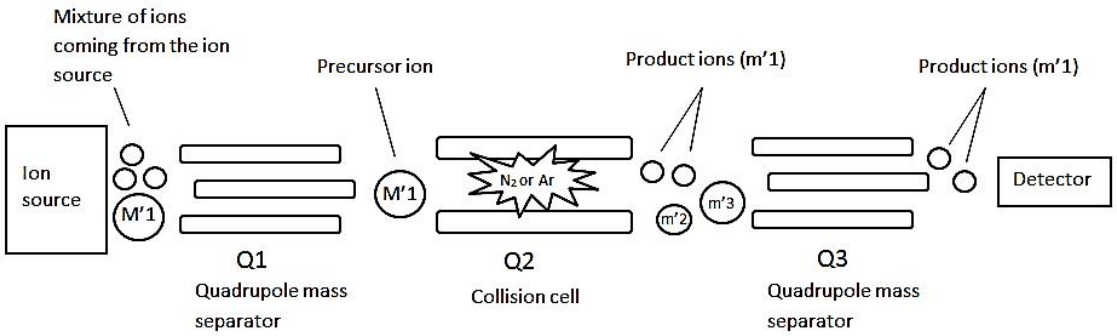


Figure 11. Schematic representation of a triple quadrupole mass spectrometer and the principle of MRM [31., p. 525].

In MRM, a mixture of ions arrives to quadrupole Q1 from the ion source. Ions with selected *m/z*, precursor ions, are allowed to pass the quadrupole. In Q2 precursor ions are collided to N_2 or Ar at very low pressure (~10^{-5} to 10^{-3} Torr). This produces characteristic molecule fragments called product ions. These ions are introduced to Q3 and the product ions of selected *m/z* pass the quadrupole and reach the detector which measures the quantity of the ions. [31, p. 524]

5 Experimental

5.1 Safety measures

Normal laboratory safety procedures were followed when working with hazardous chemicals. Due to high potency and chemical stability of fentanyl, extra attention was paid when handling the substance. Garg et al. reported that in their studies no

applicable and effective treatment to degrade fentanyl using light, base, heat or oxidation was found [35]. In this experimental all the equipment that had been in contact with the analytes was rinsed with 10 % KOH in ethanol decontamination solution. Decontamination solution waste was then collected into a plastic vessel and sent to the local waste disposal company for further processing.

5.2 Purchase of the reference standard chemicals

The substances observed in this thesis are used as pharmaceutical drugs and some of them have also importance in drug abuse. Amphetamine, fentanyl and diazepam are classified as narcotic drugs in Finnish narcotic legislation (543/2008). The Finnish Narcotic Act (373/2008) prohibits production, manufacture, import to the territory of Finland, export from the territory of Finland, distribution, trade, handling, possession of these above-mentioned narcotic drugs. Deviations from the prohibitions are allowed, for example, for research purposes. [36]

In order to purchase reference standards for amphetamine, fentanyl and diazepam, licenses to import and to handle these narcotics were applied from Finnish Medicines Agency (Fimea). It should be noted that each compound to be handled and imported has to be specified in the application, including different salt forms of the substance. For example, a license to handle or import fentanyl covers only fentanyl free base, not fentanyl salts (e.g. fentanyl citrate).

5.3 Chemicals and standards

The chemicals and reference standards used in the experimental are listed in Table 4. 0.2 M acetate buffer (pH 4) used in the urine analysis was prepared by weighing 3.775 g of ammonium acetate and dissolving it into 200 ml of ultrapure water. pH was adjusted to 4 by adding acetic acid into the solution. Water was added to obtain final volume of 250 ml. All the spiking solutions were prepared in methanol at concentrations described in the sample preparation section.

Table 4. Chemicals and standard used in the experimental.

Chemicals	Use	Manufacturer	Purity
Acetone	Solvent	Sigma Aldrich	≥99.8 %
Acetonitrile	Solvent	BDH	≥99.8 %
Dichloromethane	Solvent	VWR	HPLC grade
Methanol	Solvent	VWR	HPLC grade
Ultrapure water	Solvent	in-house	18.2 µS/cm (conductivity)
Ammonium acetate	Acetate buffer	Merck	pro analysis
Formic acid	LC eluent	Merck	98 - 100 %
Acetic acid	Acetate buffer	Merck	99.8 %
BSTFA	Silylation reagent	Alltech	-
Reference standards	Solvent / volume	Manufacturer	Concentration
Fentanyl	Methanol / 1 ml	Sigma Aldrich	1 mg/ml
Fentanyl-d_5	Methanol / 1 ml	Sigma Aldrich	100 µg/ml
Amphetamine	Methanol / 1 ml	Sigma Aldrich	1 mg/ml
Naloxone	Methanol / 1 ml	Sigma Aldrich	1 mg/ml
Diazepam	Methanol / 1 ml	Sigma Aldrich	1 mg/ml

5.4 Materials

The materials used in the experimental are listed in Table 5. In the sample preparation TurboVap LV Concentration Workstation was used for concentrating and Branson 3210 Ultrasonic Cleaner for sonicating the samples. The ultrapure water was drawn from Milli-Q (Merck Millipore, 0.22 µm filter) filter apparatus.

Table 5. Materials used in the experimental

Material	Manufacturer	Specifications
SPE cartridge	Oasis	HLB, volume 3 ml, 60 mg sorbent per cartridge
Cotton swab	-	Non-sterile wood hospital applicators, 150 mm x 2.2 mm
Cotton wipe	TexWipe	TX306, 100 % cotton, 15 cm x 15 cm
Filter paper	GE Healthcare	Whatman 50, hardened, diam. 90 mm
Disposable filter	Millex	0.2 µm, low protein binding hydrophilic (PTFE) membrane

5.5 Urine samples

Blank and standard samples were prepared into a pool of fentanyl-free urine collected from four healthy donors. The authentic urine sample was obtained from a patient who was given fentanyl intravenously prior to a surgical procedure. The sample was taken

approximately 4 hours after the injection. The authentic urine sample and pooled fentanyl-free urine were stored in the freezer at - 20 ^{o}C.

5.6 Instrumentation

5.6.1 GC–MS instrumentation

The GC–MS instrumentation and method parameters are listed in Table 6. Method 1 corresponds to the recommended GC conditions used for screening CWC-related chemicals and measuring retention indices for the OCAD [37]. This method was applied in the screening experiments. The data acquisition was operated on full scan mode in the method 1. Method 2 was applied when measuring the recoveries of the analytes from the wipe samples. The data acquisition was operated on SIM (selected ion monitoring) mode in the method 2 (the monitored ions are given in Table 7). Method 2 was set to increase the oven temperature fast after elution of amphetamine-TMS (retention time 12.70 min). This was done in order to improve the peak shapes and to reduce retention times of the late-eluting compounds fentanyl and naloxone-3TMS. Except for the data acquisition mode and temperature program, the methods 1 and 2 were identical.

Table 6. GC–MS instrumentation and method parameters.

GC–MS instrumentation		
GC	Agilent Technologies 6890N	
MS	Agilent Technologies 5975N	
Column	DB-5MS, 30 m x 250 µm x 0.25 µm	
Method	Method 1	Method 2
Injection mode	splitless	splitless
Splitless time	1 min	1 min
injection volume	1 µl	1 µl
Injection temperature	250 °C	250 °C
Carrier gas	He	He
Flow pressure	0.487 bar	0.487 bar
Temperature program	1 min at 40 °C	1 min at 40 °C
	10 °C/min to 300 °C	10 °C/min to 160 °C
	5 min at 300 °C	30 °C/min to 300 °C
		10 min at 300 °C
MS	Method 1	Method 2
Ionization	EI	EI
Electron energy	70 eV	70 eV
Tranfer line temperature	290 °C	290 °C
Ion source temperature	230 °C	230 °C
Data acquisition mode	Full scan	SIM
Scan range	40 - 600 *m/z*	See Table 7

Table 7. The ions monitored in SIM.

Analyte	Quantifier ion, Q (*m/z*)	Qualifier ions, q (*m/z*)
Fentanyl	245	146, 189
Amphetamine-TMS	91	116, 192
Naloxone-3TMS	438	528, 543

5.6.2 LC–MS/MS instrumentation

The LC–MS/MS instrumentation and method parameters are listed in Table 8. Same LC parameters were used in both wipe and urine analysis. Optimized MRM conditions for each precursor and product ion are given in Table 9. The MRM conditions were optimized by infusing 10 µg/ml analyte of interest in water into the ESI source and adjusting the cone voltages and collision energies manually.

Table 8. LC–MS/MS instrumentation and method parameters.

LC-MS instrumentation	
LC	Waters Acquity UPLC H-class
Column	Waters Acquity UPLC BEH C18 1.7 µm, 2.1 x 100 mm
Mass spectrometer	Waters TQD Xevo TQD
LC parameters	
Injection volume	5 µl
Flow rate	0.6 ml/min
Column temperature	60 ^{o}C
Mobile phase A	0.1 % HCOOH in H_2O (v/v)
Mobile phase B	0.1 % HCOOH in MeOH (v/v)
Gradient	1 % B and 99 % A for 0.6 min
	From 1 % to 100 % (B) in 0.6 - 2.3 min
	100 % B for 1.7 min
Total run time	5.5 min
MS parameters	
Ionization mode	ESI+
Capillary voltage	3.5 kV
Source temperature	120 ^{o}C
Desolvation gas	N_2
Desolvation gas flow	1000 l/h
Desolvation temperature	500 ^{o}C
Collision gas	Argon
Mass resolution	0.75 amu

Table 9. MRM transitions and conditions for amphetamine, fentanyl, fentanyl-d_5 and naloxone. Q refers to quantifier ion and q to qualifier ion.

Analyte	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (V)
Amphetamine	136	40	119 (q)	15
			91 (Q)	10
Fentanyl	337	40	216 (q)	25
			105 (q)	35
			188 (Q)	25
Fentanyl-d5	342	35	105 (q)	35
			188 (Q)	25
Naloxone	328	35	212 (q)	40
			310 (Q)	20

6 Sample preparation

6.1 Preparation of wipe samples

Preparation of the wipe samples was conducted according to the ROP of wipe samples [38]. The wipes were extracted successively with organic solvent and water. Because of the different size of the wipes, the extraction and spiking volumes varied depending on the wipe. The wipe-specific spiking and extraction volumes are shown in Table 10. Three different wipe materials (cotton swab, Whatman filter paper and cotton wipe) and two different organic extraction solvents (acetone and dichloromethane) were used in the ROP testing experiments. Acetone and dichloromethane were selected for the study because they are considered as possible non-polar organic extraction solvents in the ROP [38].

Figure 12 shows the flowchart of the sample preparation process. In total 6 sample batches were prepared and analyzed. One batch of samples included six replicate standard samples spiked with fentanyl, naloxone and amphetamine and two matrix blanks. For recovery study, one matrix blank extract was spiked with the analytes in the end of the sample preparation.

Table 10. Sample preparation specifications for different wipe materials.

	Cotton swap	Whatman filter paper	Cotton wipe
V (spiking solution)	100 µl	100 µl	200 µl
m (analyte) / wipe	4 µg	4 µg	8 µg
V (extraction vial)	8 ml	20 ml	100 ml
V (extraction solvent)	2 x 2.5 ml	2 x 10 ml	2 x 50 ml
V (volumetric flask)	5 ml	20 ml	100 ml
V (aliquot prepared)	1 ml	5 ml	10 ml
V (post-spiked spiking solution)	20 µl	25 µl	20 µl
c (analytes in the final sample with 100 % recovery)	1.6 µg/ml	2.0 µg/ml	1.6 µg/ml

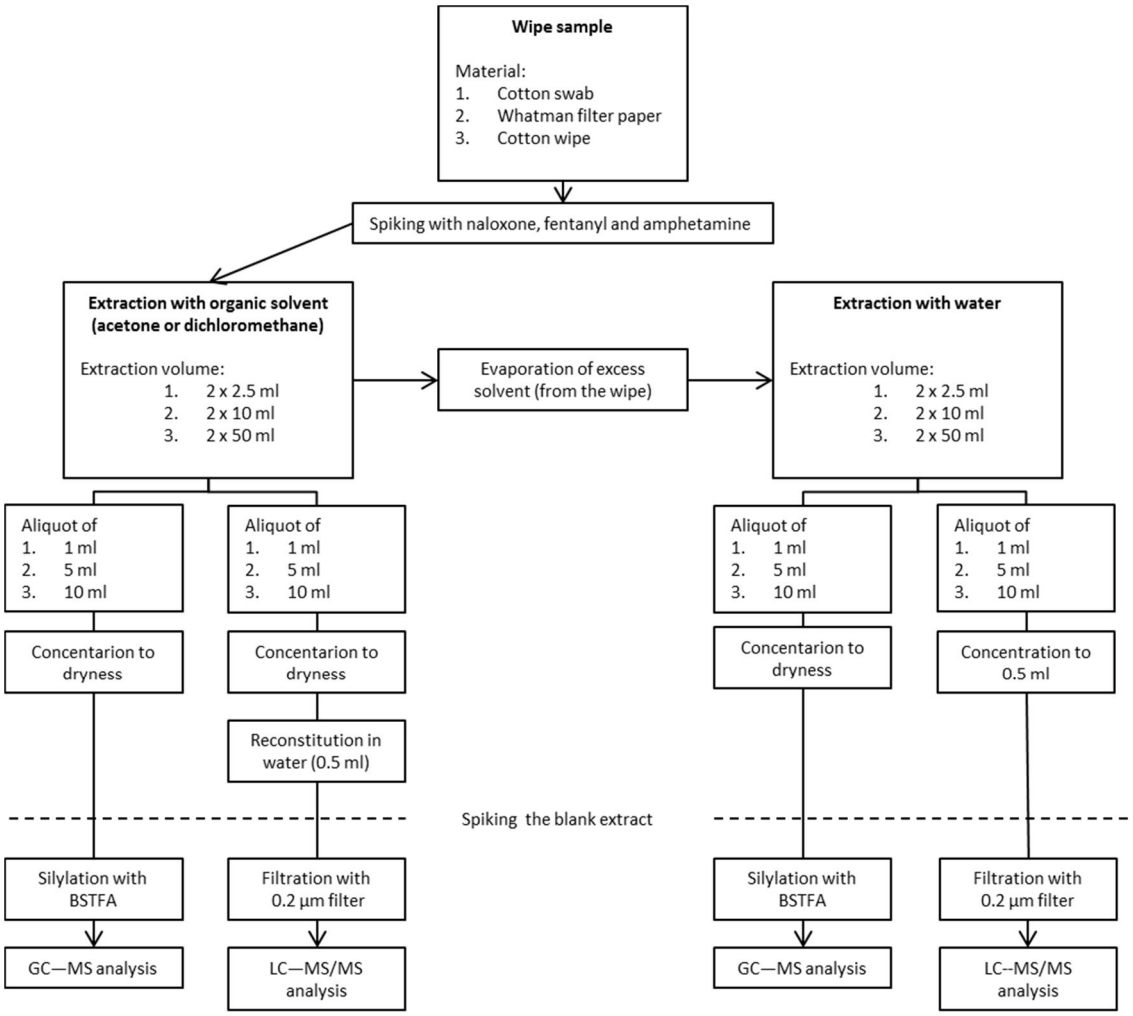


Figure 12. Sample preparation flowchart for wipe samples.

The wipe was inserted into a glass vial. The Whatman filter paper and the cotton wipe were folded multiple times before placing into the vial. The wooden rod of the cotton swab was cut off and the cotton wad was placed into the vial. The wipe was wetted with 1 ml of organic extraction solvent (except for cotton swab which was not wetted) and spiked with spiking solution (40 µg/ml fentanyl, naloxone and amphetamine in methanol). The wipe was allowed to dry for 60 min. The first portion of organic extraction solvent was added into the vial and the sample was sonicated for 3 min. After sonication the extraction solvent was transferred into a volumetric flask. The extraction procedure was repeated with another portion of organic extraction solvent. The volumetric flask was filled with solvent. The excess extraction solvent was allowed to evaporate from the wipe. After evaporation, the wipe was extracted with two portions of ultrapure water in a similar way to the organic solvent.

Organic fraction

For LC–MS/MS analysis, an aliquot of the organic extract was transferred into a test tube with screw cap and evaporated to dryness under nitrogen flow in TurboVap at 40 ^{o}C. Immediately after evaporation the residue of the aliquot was reconstituted in 500 µl of ultra-pure water. One matrix blank extract was spiked with 40 µg/ml spiking solution for recovery study. The samples were filtered with 0.2 µm disposable filter and analyzed by LC–MS/MS.

For GC–MS analysis, an aliquot of the organic fraction was evaporated to dryness in TurboVap at 40 ^{o}C. The residue of the aliquot was reconstituted in 200 µl of BSTFA and 200 µl of acetonitrile (one matrix blank extract was spiked with 40 µg/ml spiking solution prior to evaporation to dryness). The sample was incubated at 60 ^{o}C for 30 min. The sample was allowed to cool and 100 µl of dichloromethane was added prior to analysis by GC–MS.

Water fraction

Two aliquots of the aqueous extract were prepared in a similar way to the organic aliquots. For GC–MS analysis, an aliquot was evaporated to dryness and silylated with BSTFA as described before. For LC–MS/MS analysis, an aliquot was concentrated into final volume of 500 µl and filtered with 0.2 µm disposable filter.

6.2 Preparation of urine samples

The urine standard and blank samples were prepared into pooled fentanyl-free urine. 500 µl of urine was spiked with 20 µl of isotope labeled internal standard (IS) spiking solution (250 ng/ml fentanyl-d_5 in methanol). Calibrators and quality control samples (QCs) were spiked with fentanyl spiking solution into desired concentrations (Table 11). The SPE procedure used to clean the samples was based on the method of Wang & Bernert with some minor modifications [20].

Table 11. Spiking solutions and volumes for standard samples.

Standard sample	c (ng/ml)	V (fentanyl-d_5 250 ng/ml spiking solution)	V (fentanyl 50 ng/ml spiking solution)	V (fentanyl 500 ng/ml spiking solution)
Calibrator	0	20 µl	-	-
Calibrator	0.5	20 µl	5 µl	-
Calibrator	1	20 µl	10 µl	-
Calibrator	5	20 µl	50 µl	-
Calibrator	10	20 µl	-	10 µl
Calibrator	25	20 µl	-	25 µl
Calibrator	50	20 µl	-	50 µl
QC	1	20 µl	10 µl	-
Recovery standard	1	20 µl (post-spiked)	10 µl	-
Recovery standard	25	20 µl (post-spiked)	-	25 µl

The urine was diluted with 500 µl of 0.2 M ammonium acetate buffer (pH 4). The mixture was vortexed and let to equilibrate for 30 min. After equilibration the sample was loaded into an OASIS HLB cartridge preconditioned with 1 ml of methanol and 1 ml of water, respectively. The sample was washed with 1 ml of 20 % (v/v) methanol in water. Excess washing solution was expelled from the cartridge. Fentanyl was eluted from the cartridge with 1 ml of methanol. The eluate was evaporated to dryness under nitrogen flow in TurboVap at 40 ^{o}C. The residue was dissolved in 200 µl of 0.1 % (v/v) formic acid in water, filtrated with 0.2 µm disposable filter and analyzed by LC–MS/MS.

The validation experiments were conducted in four days. Three calibration curves were prepared each day, except for the last day when only two calibration curves were made for the recovery study. The seven point calibration curves were prepared at concentration levels of 0, 0.5, 1, 5, 10, 25 and 50 ng/ml. To determine extraction recovery three replicate samples were prepared by spiking pooled urine (with no IS added) with fentanyl at concentrations of 1 ng/ml and 25 ng/ml. The internal standard was added after extraction prior to evaporation to dryness. These recovery standards were quantified against normally prepared calibration curves.

Among each calibration batch a urine blank with no IS added and a solvent blank (made in water instead of urine) were analyzed. In addition, a QC sample at the concentration of 1 ng/ml was prepared each day. This QC was analyzed before, between and after each calibration batch in order to see if any variation in results occurs. The authentic samples were analyzed during the validation experiments. In total seven replicate samples were prepared from the urine of the surgical patient and analyzed.

7 Results and discussion

7.1 Mass spectra

7.1.1 EI mass spectra

One of the purposes of this thesis was to produce EI mass spectra for the selected candidate chemicals and submit the spectral data to be evaluated and included in the OCAD. The standard samples were analyzed by GC–EI–MS and the mass spectra were extracted with AMDIS software. There are certain requirements for the conditions under which the spectral data has to be recorded. The lowest recorded mass should be *m/z* 40 or lower and the highest at least 50 *m/z* above the molecular weight of the measured compound. The mass spectrum has to contain the peaks with intensity of 0.1 % or higher from the base peak. The EI mass spectra were produced for amphetamine-TMS, naloxone-3TMS, naloxone-TMS, fentanyl, fentanyl-d_5, and diazepam. Appendix 1 presents the hard copy of the accompanying information for the mass spectra to be submitted to the OCAD.

The mass spectra of TMS derivatives of amphetamine (Figure 13) and naloxone (Figure 14) show abundant peaks at *m/z* 73. This peak corresponds to a TMS cation which is very commonly found on mass spectra of the TMS derivatives. Due to poor selectivity of the fragment, it was not used as quantifier or qualifier ion. Fragment ion M–15, which is generated by the loss of methyl from the TMS groups, is shown at *m/z* 192 and at *m/z* 528 in the amphetamine-TMS and naloxone-3TMS mass spectra, respectively. [30, p.562]

The peak at *m/z* 91 in amphetamine-TMS mass spectrum corresponds to tropylium ion or benzylic cation. The base peak at *m/z* 116 results probably from the loss of methylbenzene. Naloxone-3TMS produced a large variety of fragments with relatively low abundances. The ions with high *m/z* (*m/z* 543, 528 and 438) were selected for SIM due to better selectivity of large fragments. In addition to naloxone-3TMS, three other products were formed in silylation of naloxone with BSTFA. This was due to keto-enol tautomerism and incomplete silylation of the hydroxyl groups (see paragraph 2.4.). The total ion chromatogram (TIC) of a silylated naloxone standard showing the presence of multiple silylation products of naloxone is presented in Appendix 2.

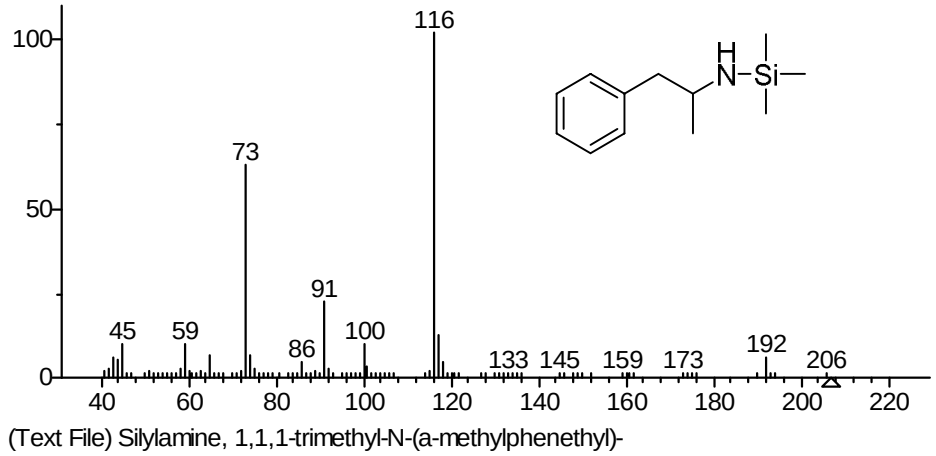


Figure 13. GC–EI–MS full scan mass spectrum of amphetamine-TMS (MW=207).

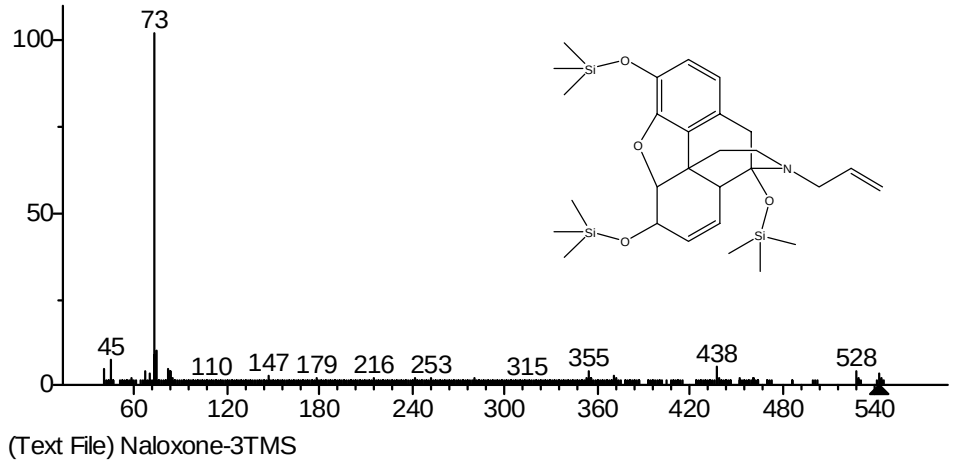


Figure 14. GC–EI–MS full scan mass spectrum of naloxone-3TMS (MW=543).

The base peak at *m/z* 245 and 250 in fentanyl (Figure 15) and fentanyl-d_5 (Figure 16) mass spectra are proposed to represent fragments generated by the loss of methylbenzene. The mass spectrum of diazepam (Figure 17) show base peak at *m/z* 256 which results from the elimination of CO molecule. The peak formed by the loss of chlorine from the fragment *m/z* 256 is present at *m/z* 221. The peak at *m/z* 283 corresponds to loss of a hydrogen radical from the molecular ion. [39]

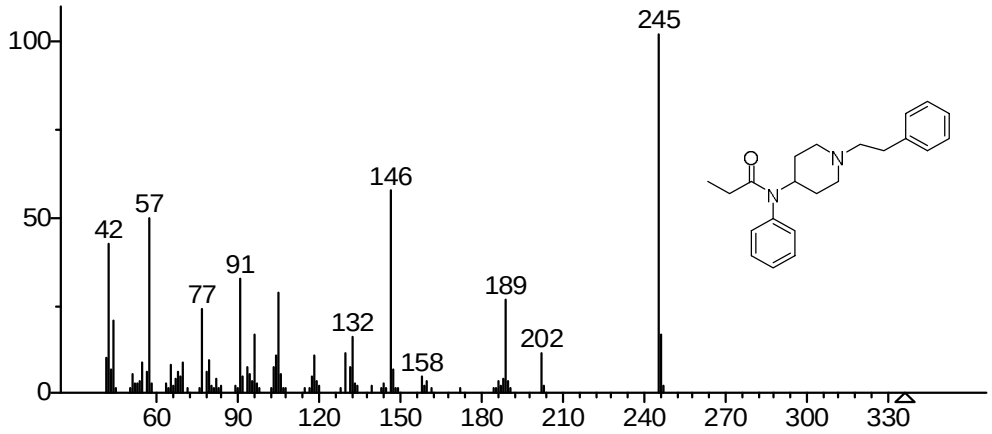


Figure 15. GC–EI–MS full scan mass spectrum of fentanyl (MW=336).

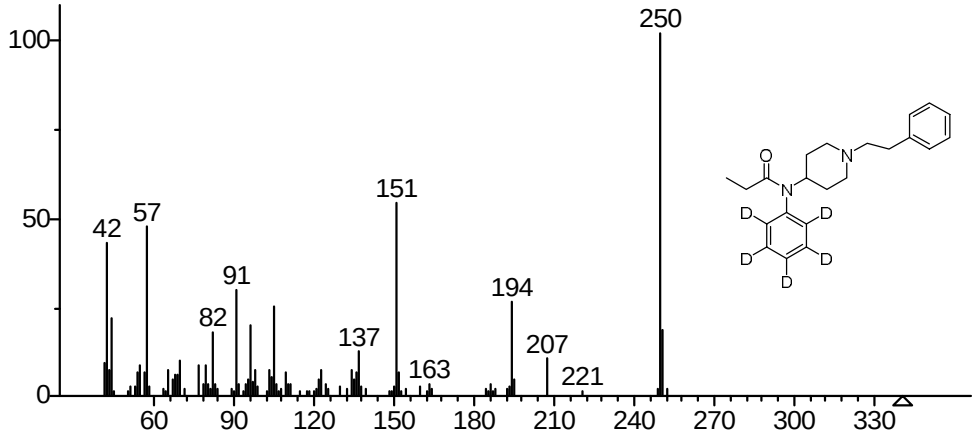


Figure 16. GC–EI–MS full scan mass spectrum of fentanyl-d_5 (MW=341).

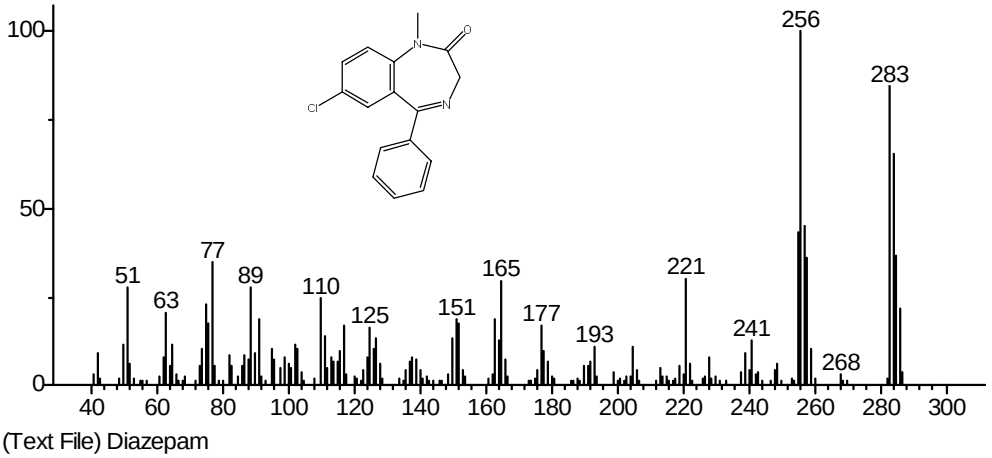


Figure 17. GC–EI–MS full scan mass spectrum of diazepam (MW=284).

7.1.2 ESI product ion mass spectra

ESI product ion mass spectra were recoded for fentanyl, fentanyl-d_5, amphetamine and naloxone with LC–MS/MS. The analytes were ionized on positive ESI and the MS/MS was operated on product ion scan. The proposed structures of the product ions and the neutral losses are given in Table 12.

Product ion mass spectra of both fentanyl and fentanyl-d_5 (Figures 18 and 19) show two major peaks at *m/z* 188 and 105. The $[M+H]^+$ can be seen at m/z 337 and 342 for fentanyl and fentanyl-d_5, respectively. The ion at *m/z* 188 is formed by the loss of N-phenylpropanamide and the ion at *m/z* 105 results from the loss of piperidine [40]. Fentanyl-d_5 showed a product ion at *m/z* 221 which is 5 units higher than corresponding product ion in the fentanyl spectrum (*m/z* 216). This indicates that the product ion contains the phenyl group labeled with 5 deuterium atoms. The structures of product ions at m/z 188, 216 and 221 are adopted from the study of Wang & Bernert [20].

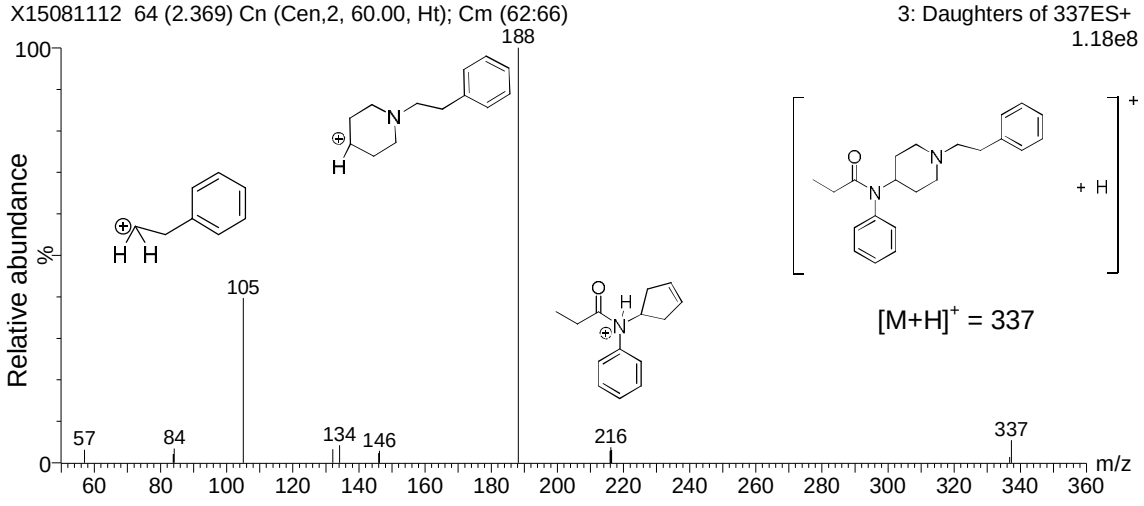


Figure 18. LC–ESI–MS/MS product ion mass spectrum of fentanyl produced with collision energy of 25 V.

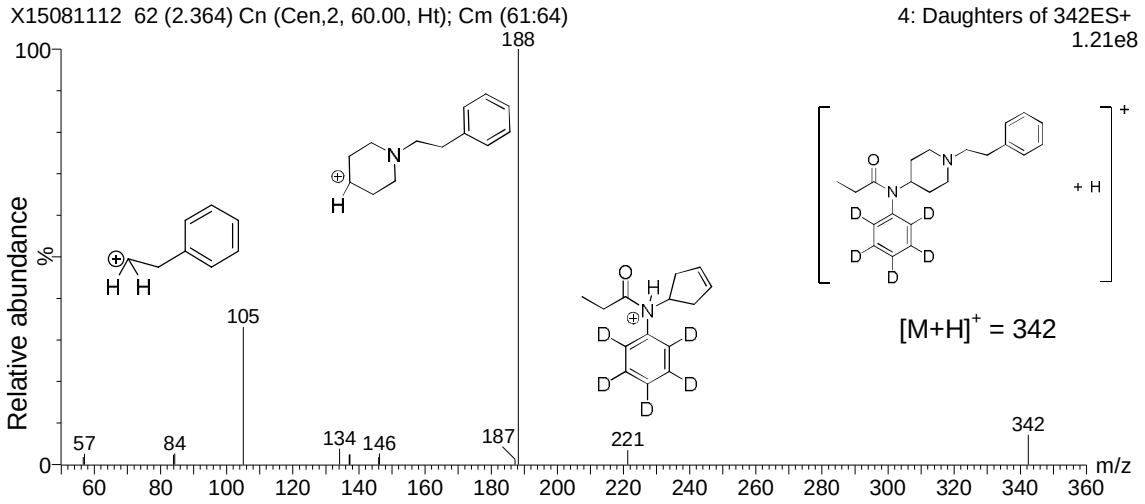


Figure 19. LC–ESI–MS/MS product ion mass spectrum of fentanyl-d_5 produced with collision energy of 25 V.

The product ion mass spectrum of naloxone (Figure 20) shows the base peak at *m/z* 310 which results from the loss of water. This product ion was selected as quantifier due to high intensity, although ROP doesn't recommend fragments formed by the loss of water to be used in MRM [41]. The peak at *m/z* 328 represents $[M+H]^+$ ion of naloxone. Naloxone produced a large number of different product ions with low relative abundances.

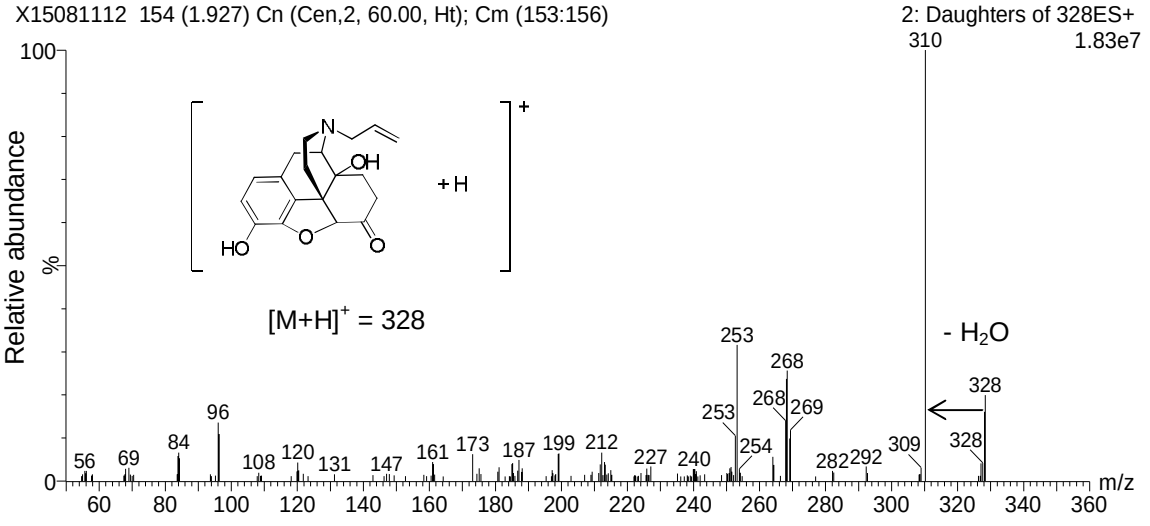


Figure 20. LC–ESI–MS/MS product ion mass spectrum of naloxone produced with collision energy of 25 V.

The product mass ion spectrum of amphetamine (Figure 21) shows two major peaks at m/z 119 and 91. The weak peak of $[M+H]^+$ ion can be seen at *m/z* 136. The fragment

ion *m/z* 119 corresponds to a neutral loss of ammonium from molecular ion of amphetamine. The fragment ion at m/z 91 is proposed to represent benzyl cation or tropylium ion $C_7H_7^+$ resulting from loss of ethylene.

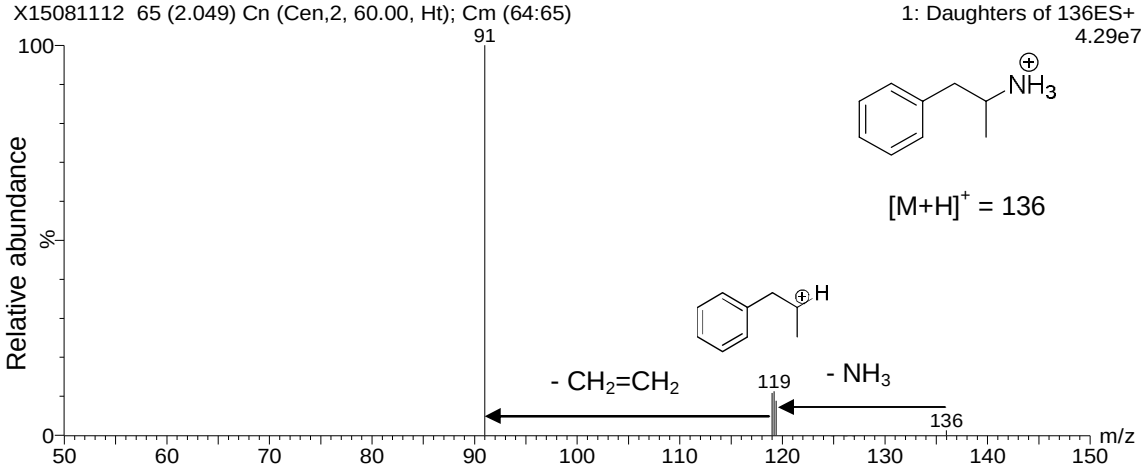
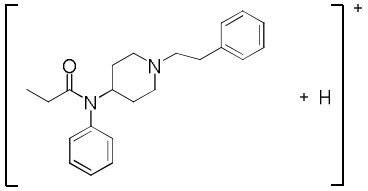
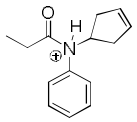
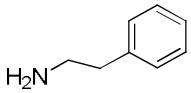
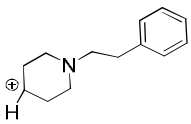
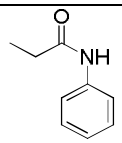
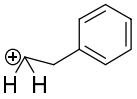
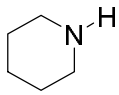
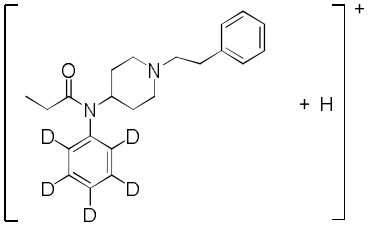
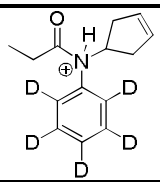
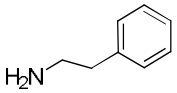
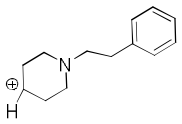
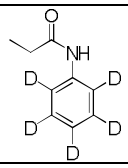
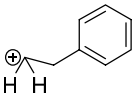
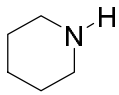
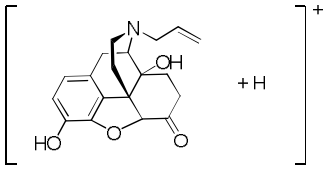
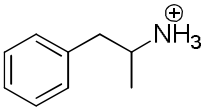
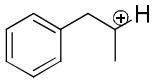
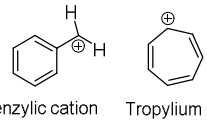


Figure 21. LC–ESI–MS/MS product ion mass spectrum of amphetamine produced with collision energy of 15 V.

Table 12. $[M+H]^+$ ions of fentanyl, fentanyl-d_5, naloxone and amphetamine and proposed structures of their product ions and neutral losses.

Analyte	$[M+H]^+$	Product ion		Neutral loss	
Fentanyl	 $[M+H]^+ = 337$		*m/z* 216		121 amu
			*m/z* 188		149 amu
			*m/z* 105		85 amu
Fentanyl-d5	 $[M+H]^+ = 342$		*m/z* 221		121 amu
			*m/z* 188		154 amu
			*m/z* 105		85 amu
Naloxone	 $[M+H]^+ = 328$	$[C_{19}H_{20}NO_3]^+$	m/z 310	H_2O	18 amu
Amphetamine	 $[M+H]^+ = 136$		*m/z* 119	NH_3	17 amu
		 Benzylic cation Tropylium ion	*m/z* *91*	$CH_2=CH_2$	28 amu

7.2 Study on wipe samples

7.2.1 Comparison of extraction solvents

Dichloromethane and acetone were tested for their efficiency to extract fentanyl, naloxone and amphetamine from different wipe materials. The efficiency was assessed by recovery of the analytes. The recovery was determined by comparing the peak area of the analyte in the standard sample extract to the peak area in the blank sample extract spiked with the analyte in the end of the sample preparation. The recoveries were calculated using the following equation:

$$Recovery\ (\%) = \frac{Integrated\ peak\ area\ of\ the\ analyte\ in\ the\ standard\ sample\ extract}{Integrated\ peak\ area\ of\ the\ analyte\ in\ the\ post\text{–}spiked\ matrix\ blank\ extract} \cdot 100\ \%$$

Table 13 shows mean recoveries and standard deviations (SD) of the analytes from wipe samples analyzed by both GC–MS and LC–MS/MS. Calculated recoveries for each sample are given in Appendix 3.

Table 13. Mean recoveries and standard deviations of amphetamine, fentanyl and naloxone from wipe samples. The highest recovery for each analyte is bolded.

	Recoveries, Mean ± SD (%) (n = 6)			
	Dichloromethane & water		Acetone & water	
	DCM fraction	Water fraction	Acetone fraction	Water fraction
	LC–MS[2] / GC–MS	LC-MS[2] / GC–MS	LC–MS[2] / GC–MS	LC–MS[2] / GC–MS
Amphetamine				
Cotton swab	45.6±4.6 / 34.1±4.3	39.4±4.5 / 9.2± 4.0	71.0±2.4 / 33.5±14.8	21.9 ±3.2 / 12.6 ±2.2
Filter paper	1.2±0.6 / -	65.7±5.2 / 27.8 ± 9.3	57.0±2.7 / 54.0±31.4	31.8 ±3.0 / 7.1 ± 2.5
Cotton wipe	3.5±1.3 / -	60.9±4.8 / 52.4 ± 12.6	63.4±4.3 / 68.1±30.1	22.8 ±4.4 / -
Fentanyl				
Cotton swab	77.9±9.6 / 68.6±7.4	7.6±0.7 / 1.9 ± 0.3	80.5 ± 3.7 / 69.3±4.8	5.4 ± 0.8 / 2.2 ± 0.4
Filter paper	6.7±2.8 / 16.9±3.7	25.7±5.7 / 10.4 ± 1.3	76.2±14.6 / 69.5±7.0	6.3 ± 2.1 / 3.2 ± 0.9
Cotton wipe	31.4±7.9 / 28.6±2.4	34.3±5.0 / 28.2 ± 9.8	76.7± 2.9 / 83.8±9.0	9.3 ± 2.3 / 5.8 ± 0.9
Naloxone				
Cotton swab	76.0±3.6 / 66.2±12.0	9.3±1.0 / 2.9 ± 0.2	80.2±1.8 / 70.8±3.4	7.2±0.9 / 3.8±0.6
Filter paper	18.2±3.2 / 10.2±2.0	45.0±4.7 / 14.9 ± 2.4	86.5±2.4 / 136.8±37.5	8.2±1.4 / -
Cotton wipe	16.0±1.9 / 16.9±1.4	46.5±5.6 / 49.8 ± 11.9	72.6±4.2 / 85.4±7.3	11.9±2.2 / 8.1±1.6

Acetone provided significantly higher recoveries for all the analytes compared to dichloromethane. In the acetone extracts, the recoveries for fentanyl and naloxone

were constantly over 70 % and for amphetamine over 50 %. Dichloromethane extracted the analytes poorly from filter paper and cotton wipe but relatively high recoveries were achieved from cotton swab. In some cases major difference in recovery results can be observed between GC–MS and LC–MS/MS analyses from the same extract. Generally, the recoveries analyzed by GC–MS were lower and the SDs higher. This may be due to lower sensitivity of GC–MS and the noisy background of the chromatogram caused by the silylation reagent. Incomplete silylation of the analytes may also have occurred. In some samples the concentrations were too small to be analyzed by GC–MS.

Both organic solvents dissolved the wipes made of cotton: cotton wipes and cotton swaps. White solid particles from the wipe matrix appeared in the sample after reconstitution of organic extract evaporation residue to water for LC–MS/MS analysis. These particles didn't exist in the filter paper extract. There may have been some loss of amphetamine during the evaporation step due to high volatility of the compound, although recovery of over 70 % was achieved for amphetamine at highest. However, the evaporation to dryness should be avoided if possible.

7.2.2 Screening

The ROPs that describe the methods for screening and identifying CWC-related chemicals were tested for the candidate chemicals. For the study, a cotton wipe containing fentanyl, amphetamine and naloxone (10 µg each) was extracted with acetone. Acetone was selected as the extraction solvent due to its high extraction efficiency (Table 13). The cotton wipe used in this experiment is the same that the OPCW uses in on-site sampling. Two aliquots of acetone extract were prepared, one for LC–MS/MS analysis and one for GC–MS analysis. The aliquot prepared for GC–MS analysis was silylated with BSTFA. Sample preparation was conducted as described earlier (see paragraph 6.1.).

7.2.2.1 GC–MS screening

GC–EI-MS analysis produces two kinds of analytical data that can be used in screening and identification of the chemical: the mass spectrum and the retention time. The identification is performed by comparing the experimental mass spectrum and retention time of an unknown chemical to the library mass spectra and retention times

of known chemicals. The retention time is usually converted into retention index (RI). The RI is calculated by comparing retention time of the analyte of interest to retention times of a group of standards. These standards are usually straight chain hydrocarbons of different lengths. The RI for each standard is determined by their carbon number (the carbon number is multiplied by 100, for example, giving RI of 800 for octane and 1200 for dodecane). The experimental retention time is compared to the retention times of adjacent standards and converted into RI by interpolation. [41]

The degree that describes how closely the experimental unknown spectrum matches the library spectrum is expressed as match factor (MF), reversed match factor (RMF) and net match factor (NMF). These match factors give a value ranging from 0 to 1000 (or from 0 to 100, depending on the numerical scale). The MF calculation is based on similarity of the *m/z* values and intensities of the peaks in both the unknown spectrum and the library spectrum. The difference between RMF and MF is that RMF do not take account the extra peaks that are found in the search spectrum but do not exist in the library spectrum. This is useful when analyzing compounds in complex matrices, although RMF is more likely to give false positive identification. NMF is determined by combining both MF and NMF as follows:

$$Net\ Match\ Factor\ (NMF) = 0.75MF + 0.25RMF.$$ [41]

AMDIS (the Automated Mass Spectral Deconvolution and Identification System) software was employed in screening and identification of the candidate chemicals from the GC–EI–MS data. The software is able to calculate RIs and to process the GC–MS data by extracting spectra for individual components and performing automated spectral cleaning. AMDIS searches and identifies target chemicals by comparing the found mass spectra to library spectra. The reference mass spectra were recorded and the spectral library was built for these candidate chemicals before the screening experiment. [42]

The silylated wipe extract was analyzed with GC–MS on full scan mode (Figure 22). The produced data was then analyzed using AMDIS software. The software performed spectral cleaning, gave NMF for the found mass spectra and calculated the RIs. Each analyte of interest was found by AMDIS. The mass spectra extracted by AMDIS were also searched against NIST database which includes a large variety of mass spectra produced by different laboratories. Figures 23, 24 and 25 show extracted ion

chromatograms and mass spectra of the found compounds. Table 14 lists the determined RIs, match factors and total S/N (signal-to-noise) of the extracted ions.

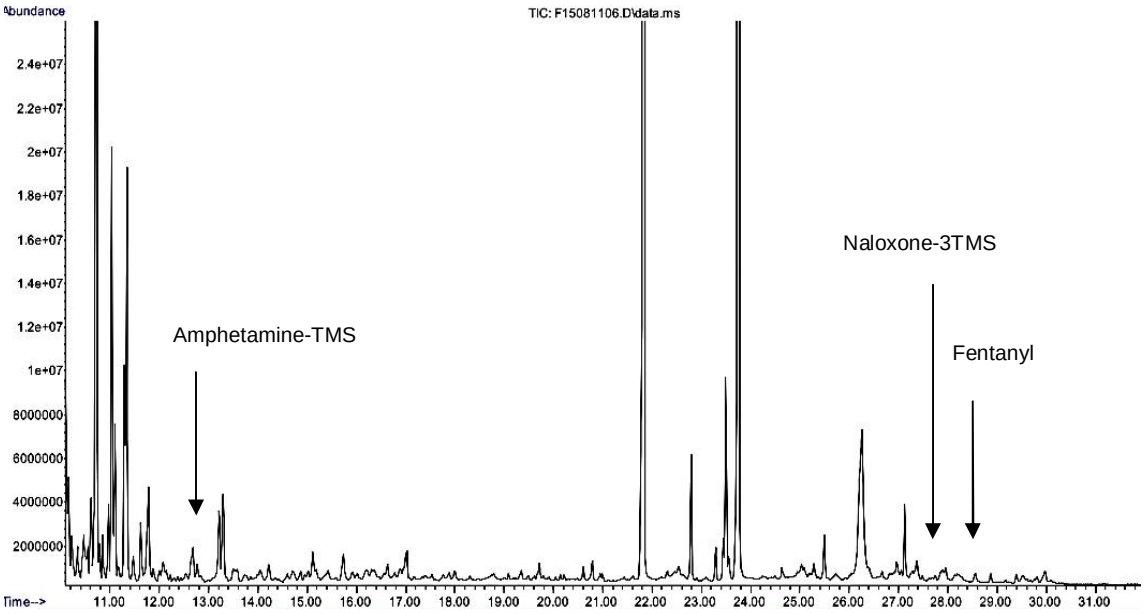


Figure 22. GC–EI–MS TIC of the silylated cotton wipe extract

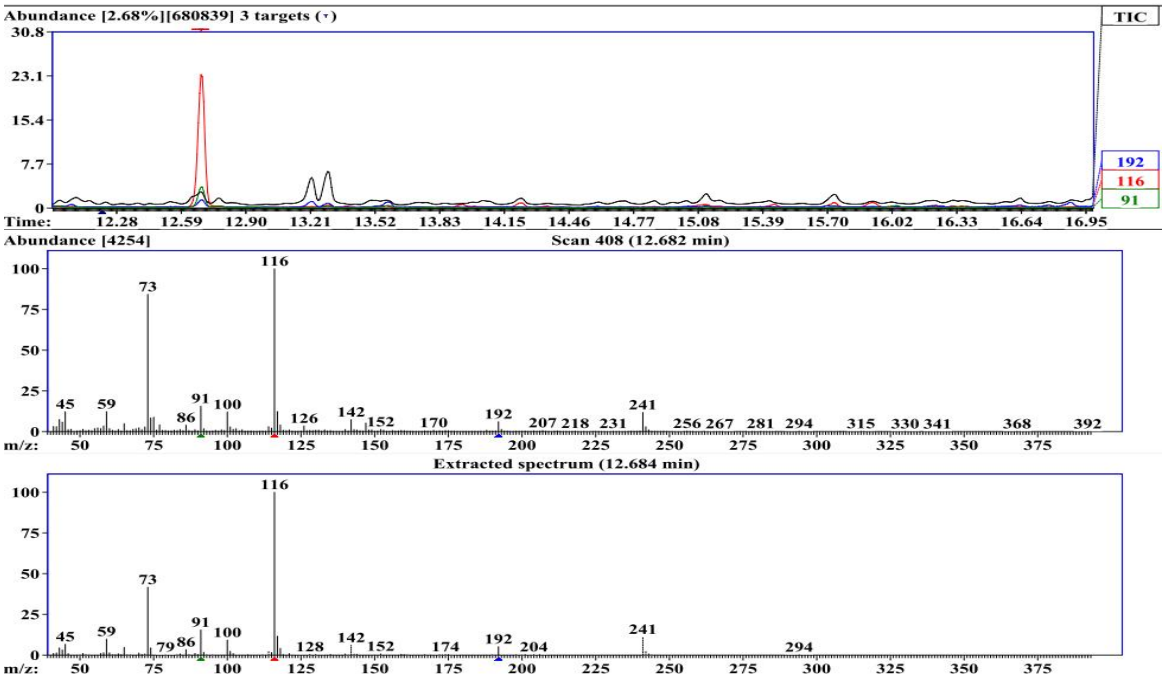


Figure 23. GC–EI–MS TIC of the silylated cotton wipe extract analyzed by AMDIS showing the presence of amphetamine-TMS. *Top*: TIC with extracted ions *m/z* 192, 116 and 91, *middle*: scanned mass spectrum at 12.682 min, *bottom*: extracted mass spectrum at 12.682 min after automatic cleanup by AMDIS.

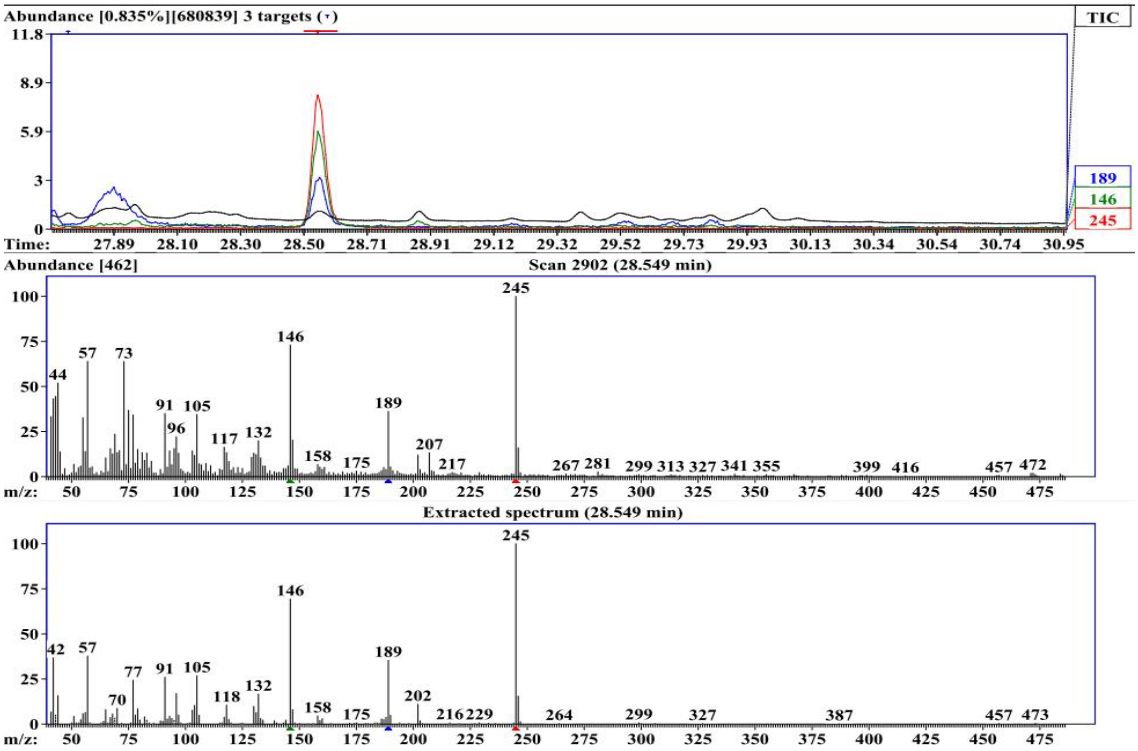


Figure 24. GC–EI–MS TIC of the silylated cotton wipe extract analyzed by AMDIS showing the presence of fentanyl. *Top*: TIC with extracted ions *m/z* 189, 146 and 245, *middle*: scanned mass spectrum at 28.549 min, *bottom*: extracted spectrum at 28.549 min after automatic cleanup by AMDIS.

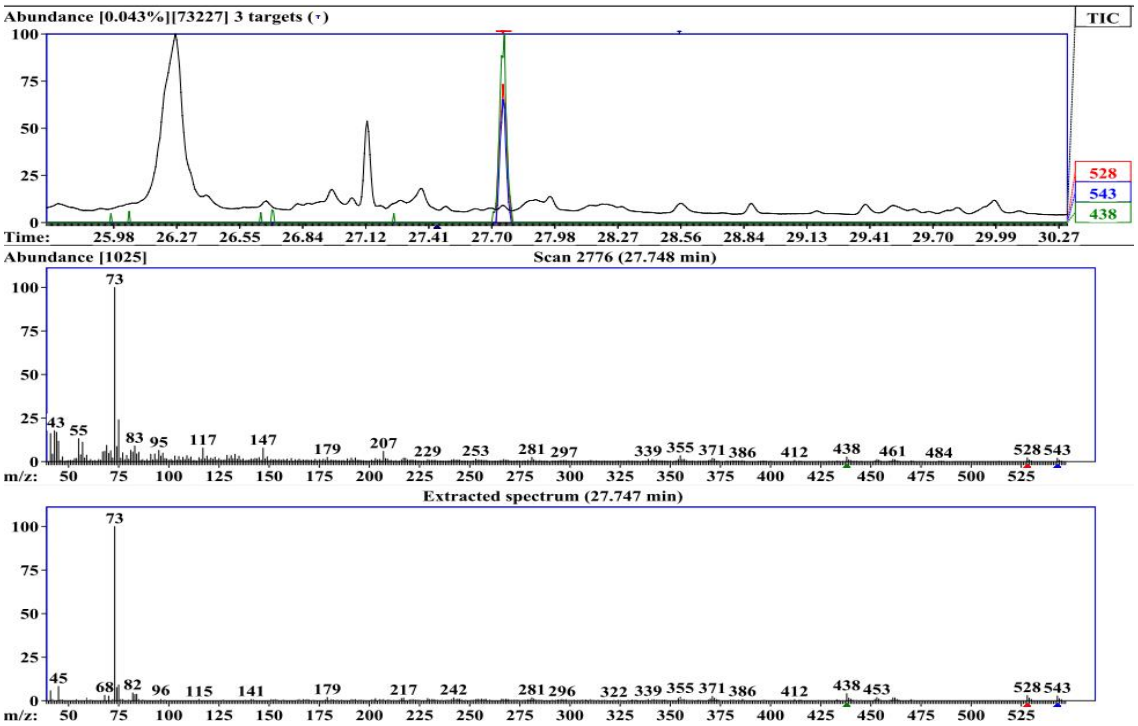


Figure 25. GC–EI–MS TIC of the silylated cotton wipe extract analyzed by AMDIS showing the presence of naloxone-3TMS. *Top*: TIC with extracted ions m/z 543, 528 and 438, *middle*: scanned mass spectrum at 27.748 min, *bottom*: extracted spectrum at 27.747 min after automatic cleanup by AMDIS.

Table 14. RTs, RIs, match factors and S/N of the target analytes in the silylated wipe extract.

Chemical	RT (min)	RI	NMF (AMDIS)	MF (NIST)	RMF (NIST)	NMF (NIST)	Total S/N of extracted ions
Fentanyl	28.55	2770	95	927	969	938	125
Amphetamine-TMS	12.68	1303	93	841	936	865	236
Naloxone-3TMS	27.75	2682	86	-	-	-	91

The NMFs computed by AMDIS were high, over 90 for fentanyl and amphetamine-TMS and 86 for naloxone-3TMS (maximum 100). The search against NIST database gave NMF of 865 and 938 for amphetamine-TMS and fentanyl, respectively (maximum 999). No reference spectrum for naloxone-3TMS was found in the NIST database. In OPCW Proficiency Test the identification criterion is defined as minimum value of 80 or 800 for match factors [41]. Because of the late elution of naloxone-3TMS and fentanyl, the retention times were converted to RI by extrapolation. The GC–MS was found to be valid technique for screening and identification of the chemicals in question at parts-per-million (ppm, µg/g) concentration levels.

7.2.2.2 LC–MS/MS screening

The first step in LC–MS/MS screening was to analyze the wipe sample extract on LC-MS full scan mode. The $[M+H]^+$ ions of the analytes were then extracted from TIC according to their *m/z*. TIC is shown in Figure 26 and the extracted precursor ion chromatograms are seen in Figure 27. The extracted precursor ions distinguished clearly from the background offering S/N of 754, 573 and 1882 for fentanyl, naloxone and amphetamine, respectively. Also, the precursor ion peaks can be visually detected in the TIC.

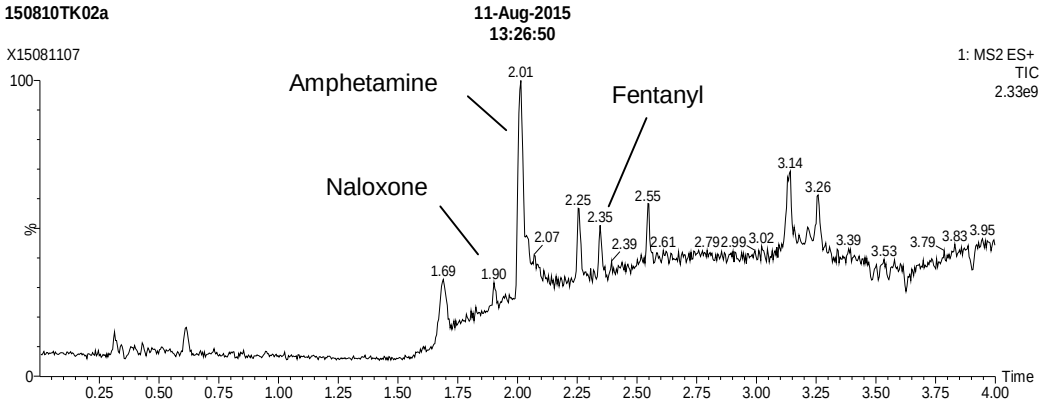


Figure 26. LC–ESI–MS TIC of the wipe extract.

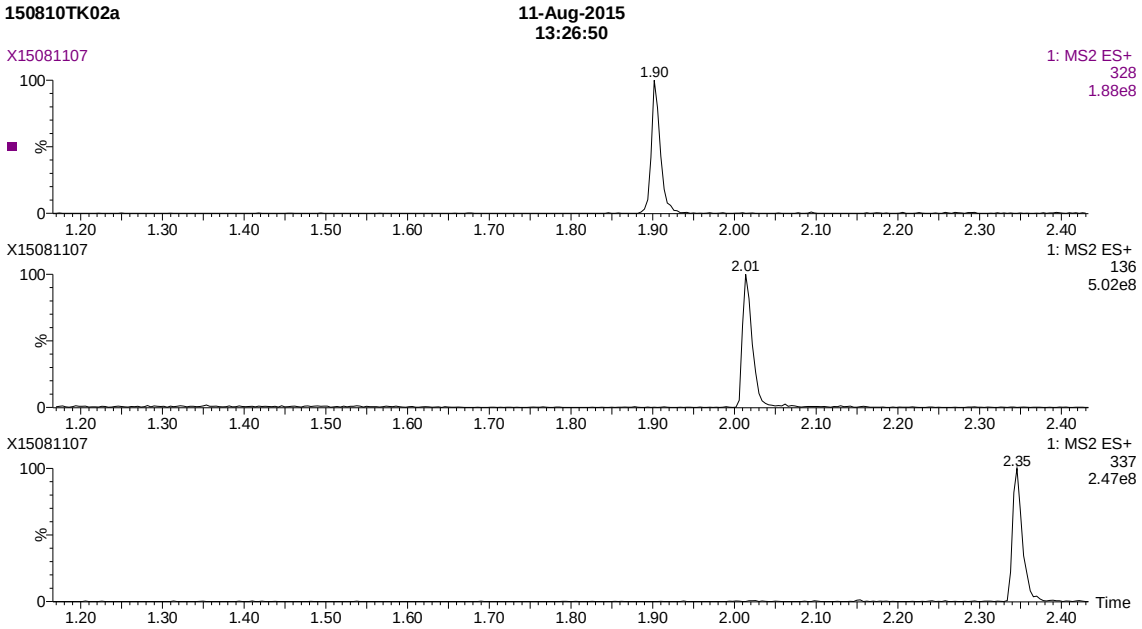


Figure 27. Precursor ions of naloxone (*m/z* 328), amphetamine (*m/z* 136) and fentanyl (*m/z* 337) extracted from the LC–ESI–MS TIC.

After the full scan, targeted screening using MRM was applied for the candidate chemicals. This screening is based on searching chemicals with known molecular weights, retention times and product ions. The MRM method (Table 9) was developed by selecting the product ions to be monitored and optimizing cone voltages for precursor ions and collision energies for selected product ions. The ions to be monitored were selected according to the product ion spectra of the analytes (see Figures 18, 19, 20 and 21). In total seven transitions were monitored. The transitions monitored offered excellent S/N of 20 000 or higher (Figure 28).

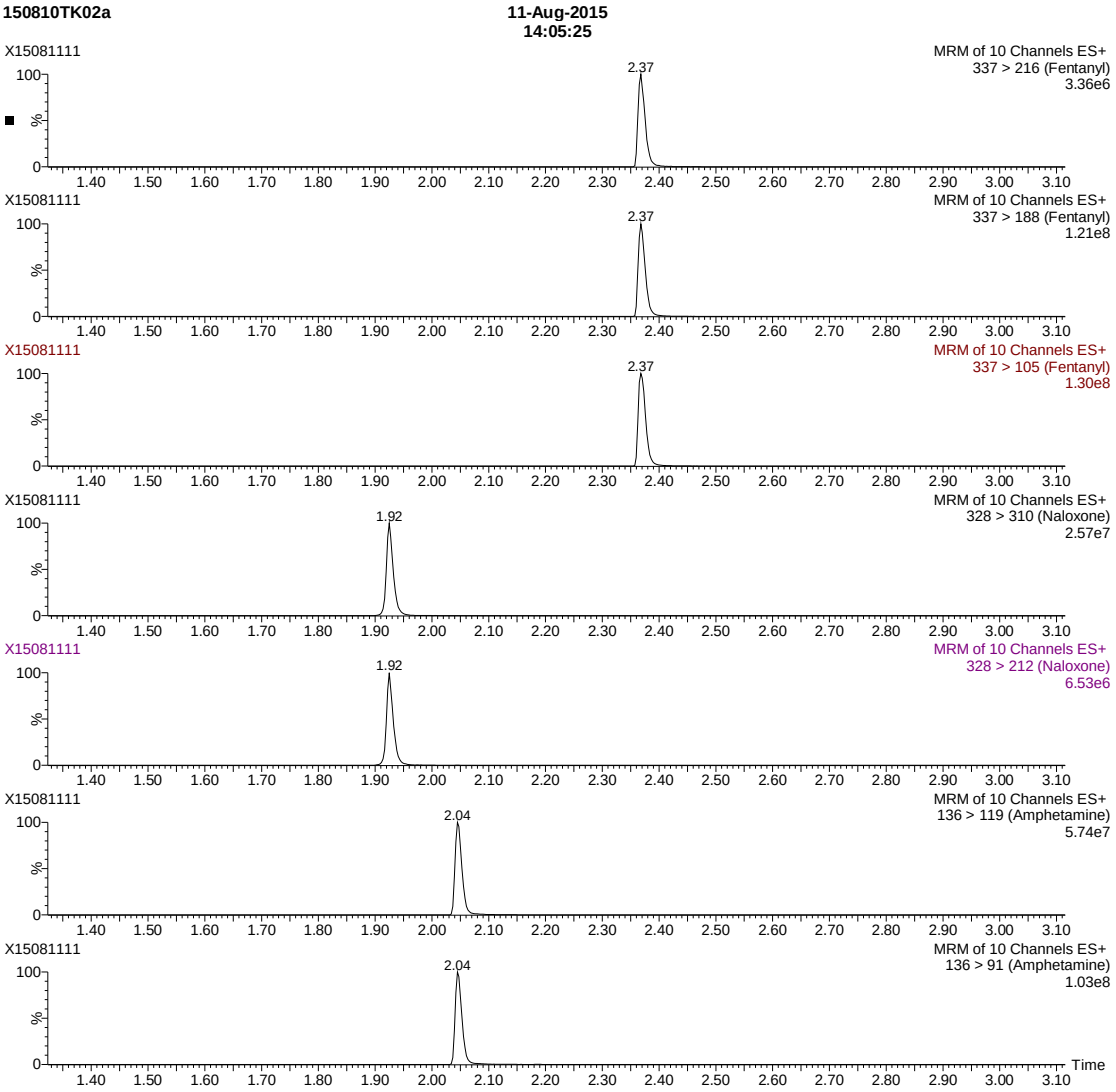


Figure 28. LC–ESI–MS/MS MRM chromatograms of the wipe extract. The transitions monitored were (from top to bottom) *m/z* 337 → 216, *m/z* 337 → 188 and *m/z* 337 → 105 for fentanyl, *m/z* 328 → 310 and *m/z* 328 → 212 for naloxone and *m/z* 136 → 119 and *m/z* 136 → 91 for amphetamine.

The LC–MS/MS was discovered to be valid technique for screening and identification of the chemicals in question at parts per million concentration levels. Compared to GC–MS, the LC–MS/MS was found to be more appropriate technique for analysis of these candidate chemicals. Significantly better sensitivity was achieved and the analytes did not require derivatization prior to analysis by LC–MS/MS. In addition, the LC–MS/MS analysis saved time compared to GC–MS analysis in terms of shorter run times and simpler sample preparation.

7.3 Analysis of fentanyl in urine

7.3.1 Linearity

Linearity was assessed by correlation coefficient (R^2) and visual inspection of residual plots of the measured calibration curves (n = 9). Figure 29 represents a typical calibration curve measured. Good linearity was achieved for fentanyl in the concentration range of 0.5–50 ng/ml. Each calibration curve showed correlation coefficient of over 0.9995 and as can be seen in residual plot (Figure 30) the residuals are dispersed randomly on both sides of x-axis indicating that the calibration points are associated linearly. All the calibration curves and residual plots are given in Appendix 4.

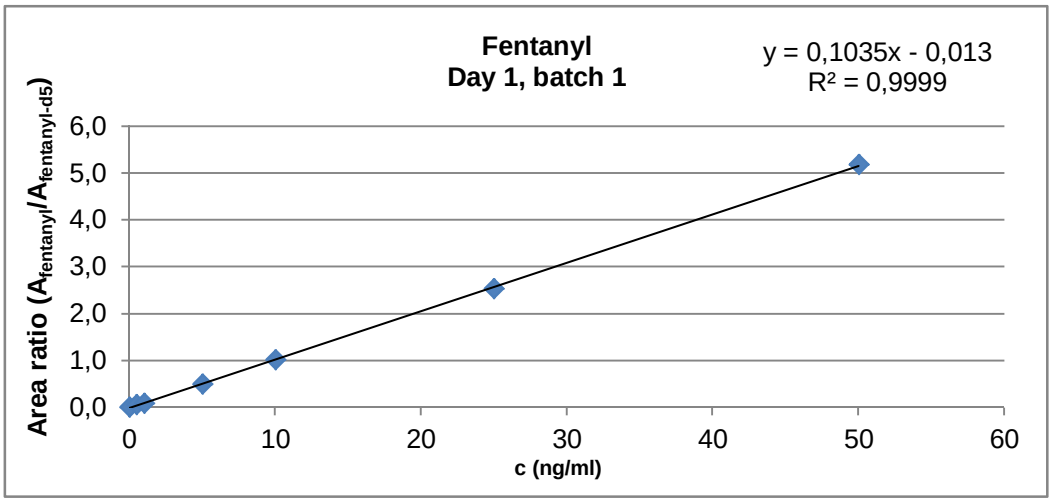


Figure 29. Typical calibration curve for fentanyl.

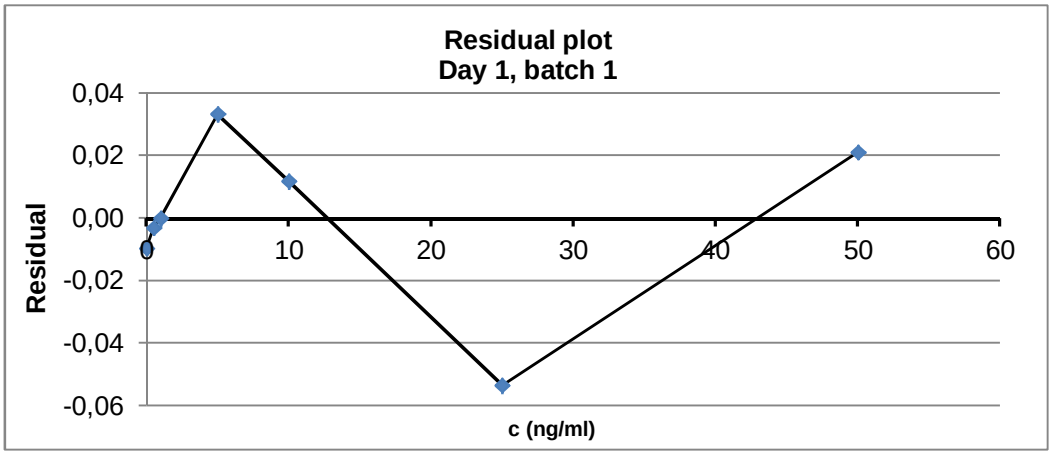


Figure 30. Typical residual plot of a fentanyl calibration curve.

7.3.2 LOD and LOQ

Limit of detection (LOD) describes the lowest concentration of analyte at which the detection and identification is feasible and the analyte can be reliably distinguished from the background noise [43, p.10]. LOD can be calculated from the calibration curve (y = mx + n) using equation

$$LOD = \frac{3.3 \cdot S_n}{m},$$

where
S_n = standard deviation of y-intercepts,
m = slope [44, p. 11].

Limit of quantification (LOQ) is the lowest concentration of analyte that can be quantitated with acceptable precision and accuracy [45]. LOD can be calculated from the calibration curve (y = mx + n) using equation

$$LOQ = \frac{10 \cdot S_n}{m},$$

where
S_n = standard deviation of y-intercepts,
m = slope [44, p. 12].

LOD and LOQ were calculated for each calibration curve (results are given in Appendix 5) and method's LOD and LOQ for fentanyl were expressed as the mean values. LOD and LOQ were determined to be 0.4 ng/ml and 1.3 ng /ml, respectively. The calculated limits were relatively high and, for instance, the LOQ was higher than the two lowest calibration levels. These high limits could be explained by the wide concentration range of the calibration curve. Although the linearity was evaluated to be good at range of 0.5–50 ng/ml fentanyl in urine, the wide concentration range causes relatively large deviation on the measured y-intercept values which can be observed as high LOD and LOQ. This could be avoided by preparing separate calibration curves for low and high concentrations.

However, as can be seen in the chromatogram of a urine standard at 0.5 ng/ml (Figure 31), the peaks for transitions *m/z* 337 → 188 and *m/z* 337 → 105 are distinguished

clearly from the background. The peaks offered mean S/N of 280 for *m/z* 188 and 60 for *m/z* 105. The minimum S/N criteria for LOD and LOQ are typically 3 and 10, respectively [44, pp. 11–12]. In addition, the accuracy and precision criteria set for the LOQ were satisfied (see paragraph 7.3.5). Hence, the method's LOD and LOQ for fentanyl were determined to be 0.5 ng/ml. It is probable that significantly lower LOD and LOQ could be achieved for fentanyl by using lower concentration level calibration curve.

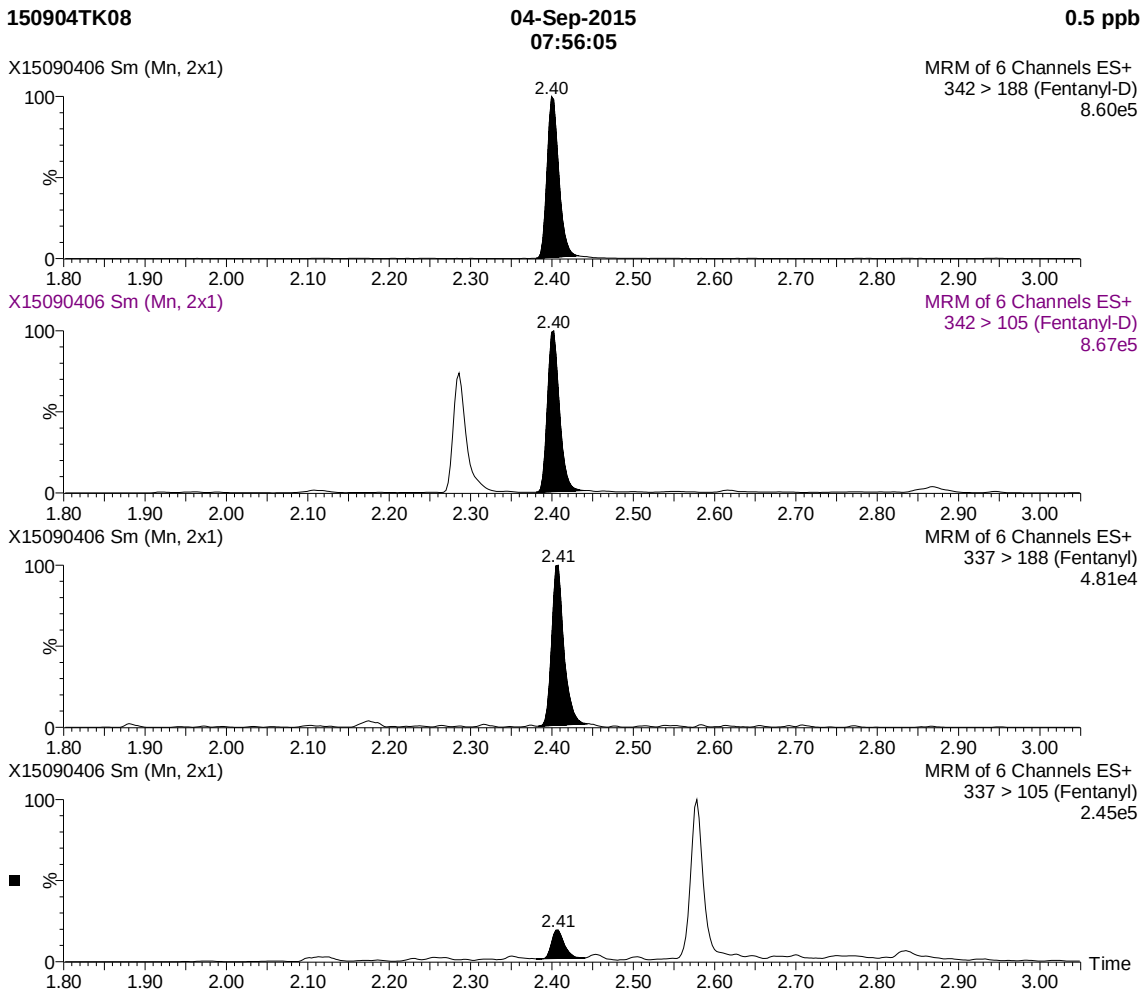


Figure 31. LC–ESI–MS/MS MRM chromatograms of a urine standard at 0.5 ng/ml. The transitions monitored were (from top to bottom) *m/z* 342 → 188, *m/z* 342 → 105 for fentanyl-d5 and *m/z* 337 → 188, *m/z* 337 → 105 for fentanyl.

7.3.3 Selectivity

Selectivity describes the method's ability to differentiate the analyte from the other interfering components in the sample matrix. Selectivity of the method was evaluated

by blank samples (no fentanyl or IS spiked) which were analyzed among the validation batches. The MRM chromatograms (Figure 32) show no interferences on observed transitions in urine blank.

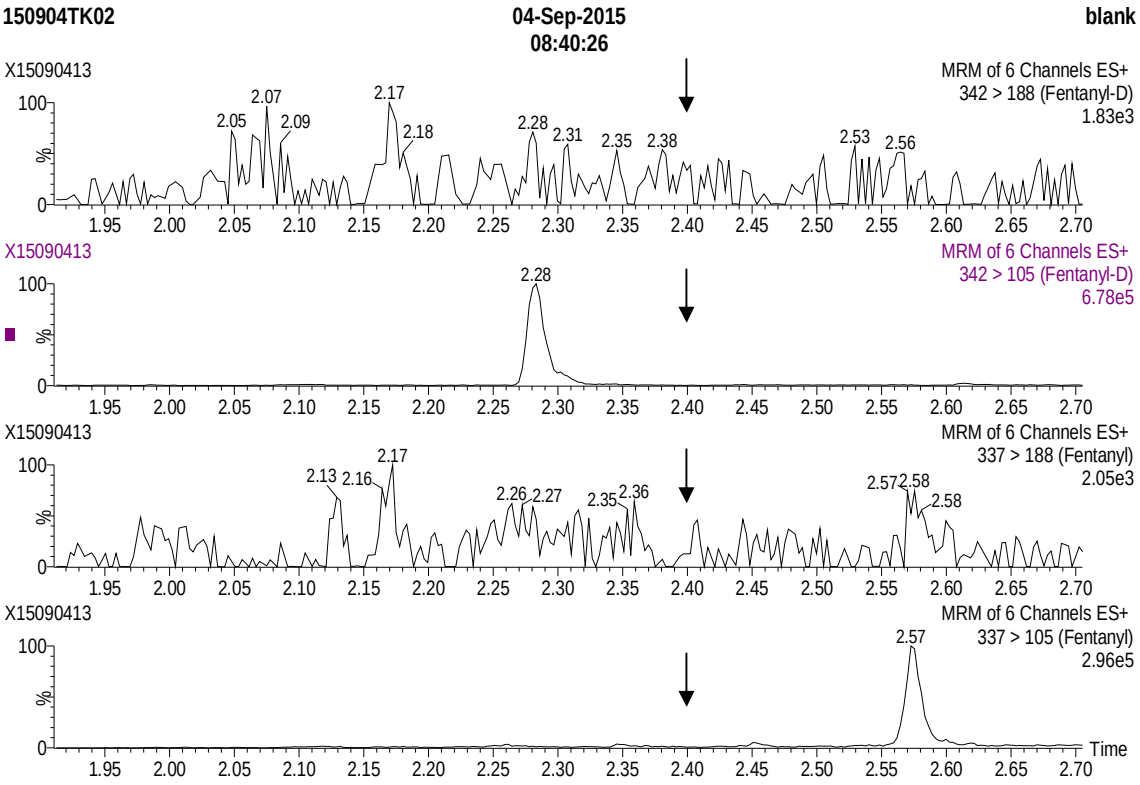


Figure 32. LC–MS/MS MRM chromatograms of a urine blank extract. The arrows point out the retention time of fentanyl.

7.3.4 Recovery

Recovery can be defined as the percentage of analyte in the sample that reaches the end of the sample preparation procedure [43, p.12]. The recovery of the analyte can be determined by comparing the measured concentration of spiked sample to the true reference concentration. Because IS was used in the experiments, the recovery standards were prepared by spiking IS in the end of the sample preparation. These standards were quantitated against normally prepared calibration curve. Recovery was determined at two concentration levels, 1 ng/ml and 25 ng/ml, and was calculated using the following equation:

$$Recovery\ (\%) = \frac{c_r}{c_s} \cdot 100\ \%,$$

where
c_r = observed concentration of fentanyl in recovery standard and
c_s = true reference concentration of fentanyl in recovery standard.

Calculated mean recoveries and SDs of three replicate recovery samples at concentrations of 1 and 25 ng/ml were 83.8 ± 4.5 % and 90.1 ± 3.8 % respectively. The recoveries were relatively high which indicates that no major loss of fentanyl occurred during the SPE. The measured recoveries are given in Appendix 5.

7.3.5 Accuracy and precision

Accuracy can be defined as closeness of an observed result to a true reference value of a sample. Accuracy is sometimes referred as trueness. It expresses the systematic error that occurs in measurements and can be reported as bias. The relative bias is calculated using the equation

$$b(\%) = \frac{\bar{x} - x_{ref}}{x_{ref}} \cdot 100\%,$$

where
b(%) = relative bias in per cent
$\bar{x}$ = mean of the observed value
x_{ref} = true reference value. [46, p.31]

Precision describes the closeness of results to one another. It usually expresses the random error that occurs in the method and can be reported as variance, standard deviation or relative standard deviation. Precision can be divided into different components: repeatability, reproducibility and intermediate precision. To calculate those components, variance results from one-way analysis of variance (ANOVA) by Microsoft Excel were used (Appendix 6). Repeatability describes method's ability to give results as close as possible to the same value when measurements are made during a short timescale and the conditions are unchanged. Repeatability standard deviation can be determined using equation

$$s_w = \sqrt{MS_w},$$

where
s_w = repeatability standard deviation
MS_w = mean square within group (obtained from ANOVA). [46, 35]

Reproducibility describes variability of results between laboratories and is considered to give the maximum variation in results. It can be determined only in interlaboratory experiments. Intermediate precision (within-laboratory reproducibility) combines within- and between-run variations and describes the variation in results when measurements are performed in a single laboratory but the conditions are changed. In this method validation day-to-day variability was evaluated. The between-run standard deviation was calculated using equation

$$s_b = \sqrt{\frac{MS_b - MS_w}{n}},$$

where
MS_b = mean square between groups (obtained from ANOVA),
MS_w = mean square within group (obtained from ANOVA) and
n = number of replicate samples in a group [46, p. 35].

The intermediate precision was calculated combining the within- and between-run variances using equation

$$s_{tot} = \sqrt{s_w^2 + s_b^2}\ .$$

Precision results were expressed as relative standard deviations using equation

$$\%RSD = \frac{s_x}{\bar{x}} \cdot 100\ \%,$$

where
s_x = within-run, between-run or intermediate precision and
$\bar{x}$ = mean result at observed concentration level.

Standard samples used in accuracy and precision calculations were quantitated against a calibration curve with three points per calibration level prepared each day (Figure 33). For each concentration level, three separate batches (n = 3 each) were analyzed and the within- and between-day variations were determined. The accuracy and precision results for each concentration level are summarized in Table 15.

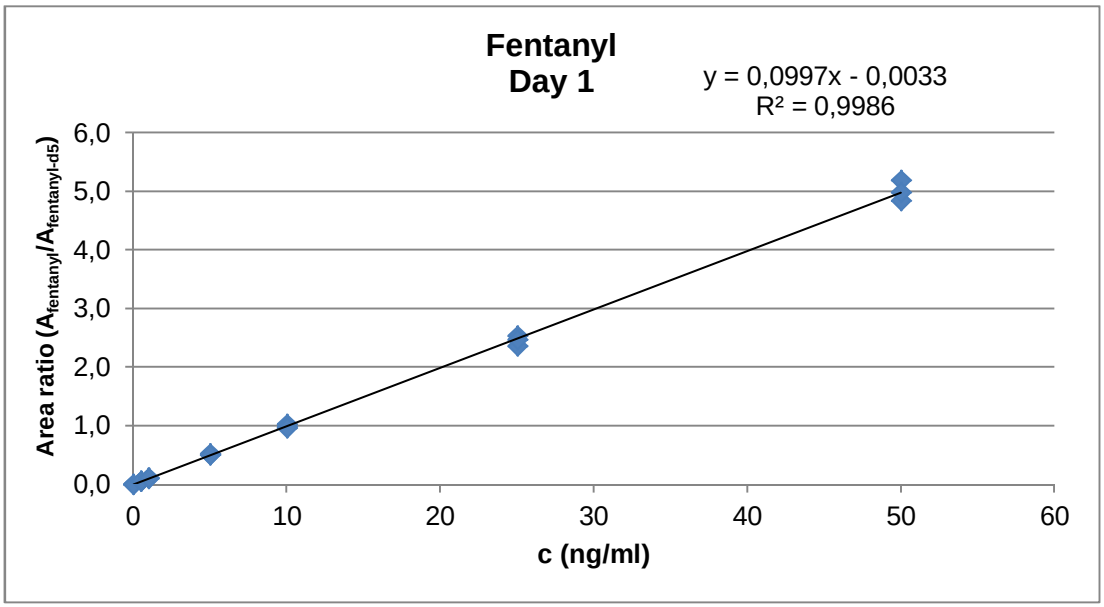


Figure 33. Typical calibration curve with triplicate calibration points prepared each day for quantitation of the standard samples.

Table 15. The accuracy and precision results for fentanyl.

Standard level (ng/ml)	Observed mean c (ng/ml)	SD (ng/ml)	RSD (%)	Bias (%)	Within-day variation (%)	Between-day variation (%)	Intermediate precision (%)
0.5	0.57	0.028	4.9	14.3	5.0	1.1	5.2
1	1.03	0.063	6.1	3.3	6.0	1.4	6.1
5	5.07	0.11	2.1	1.3	2.0	0.9	2.2
10	9.88	0.22	2.2	-1.2	2.4	1.2	2.7
25	24.75	0.67	2.7	-1.0	2.7	0.4	2.7
50	50.14	1.05	2.1	0.3	2.4	1.3	2.7

Accuracy, expressed as relative bias, ranged from -1.2 % to 14.3 %. US Food and Drug Administration (FDA) has set criteria for method accuracy in bioanalytical method validation. According to those criteria the measured mean value should not deviate more than 15 % from the true value (except at LOQ the mean should be within 20 %

from the actual value). The measured mean values were inside the 15 % tolerance window at every concentration level. Overall the method showed good accuracy. The highest calculated intermediate precision was 6.1 % at concentration level of 1 ng /ml. According to FDA, the determined precision should not exceed 15 % of RSD. Neither the calculated RSD nor the intermediate precision exceeded that limit at any concentration level. Generally, excellent precision was achieved. [47, p. 5]

7.3.6 Relative ion abundances

In order to prevent the risk of false positive identification of fentanyl, the detected product ion abundance ratios were determined in standard samples and unknown samples and compared. The peak area ratio between qualifier (q) and quantifier ion (Q) is calculated by dividing the peak area of the less abundant ion by the peak area of the more abundant ion and multiplying with 100 %. World Anti-doping Agency (WADA) has set criterion for the maximum permitted difference in relative ion abundances between standard and unknown samples. When the peak area ratio between detected ions in standard samples is 50 % or higher, the maximum permitted tolerance for relative ion abundance in the unknown sample is ±10 % (absolute) [48].

Relative ion abundances may vary depending on the concentration of analyte. The ion ratio for fentanyl (q/Q) was calculated from the standard samples at level 25 ng/ml. The integrated peak areas and the calculated relative ion abundances are given in Appendix 5. The mean ion ratio in standard samples was 88.4 % and, consequently, the tolerance window was 78.4–98.4 % for an unknown sample.

7.3.7 Authentic sample

Seven replicate samples were prepared from the urine of the surgical patient. The samples were analyzed within four days during the validation experiments. The measured mean concentration (± SD) for fentanyl in urine was 20.9 ± 1.2 ng/ml (Table 16). Representative MRM chromatograms of the authentic urine sample are shown in Figure 34. The relative abundance of fentanyl product ions in each sample was inside the determined tolerance window (88.4 ± 10 %) ensuring with appropriate confidence that the compound detected is fentanyl.

Table 16. RTs, ion ratios, S/N and observed concentration of fentanyl in the surgical patient's urine sample.

Sample ID	Integrated peak area		Ion ratio (%)	RT (min)	S/N (m/z 188)	Observed c (ng/ml)
	m/z 105	m/z 188				
1	16497	14022	85.0	2.42	701	20.2
2	17314	16210	93.6	2.41	609	22.1
3	16830	15463	91.9	2.41	622	22.7
4	15187	14342	94.4	2.40	657	21.6
5	12556	11531	91.8	2.40	465	20.1
6	19709	18491	93.8	2.40	795	20.6
7	16835	15731	93.4	2.40	627	19.3
				Mean concentration (ng/ml)		20.9
				SD (ng/ml)		1.2

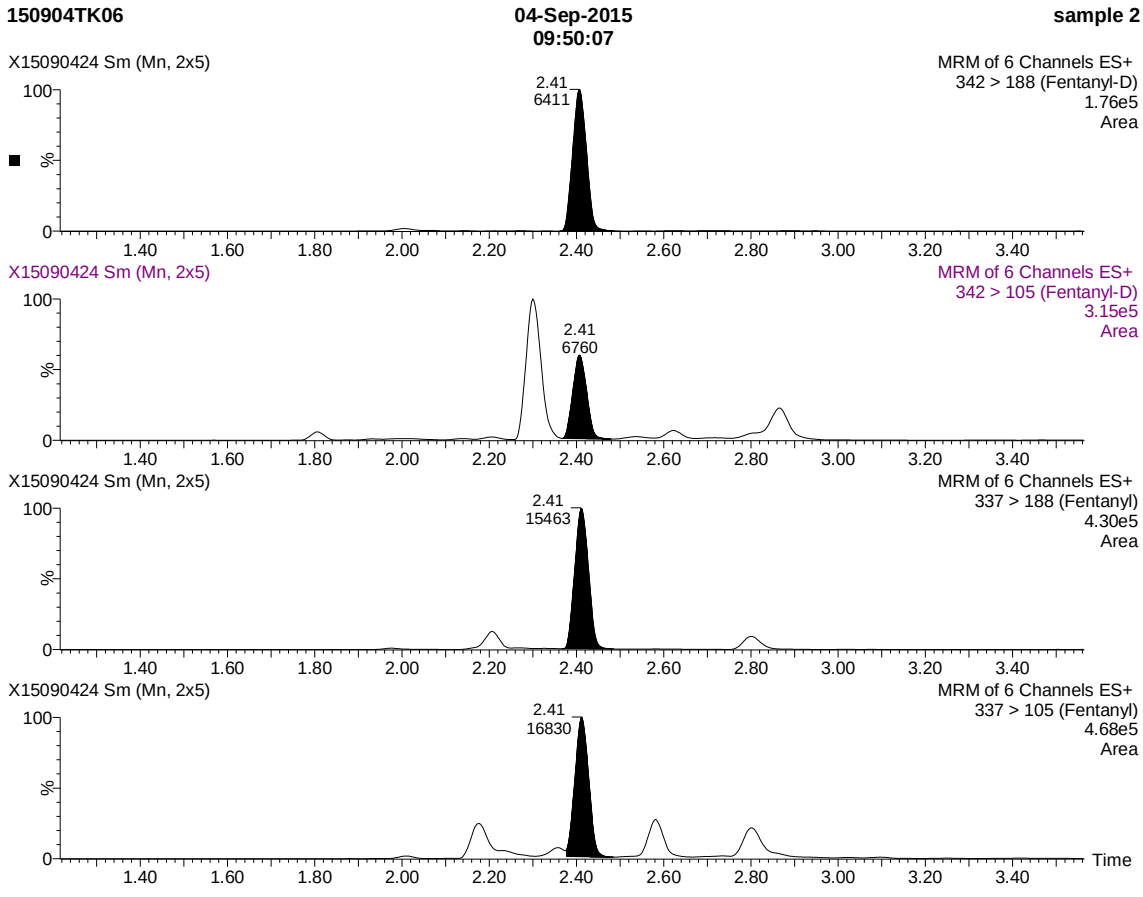


Figure 34. LC–MS/MS MRM chromatograms of the surgical patient's urine sample. The transitions monitored were (from top to bottom) *m/z* 342 → 188, *m/z* 342 → 105 for fentanyl-d5 and *m/z* 337 → 188, *m/z* 337 → 105 for fentanyl.

8 Conclusion

The present thesis describes a study on analytical methods for detecting CNS acting chemicals. The study includes a review of existing literature on selected candidate chemicals, testing of the ROPs for sample preparation, analysis, screening and identification of the substances in wipe samples and a validation of a method for determining fentanyl in human urine. Chemicals selected for the study were CNS acting drugs: amphetamine, diazepam, naloxone and fentanyl. The availability of the reference chemicals was investigated. Diazepam had to be left outside of the ROP testing experiments due to delivery problems of the reference chemical. Fentanyl, amphetamine and diazepam are drugs with narcotic properties, and they are covered under Finnish narcotic legislation. Therefore, the purchase of the reference chemicals required applying licenses from Fimea for importing and handling the narcotics in question. This was somewhat time consuming process.

The experimental can be divided into two sections. In the first part, a study on detecting amphetamine, naloxone and fentanyl in wipe samples was carried out. Different organic solvents, dichloromethane and acetone, and water were tested for their efficiency to extract selected candidate chemicals from spiked wipe samples. The wipe samples were extracted successively with organic and aqueous solvents and the extraction efficiency was assessed by recovery of the analytes. Three different wipe materials were used in this ROP testing experiment. Acetone was found to give significantly higher recoveries for all the candidate chemicals compared to dichloromethane. The water extraction performed after dichloromethane extraction provided relatively high recoveries for the analytes. According to these results, acetone is highly recommended solvent to be used for extraction of the chemicals in question from wipe samples.

The candidate chemicals were screened and identified from acetone extract of a spiked cotton wipe sample. The extract was screened using both LC–MS/MS and GC–MS techniques and methods described in the ROPs. The GC–MS analysis required derivatization of amphetamine and naloxone with BSTFA. The GC–MS screening based on analysis of the produced full scan data by AMDIS software. In the LC–MS/MS screening, the precursor ions were extracted from the LC–MS full scan TIC. After that, the transitions characteristic to the target chemicals were detected using MRM. Both GC–MS and LC–MS/MS techniques and the tested ROPs were discovered

to be valid for screening and identification of the candidate chemicals at parts-per-million (ppm, µg/g) concentration levels. From these two analysis techniques, the LC–MS/MS was found to be more appropriate technique for analysis of the chemicals in question. The recorded EI mass spectra of the analytes will be submitted for evaluation and inclusion in the OCAD in the future.

The second section of experimental part included a validation of a quantitative analysis of fentanyl in human urine by LC–ESI–MS/MS. The assay was based on extracting fentanyl from urine by SPE. The validation parameters studied were linearity, selectivity, LOD, LOQ, recovery, accuracy and precision. The method showed good linearity in the range of 0.5–50 ng/ml fentanyl in human urine. LOD and LOQ were calculated from the calibration curve. According to the calculations, LOQ was estimated to be higher than the lowest concentration levels used in the validation experiments. It is possible that these high limits result from the excessively wide concentration range of the calibration curve. Hence, it is recommended that calibration curves should be prepared for low and high concentrations separately. However, the S/N, accuracy and precision at the lowest observed concentration level satisfied the criteria set for LOQ and therefore LOD and LOQ for fentanyl in urine were determined to be 0.5 ng/ml. It is very likely that by using lower concentration level calibration curve, significantly lower LOD and LOQ would be achieved.

The SPE procedure yielded recoveries of over 80 %. The high recoveries indicated that the extraction procedure did not a cause significant loss of fentanyl. Accuracy (bias) was determined to be lower than 5 % at every concentration level except for the lowest level (14.3%). The intermediate precision was maximum 6.1 %. The method accuracy and precision were inside the acceptance criteria of 15 % set in the guidelines for bioanalytical method validation by FDA. Overall the method showed excellent accuracy and precision.

In addition to the validation, an authentic urine sample from a surgical patient who was given fentanyl intravenously prior to the operation was analyzed. In total, seven replicate samples were prepared and analyzed. The mean concentration of fentanyl in urine was observed to be 20.9 ng/ml. The relative ion abundance in each measured replicate sample was inside the determined tolerance window assuring that the compound detected is fentanyl.

References

1 VERIFIN homepage. Webpage. <http://www.helsinki.fi/verifin/VERIFIN/english/>. Accessed 10.8.2015.

2 International Committee of Red Cross, ICRC. 2010. Expert Meeting: Incapacitating Chemical Agents, Implications for International Law. Montreux, Switzerland.

3 Davison N. 2007. 'Off the Rocker' and 'On the Floor: The Continued Development of Biochemical Incapacitating Weapons. University of Bradford.

4 Vanninen P. 2011. Introduction in Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament edited by Vanninen P, pp.1-3. University of Helsinki. Helsinki, Finland.

5 United States Environmental Protection Agency, EPA. 2013. Voluntary Guidelines for Methamphetamine Laboratory Cleanup.

6 Mesilaakso M. 2005. Chemical Weapon Convention Chemicals Analysis: Sample Collection, Preparation and Analytical Methods. John Wiley & Sons Ltd. West Sussex, England.

7 Neill D. 2007. Riot Control and Incapacitating Chemical Agents under the Chemical Weapons Convention. Defence R&D Canada, Center for Operational Research & Analysis. Canada.

8 Crowley M. 2009. Dangerous Ambiguities: Regulations of Riot Control Agents and Incapacitants under the Chemical Weapons Convention. Bradford Non-lethal Weapons Research Project, University of Bradford.

9 Lakoski JM, Murray BW, Kenny JM. 2000. The Advantages and Limitations of Calmatives for Use as a Non-lethal Technique. College of Medicine, Applied Researc Laboratory, The Pennsylvania State University.

10 Rang HP, Dale MM, Ritter JM, Flower RJ. 2007. Rang and Dale's Pharmacology. Churchill Livingstone Elsevier. Philadelphia, USA.

11 Riches JR, Read RW, Black RM, Cooper NJ, Timperley CM. 2012. Analysis of Clothing and Urine from Moscow Theatre Siege Casualties Reveals Carfentanil and Remifentanil Use. J Anal Toxicol. 2012; 36(9): 647-656.

12 Glasser SB, Baker P. 2002. Russia Confirms Suspicions About Gas Used in Raid; Potent Anesthetic Pumped Into Theater, 2 More Hostages Die From Drug's Effects. Washington Post 2002; A15.

13 Sutherland RG. 2008. Chemical and Biochemical Non-lethal Weapons, Political and Technical Aspects. Stockholm International Peace Research Institute. Solna, Sweden.

14 Stanley TH, Talmage ED, Van Aken H. 2008. A Tribute to Dr. Paul A. J. Janssen: Entrepreneur Extraordinaire, Innovative Scientist, and Significant Contributor to Anesthesiology. Anesth Analg 2008; 106: 451-462.

15 European Monitoring Centre for Drugs and Drug Addicts, EMCDDA. Fentanyl Drug Profile. Webpage.< http://www.emcdda.europa.eu/publications/drug-profiles/fentanyl>. Accessed 13.7.2015.

16 Merck &CO. INS. 2006. The Merck Index: an Encyclopedia of Chemicals, Drugs and Biologicals. Whitehouse Station, USA.

17 Malkawi AH, Al-Ghananeem AM, Crooks PA. 2008. Development of a GC–MS assay for the determination of fentanyl pharmacokinetics in rabbit plasma after sublingual spray delivery. AAPS J. 2008; 10(2); 261-267.

18 Huynh N, Tyrefors N, Ekman L, Johansson M. 2005. Determination of fentanyl in human plasma and fentanyl and norfentanyl in human urine using LC–MS/MS. J Pharm Biomed Anal. 2005; 37(5): 1095-1100.

19 Berg T, Benedicte J, Strand DH. 2013. Determination of Buprenrphine, Fentanyl and LSD in Whole Blood by UPLC-MS-MS. J Anal Toxic. 2013; 37(3):159-165.

20 Wang L, Bernert JT. 2006. Analysis of 13 Fentanils, Including Sufentanil and Carfentanil, in Human Urine by Liquid Chromatography-Atmospheric-Pressure Ionization-Tandem Mass Spectrometry. J Anal Toxicol. 2006; 30(5): 335-341.

21 Gardner MA, Owens JE, Sampsel S, Jenkins WW. 2015. Analysis of fentanyl in urine by DLLME-GC–MS. J Anal Toxicol. 2015; 39(2): 118-125.

22 Van Nimmen NFJ, Veulemans HAF. 2004. Development and validation of a highly sensitive gas chromatographic-mass spectrometric screening method for the simultaneous determination of nanogram levels of fentanyl, sufentanil and alfentanil in air and surface contamination wipes. J Chromatogr A. 2004; 1035(2): 249-259.

23 United Nations Office on Drugs and Crime. 2006. The recommended methods for the identification and analysis of amphetamine, methamphetamine and their ring-substituted analogues in seized materials. New York, USA.

24 Melgar R, Kelly RC. 1993. A novel GC–MS derivatization method for amphetamines. J Anal Toxicol. 1993; 17(7): 399-402.

25 Fernandez MdMR, Samyn N. 2011. Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Analysis of Amphetamines in Plasma. J Anal Toxicol. 2011; 35(8); 577-582.

26 Madireddy SB, Bodeddula VR, Mansani SK, Wells MJM, Boles JO. 2013. Wipe sampling of amphetamine-type stimulants and recreational drugs on selected household surfaces with analysis by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. J Hazard Mater. 2013; 254-255: 46-56.

27 Fang WB, Chang Y, McCance-Katz EF, Moody DE. 2009. Determination of Naloxone and Nornaloxone (Noroxymorphone) by High-Performance Liquid Chromatography-Electrospray Ionization- Tandem Mass Spectrometry. J Anal Toxic. 2009; 33(8): 409-417.

28 Tzatzarakis MN, Vakonaki E, Belivanis S, Alegakis A, Tsatsakis AM, Kovatsi L. 2015. Determination of buprenorphine, norbuprenorphine and naloxone in fingernail clippings and urine of patients under opioid substitution therapy. J Anal Toxicol. 2015; 39(4):313-320.

29 Mehrdad R, Khosrou A, Rassoul D, Sanaz V, Mohsen A. 2009. A Simple and Sensitive Analytical Method for Determination of Naltrexone Level in Plasma by GC–MS. Chromatographia. 2009; 70(9/10): 1491-1494.

30 Moldoveanu SC, David V. 2002. Sample Preparation in Chromatography. Elsevier Science B.V. Amsterdam, the Netherlands.

31 Harris DC. 2010. Quantitative Chemical Analysis. Freeman and Company. New York, USA.

32 Bouchonnet S. 2013. Introduction to GC–MS Coupling. CRC Press, Taylor &Francis Group. Boca Raton, FL, USA.

33 Ardrey B. 2003. Liquid Chromatography-Mass Spectrometry: an Introduction. John Wiley & Sons Ltd. West Sussex, England.

34 Banerjee S, Mazumdar S. 2012. Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte. Int J Anal Chem. Article ID 285574.

35 Garg A, Solas DW, Takashi LH, Cassela JV. 2010. Forced degradation of fentanyl: Identification and analysis of impurities and degradants. J Pharm Biomed Anal. 2010; 53(3): 325-334.

36 Finnish Medicines Agency, Fimea. Narcotics control. Webpage. <http://www.fimea.fi/supervision/narcotics_control>. Accessed 10.8.2015.

37 Häkkinen V, Söderström M. 2011. Gas chromatography-electron ionization/mass spectrometry (GC-EI/MS) in Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament edited by Vanninen P, pp. 235-245. University of Helsinki. Helsinki, Finland.

38 Kuitunen M. 2011. Wipe samples in Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament edited by Vanninen P, pp. 207-211. University of Helsinki. Helsinki, Finland.

39 Zayed MA, Fahmey MA, Hawash MF. 2005. Investigation of Diazepam Drug Using Thermal Analyses, Mass Spectrometry and Semi-empirical MO Calculations. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2005; 61(5): 799-805.

40 Peer CJ, Shakleya DM, Yonis IR, Kraner JC, Callery PS. 2007. Direct-injection mass spectrometric method for the rapid identification of fentanyl and norfentanyl in postmortem urine of six drug-overdose cases. J Anal Toxicol. 2007; 31(8): 515-521.

41 Maillard G, Söderström M. 2011. False positive identification if Chemical Weapons Convention-related compounds in Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament edited by Vanninen P, pp. 65-87. University of Helsinki. Helsinki, Finland.

42 Söderström M, Kostiainen O, Koskela Harri, Bossee A, Juillet Y, Xie J, Schaer M, Stiegenthaler P, Jensen JS. 2011. Screening in Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament edited by Vanninen P, pp. 31-59. University of Helsinki. Helsinki, Finland.

43 United Nations Office of Drugs and Crimes. 2009. 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. New York, USA.

44 International Confrence on Harmonisation, ICH. 1994. Validation of Analytical Procedures: Text and Methodology Q2 (R1).

45 Armbruster DA, Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 2008; 29(Suppl 1): S49-52.

46 Magnusson B, Örnemark U. 2014. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, 2nd edition. Eurachem.

47 US Department of Health and Human Services, Food and Drug Administrator. 2001. Guidance for Industry, Bioanalytical Method Validation. USA.

48 World Anti-Doping Angency, WADA. 2010. Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry. Document number TD2010IDCR.

Appendix 1. Hard copy of the accompanying information for the mass spectra submitted to the OCAD

ANALYTICAL CONDITIONS FOR MASS SPECTRA xx-x-xxxx to xx-x-xxx

Common information:

Data identification:

Contributor's name and address:
Tatu Köli
VERIFIN Finnish Institute for Verification of the Chemical Weapons Convention
A. I. Virtasen aukio 1 (P.O. Box 55)
Department of Chemistry
FI-00014 University of Helsinki, Finland

Instrument information:

Manufacturer:	Agilent Technologies
Model:	5975N
Data System:	AMDIS
Software version:	2.66

Sample information:

Sample source:	Commercial, purchased from Sigma Aldrich

Experiment information:

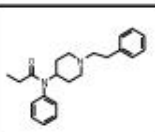
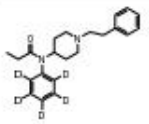
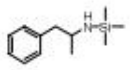
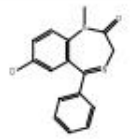
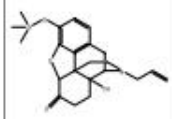
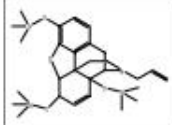
Inlet system	GC, splitless
GC	
Column:	DB-5MS, 30 m x 250 µm x 0.25 µm
Temperature program:	40 °C (1 min) – 10 °C/min – 300 °C (5 min)
Carrier gas:	Helium
Injection temperature:	250 °C
MSD inlet temperature:	290 °C
Ion source temperature:	230 °C
Electron energy:	70 eV
Scan Range:	40–600 m/z
Date of experiment:	June–August 2015

Electric format:

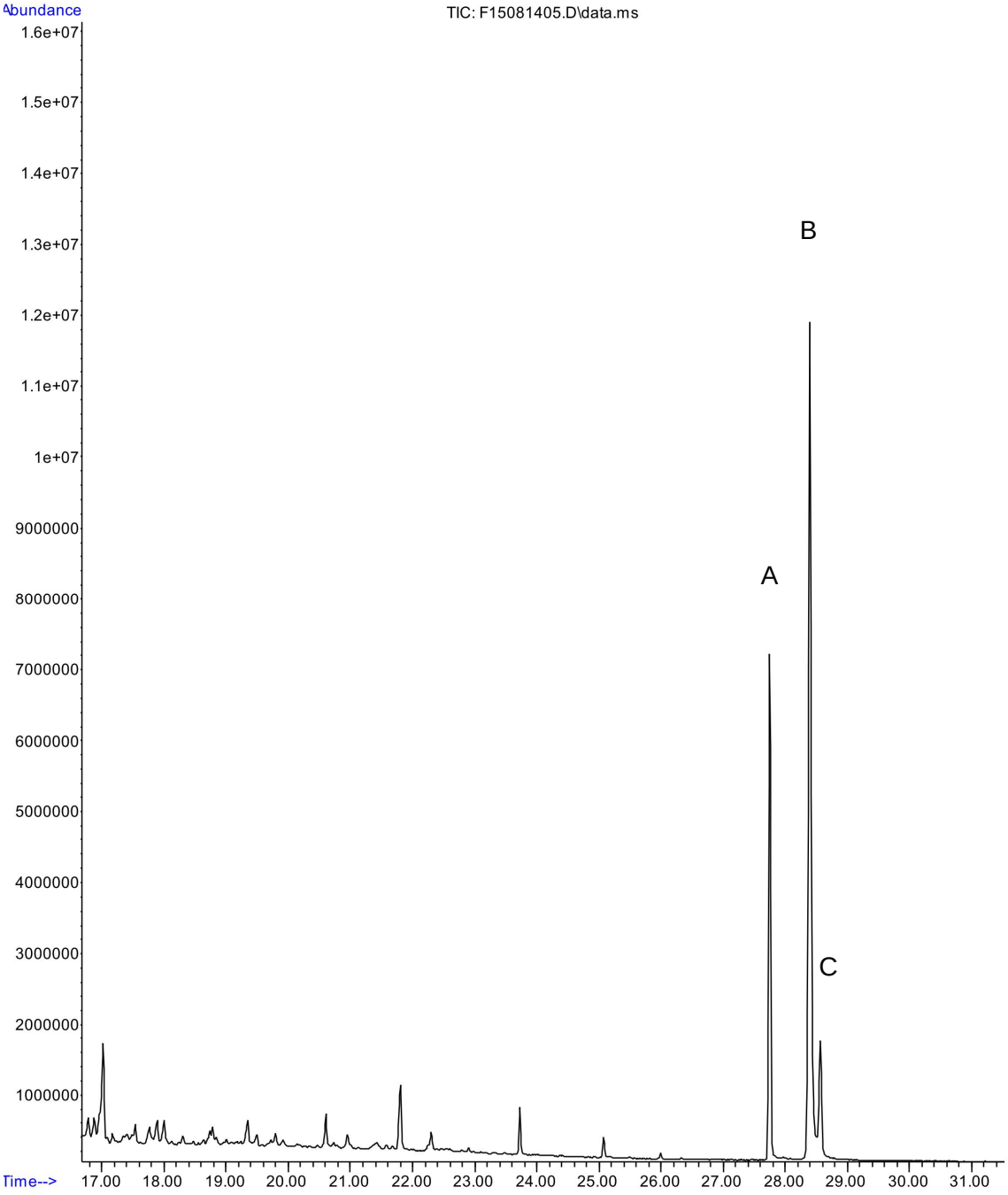
Type of electric format:	*.msp (mass spectral), *.mol (structure)

1

Spectrum-specific information:

OPCW Code	Schedule Number	Name	Synonyms	Structure	Formula	CAS No.
05-02-0000	Not scheduled	N-Phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide	Fentanyl		$C_{22}H_{28}N_2O$	437-38-7
05-02-0000	Not scheduled	N-Phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide	Fentanyl-D5		$C_{22}D_5H_{23}N_2O$	118357-29-2
05-02-0000	Not scheduled	N-(α-Methylphenethyl)trimethylsilylamine	Amphetamine-TMS		$C_{12}H_{21}NSi$	14629-65-3
05-02-0000	Not scheduled	7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one	Diazepam		$C_{16}H_{13}ClN_2O$	439-14-5
05-02-0000	Not scheduled	3-[(trimethylsilyl)oxy]-4,5α-epoxy-14-hydroxy-17-(2-propenyl)morphinan-6-one	Naloxone-TMS		$C_{22}H_{29}NO_4Si$	n/a
05-02-0000	Not scheduled	(5α,6α)-17-allyl-7,8-didehydro-4,5-epoxy-3,6,14-tris[(trimethylsilyl)oxy]morphinan	Naloxone-3TMS		$C_{28}H_{45}NO_4Si_3$	n/a

Appendix 2. Total ion chromatogram of a silylated naloxone standard



The 100 µg/ml naloxone standard was silylated with BSTFA. Peak A is pertrimethylsilated naloxone-3TMS. Two silylation products (naloxone-TMS and naloxone-2TMS) elute with the same retention time in peak B. Peak C represents the fourth silylation product of naloxone with two TMS groups attached.

Appendix 3. Recoveries from the wipe samples

Wipe: Whatman filter paper
Solvents: Acetone & water
Analysis: LC–MS/MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150721TK01e	58.7	90,7	90,8
150721TK02e	61.4	87,8	54,6
150721TK03e	54,1	84,9	62,8
150721TK04e	57,1	84,7	86.5
150721TK05e	54.6	86.5	76.4
150721TK06e	56.2	84.3	86.1
Mean	57.0	86.5	76.2
SD	2.7	2.4	14.6
RSD	4.8	2.8	19.1
Recovery (%). water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150721TK01c	27.1	6.8	9.1
150721TK02c	34.3	8.0	6.0
150721TK03c	29.4	6.3	4.8
150721TK04c	34.0	9.6	4.9
150721TK05c	33.9	9.1	7.3
150721TK06c	32.2	9.2	5.8
Mean	31.8	8.2	6.3
SD	3.0	1.4	1.6
RSD	9.3	16.8	25.9

Wipe: Whatman filter paper
Solvents: Acetone & water
Analysis: GC–MS

Recovery (%). acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150721TK01b	54.3	157.3	58.4
150721TK02b	112.8	159.1	76.4
150721TK03b	35.7	189.9	77.0
150721TK04b	45.0	113.4	65.3
150721TK05b	21.4	109.0	69.2
150721TK06b	54.6	92.4	70.5
Mean	54.0	136.8	69.5
SD	31.4	37.5	7.0
RSD	58.2	27.4	10.1
Recovery (%). water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150721TK01d	5.1	-	3.0
150721TK02d	6.9	-	2.8
150721TK03d	4.8	-	2.4
150721TK04d	10.9	-	3.3
150721TK05d	0.0	-	4.9
150721TK06d	7.6	-	2.5
Mean	7.1	-	3.2
SD	2.5	-	0.9
RSD	34.7	-	28.3

Wipe: Whatman filter paper
Solvents: Dichloromethane & water
Analysis: LC–MS/MS

Recovery (%), dichloromethane fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150727TK01a	1.3	20.5	10.0
150727TK02a	0.9	20.3	8.2
150727TK03a	1.5	15.2	5.1
150727TK04a	0.8	14.9	3.9
150727TK05a	0.7	16.0	3.8
150727TK06a	2.1	22.3	9.4
Mean	1.2	18.2	6.7
SD	0.6	3.2	2.8
RSD	45.4	17.8	41.9
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150727TK01c	65.3	45.8	30.6
150727TK02c	67.2	43.6	22.4
150727TK03c	67.5	46.6	24.0
150727TK04c	71.9	50.3	33.5
150727TK05c	56.1	36.6	17.7
150727TK06c	65.9	47.3	26.2
Mean	65.7	45.0	25.7
SD	5.2	4.7	5.7
RSD	8.0	10.4	22.1

Wipe: Whatman filter paper
Solvents: Dichloromethane & water
Analysis: GC–MS

Recovery (%), dichloromethane fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150727TK01b	-	11.2	15.0
150727TK02b	-	12.9	22.7
150727TK03b	-	11.1	20.2
150727TK04b	-	8.8	15.9
150727TK05b	-	7.1	12.8
150727TK06b	-	10.0	14.9
Mean	-	10.2	16.9
SD	-	2.0	3.7
RSD	-	19.7	22.2
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150727TK01d	21.9	15.5	12.5
150727TK02d	38.3	12.2	9.2
150727TK03d	24.5	16.2	10.2
150727TK04d	39.6	14.8	10.2
150727TK05d	26.8	12.3	8.9
150727TK06d	16.0	18.6	11.1
Mean	27.8	14.9	10.4
SD	9.3	2.4	1.3
RSD	33.5	16.4	12.8

Wipe: Cotton swab
Solvents: Acetone & water
Analysis: LC–MS/MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150729TK01a	69.6	78.4	83.1
150729TK02a	69.8	78.1	75.9
150729TK03a	72.0	82.3	83.1
150729TK04a	75.3	81.9	84.2
150729TK05a	69.0	80.9	80.3
150729TK06a	70.3	79.4	76.0
Mean	71.0	80.2	80.5
SD	2.4	1.8	3.7
RSD	3.3	2.2	4.6
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150729TK01c	23.1	6.4	5.1
150729TK02c	23.3	7.9	6.2
150729TK03c	25.4	7.2	6.2
150729TK04c	16.0	5.9	4.1
150729TK05c	22.1	8.4	5.5
150729TK06c	21.5	7.2	5.0
Mean	21.9	7.2	5.4
SD	3.2	0.9	0.8
RSD	14.5	13.2	15.1

Wipe: Cotton swap
Solvents: Acetone & water
Analysis: GC–MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150729TK01b	57.0	72.1	68.0
150729TK02b	35.0	69.8	76.7
150729TK03b	28.4	67.7	62.0
150729TK04b	19.2	74.5	68.9
150729TK05b	42.7	74.2	71.3
150729TK06b	18.7	66.4	69.1
Mean	33.5	70.8	69.3
SD	14.8	3.4	4.8
RSD	44.1	4.8	6.9
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150729TK01d	12.2	2.9	2.3
150729TK02d	11.1	3.6	3.0
150729TK03d	12.9	3.8	2.1
150729TK04d	13.7	4.4	1.9
150729TK05d	16.2	4.0	1.9
150729TK06d	9.7	4.3	2.2
Mean	12.6	3.8	2.2
SD	2.2	0.6	0.4
RSD	17.5	14.7	18.5

Wipe: Cotton swab
Solvents: Dichloromethane & water
Analysis: LC–MS/MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150723TK01a	52.5	79.0	67.0
150723TK02a	48.6	80.8	84.4
150723TK03a	43.9	72.9	85.6
150723TK04a	39.1	72.9	88.4
150723TK05a	45.5	77.5	67.0
150723TK06a	44.1	72.9	74.9
Mean	45.6	76.0	77.9
SD	4.6	3.6	9.6
RSD	10.0	4.7	12.3
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150723TK01c	37.2	8.7	7.2
150723TK02c	43.5	9.3	7.3
150723TK03c	34.3	8.2	6.7
150723TK04c	34.9	8.6	7.7
150723TK05c	42.3	10.8	8.7
150723TK06c	44.1	10.1	7.9
Mean	39.4	9.3	7.6
SD	4.5	1.0	0.7
RSD	11.3	10.7	9.2

Wipe: Cotton swab
Solvents: Dichloromethane & water
Analysis: GC–MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150723TK01b	30.7	42.3	59.6
150723TK02b	35.6	70.5	77.0
150723TK03b	34.4	73.4	66.6
150723TK04b	39.8	74.2	73.2
150723TK05b	27.8	69.7	74.5
150723TK06b	36.4	66.7	60.6
Mean	34.1	66.2	68.6
SD	4.3	12.0	7.4
RSD	12.6	18.1	10.8
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150723TK01d	7.9	3.1	2.0
150723TK02d	7.0	3.1	2.3
150723TK03d	9.4	2.6	1.6
150723TK04d	7.7	2.6	2.0
150723TK05d	6.5	3.0	1.9
150723TK06d	17.0	2.6	1.4
Mean	9.2	2.9	1.9
SD	4.0	0.2	0.3
RSD	42.7	8.3	17.3

Wipe: Cotton wipe
Solvents: Acetone & water
Analysis: LC–MS/MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150804TK01a	70.6	79.4	78.6
150804TK02a	65.4	72.6	81.6
150804TK03a	60.6	68.1	73.7
150804TK04a	58.5	69.8	75.4
150804TK05a	61.7	70.1	76.1
150804TK06a	63.9	75.5	74.9
Mean	63.4	72.6	76.7
SD	4.3	4.2	2.9
RSD	6.7	5.8	3.8
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150804TK01c	15.4	8.2	5.5
150804TK02c	20.8	12.2	8.6
150804TK03c	21.8	11.6	9.2
150804TK04c	27.2	13.3	11.7
150804TK05c	26.2	14.6	11.8
150804TK06c	25.2	11.8	9.3
Mean	22.8	11.9	9.3
SD	4.4	2.2	2.3
RSD	19.2	18.0	24.9

Wipe: Cotton wipe
Solvents: Acetone & water
Analysis: GC–MS

Recovery (%), acetone fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150804TK01b	106.9	82.5	76.9
150804TK02b	73.71	77.1	76.4
150804TK03b	32.9	81.9	76.4
150804TK04b	88.1	82.4	83.6
150804TK05b	75.1	97.3	96.1
150804TK06b	31.9	90.9	93.7
Mean	68.1	85.4	83.8
SD	30.1	7.3	9.0
RSD	44.2	8.6	10.8
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150804TK01d	-	8.0	5.6
150804TK02d	-	6.2	4.9
150804TK03d	-	6.8	5.2
150804TK04d	-	10.6	6.8
150804TK05d	-	9.2	7.0
150804TK06d	-	7.5	5.4
Mean	-	8.1	5.8
SD	-	1.6	0.9
RSD	-	19.8	14.8

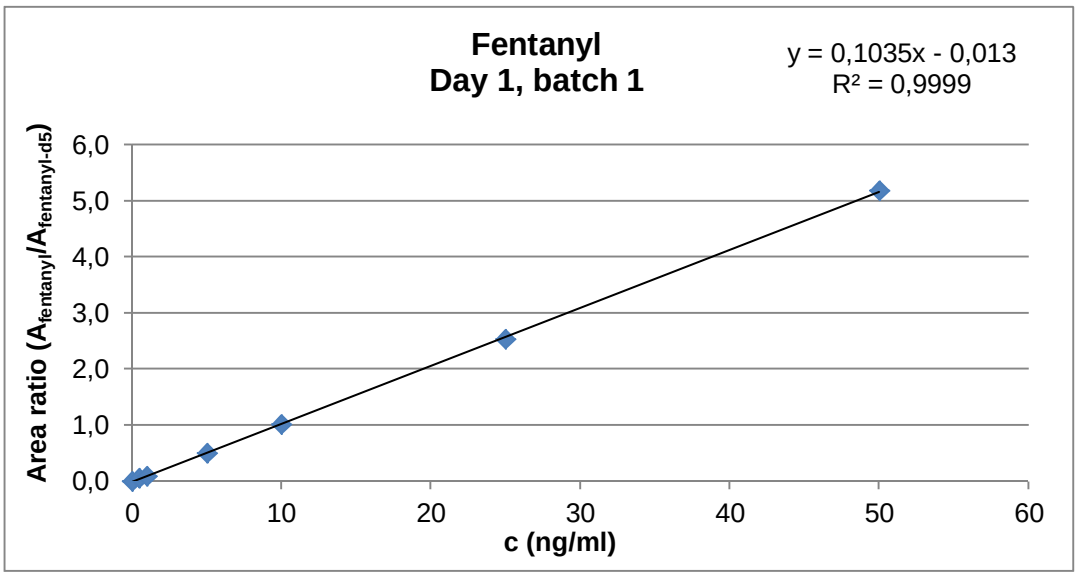
Wipe: Cotton wipe
Solvents: Dichloromethane & water
Analysis: LC–MS/MS

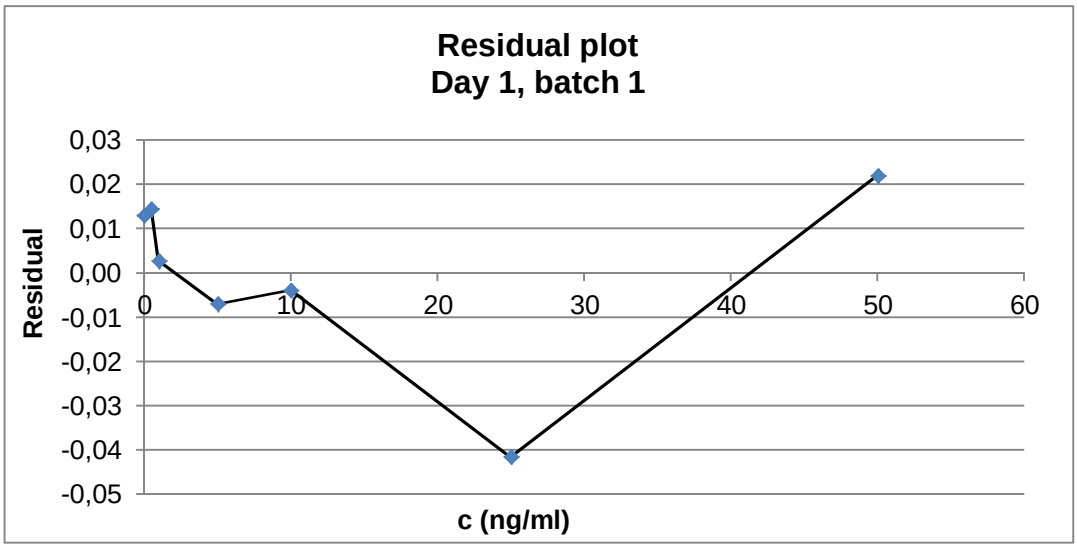
Recovery (%), dichloromethan fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150730TK02a	3.3	15.8	23.2
150730TK03a	2.8	16.3	35.8
150730TK04a	2.1	19.4	39.3
150730TK05a	2.8	15.4	24.1
150730TK06a	5.7	14.3	25.8
150730TK07a	4.1	14.5	40.3
Mean	3.5	16.0	31.4
SD	1.3	1.9	7.9
RSD	36.6	11.7	25.2
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150730TK02c	64.9	41.7	29.2
150730TK03c	60.5	46.3	34.6
150730TK04c	56.5	47.7	39.2
150730TK05c	68.6	56.8	40.4
150730TK06c	57.9	41.7	28.1
150730TK07c	57.3	44.5	34.2
Mean	60.9	46.5	34.3
SD	4.8	5.6	5.0
RSD	8.0	12.1	14.6

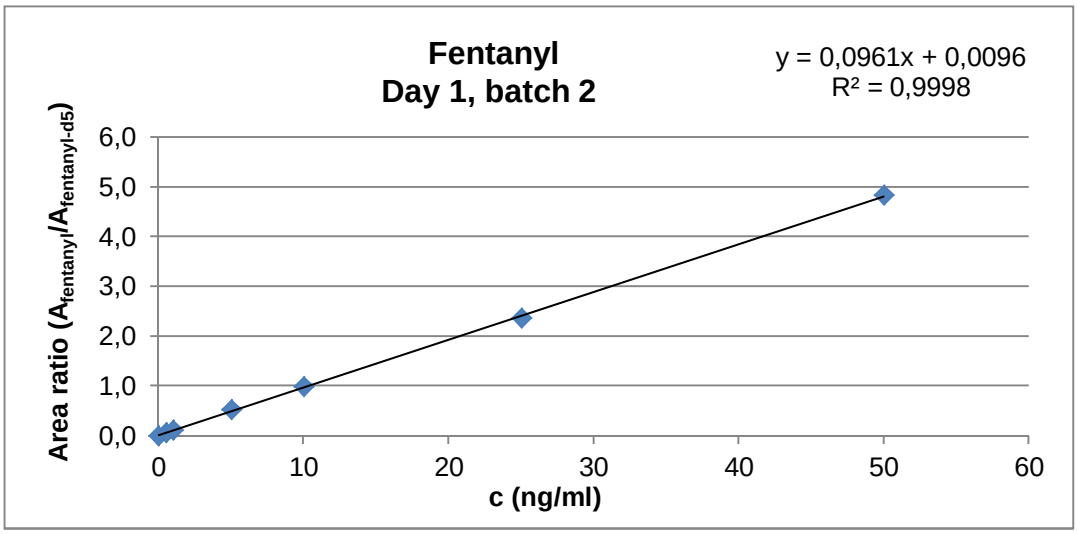
Wipe: Cotton wipe
Solvents: Dichloromethane & water
Analysis: GC–MS

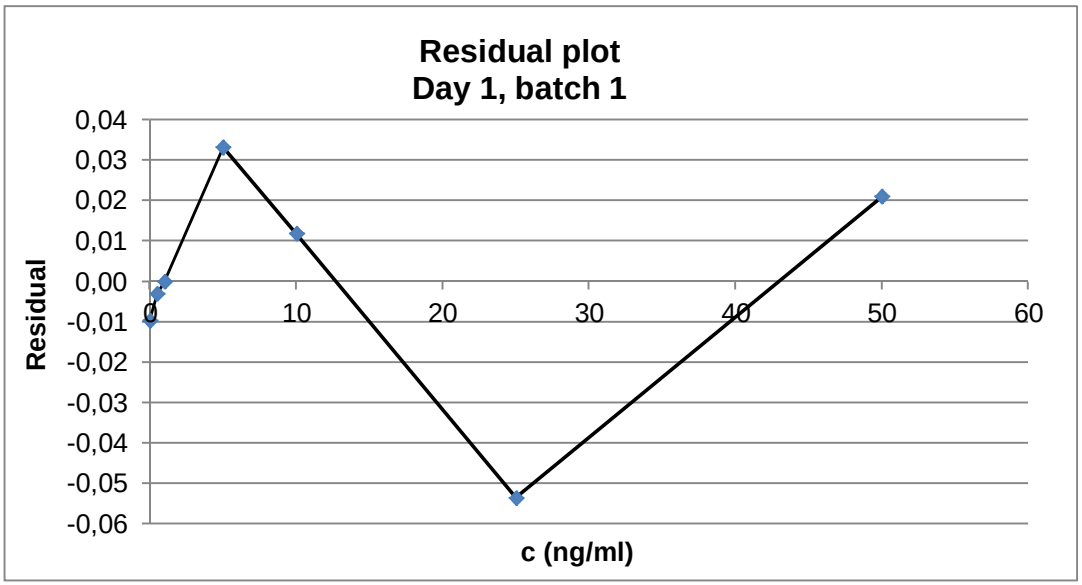
Recovery (%), dichloromethane fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150730TK02b	-	17.3	25.6
150730TK03b	-	19.4	30.5
150730TK04b	-	16.9	29.9
150730TK05b	-	15.5	31.5
150730TK06b	-	15.8	27.2
150730TK07b	-	16.3	26.5
Mean	-	16.9	28.6
SD	-	1.4	2.4
RSD	-	8.5	8.5
Recovery (%), water fraction			
Sample ID	Amphetamine	Naloxone	Fentanyl
150730TK02d	51.8	29.7	12.8
150730TK03d	49.9	42.8	24.1
150730TK04d	59.3	52.4	33.0
150730TK05d	40.9	60.4	36.8
150730TK06d	39.2	52.2	23.8
150730TK07d	73.1	61.0	38.7
Mean	52.4	49.8	28.2
SD	12.6	11.9	9.8
RSD	24.0	23.8	34.7

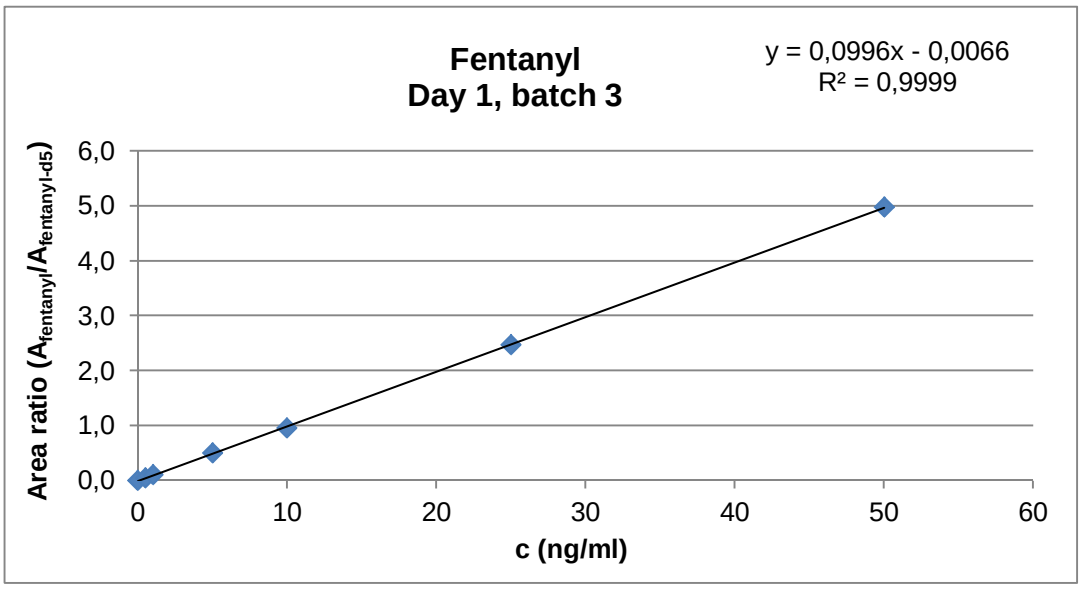
Appendix 4. Calibration curves and residual plots

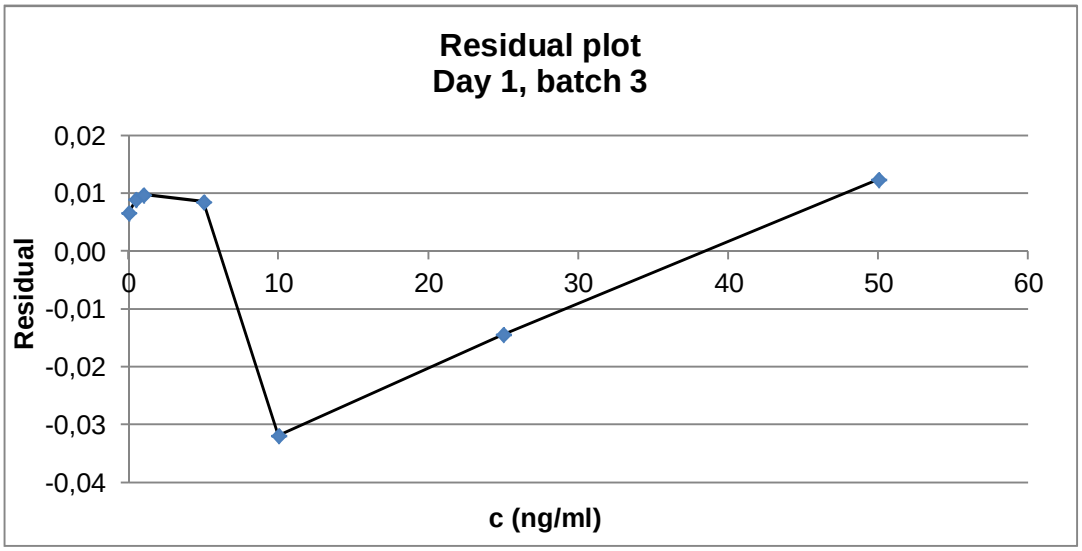


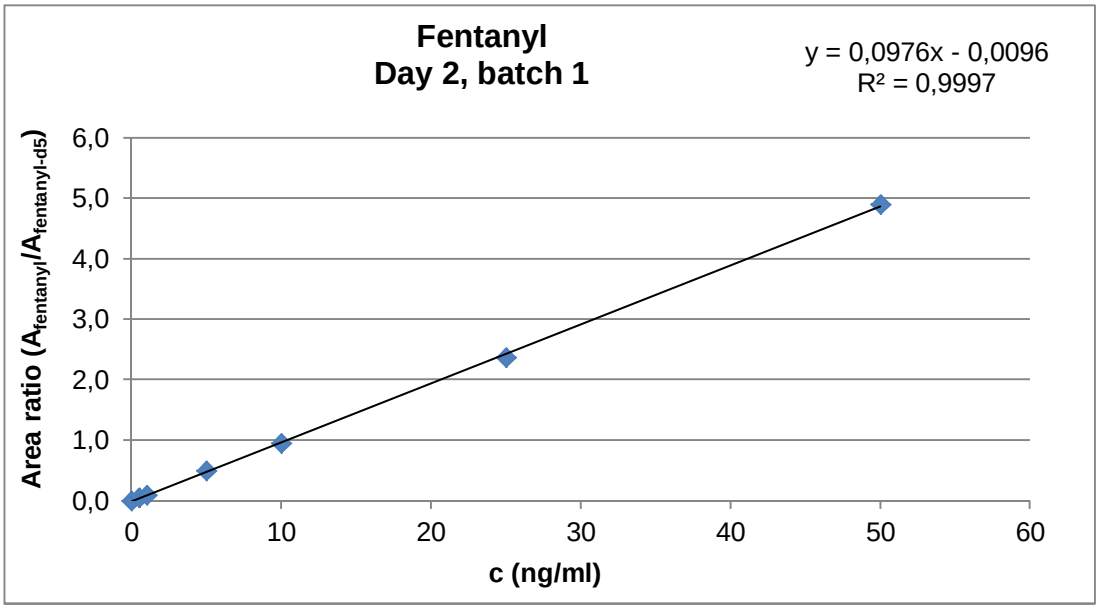


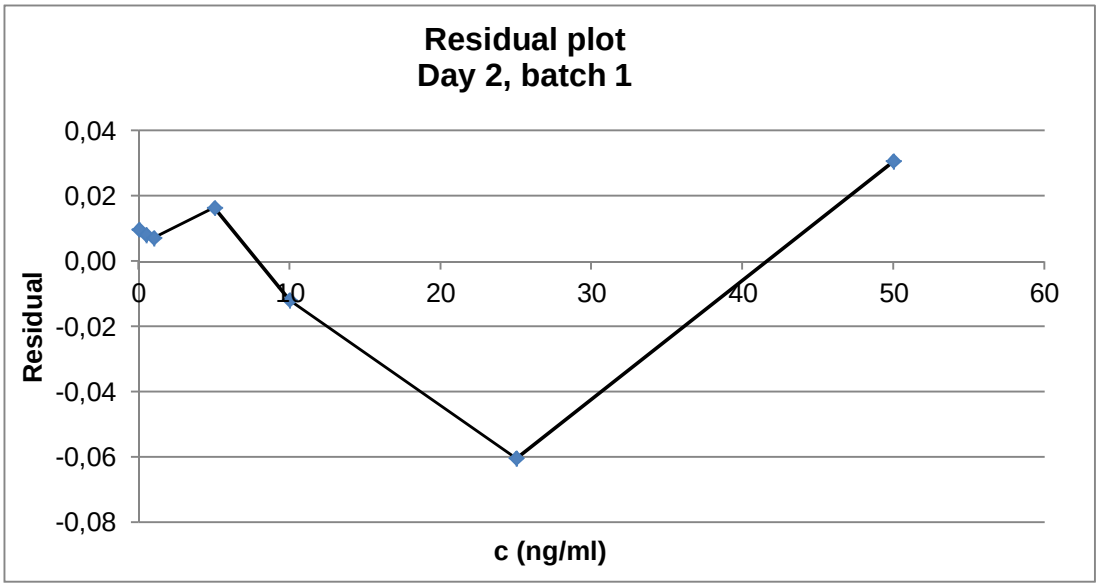


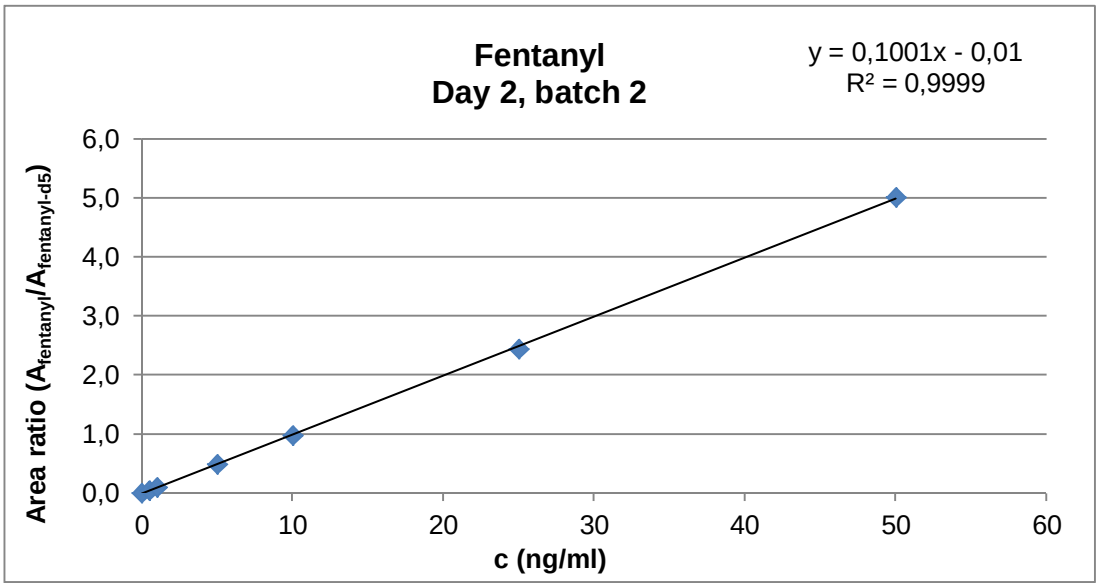

Residual plot
Day 1, batch 1
Residual
0,04
0,03
0,02
0,01
0,00
-0,01
-0,02
-0,03
-0,04
-0,05
-0,06
0
10
20
30
40
50
60
c (ng/ml)

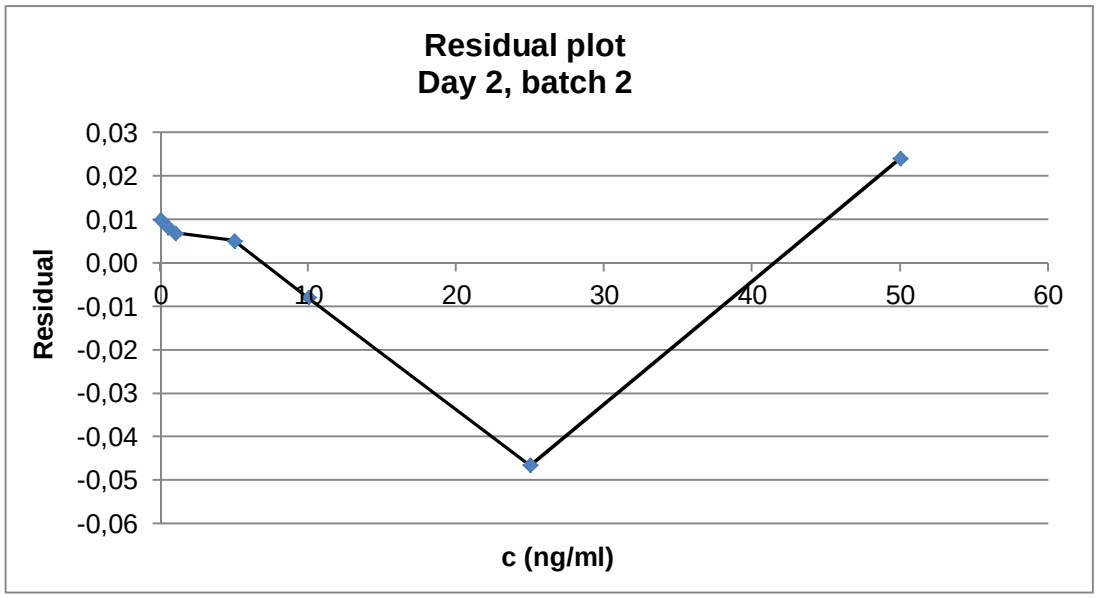

Fentanyl
Day 1, batch 3
y = 0,0996x - 0,0066
R² = 0,9999
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)

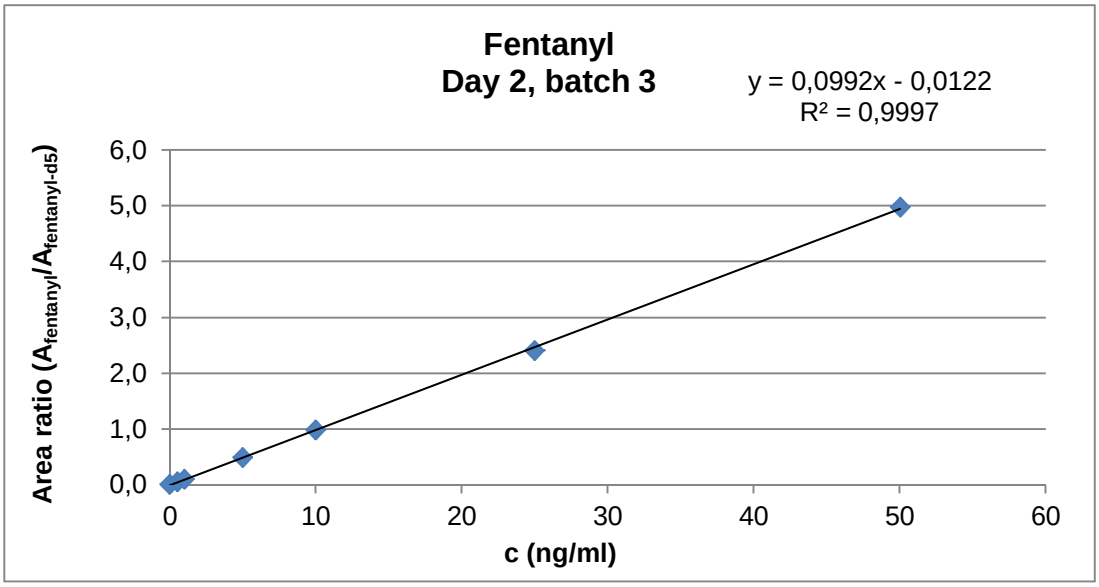

Residual plot
Day 1, batch 3
Residual
0,02
0,01
0,00
-0,01
-0,02
-0,03
-0,04
0
10
20
30
40
50
60
c (ng/ml)

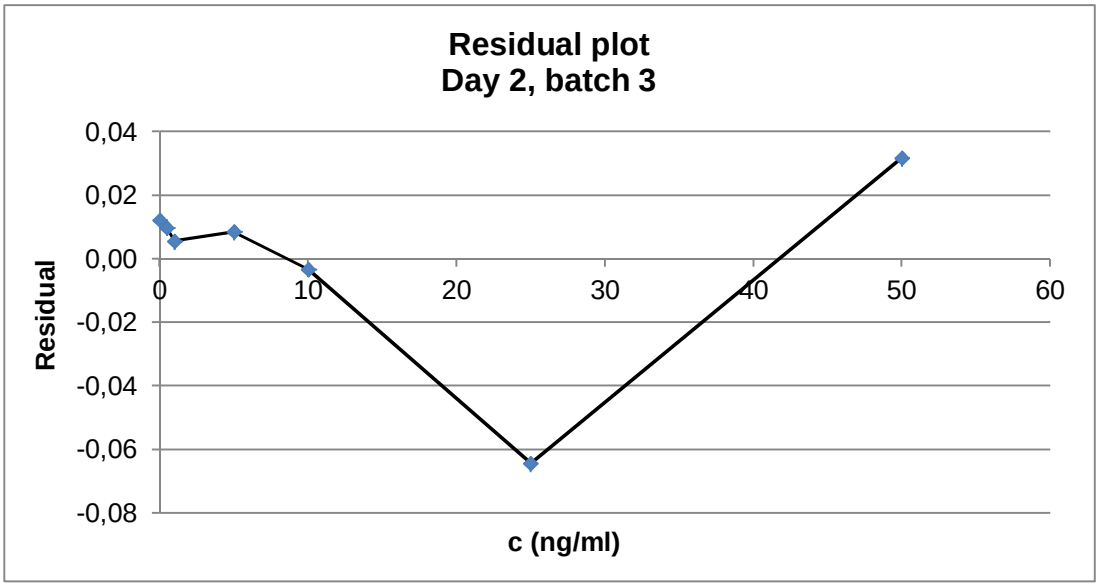

Fentanyl
Day 2, batch 1
y = 0,0976x - 0,0096
R² = 0,9997
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)

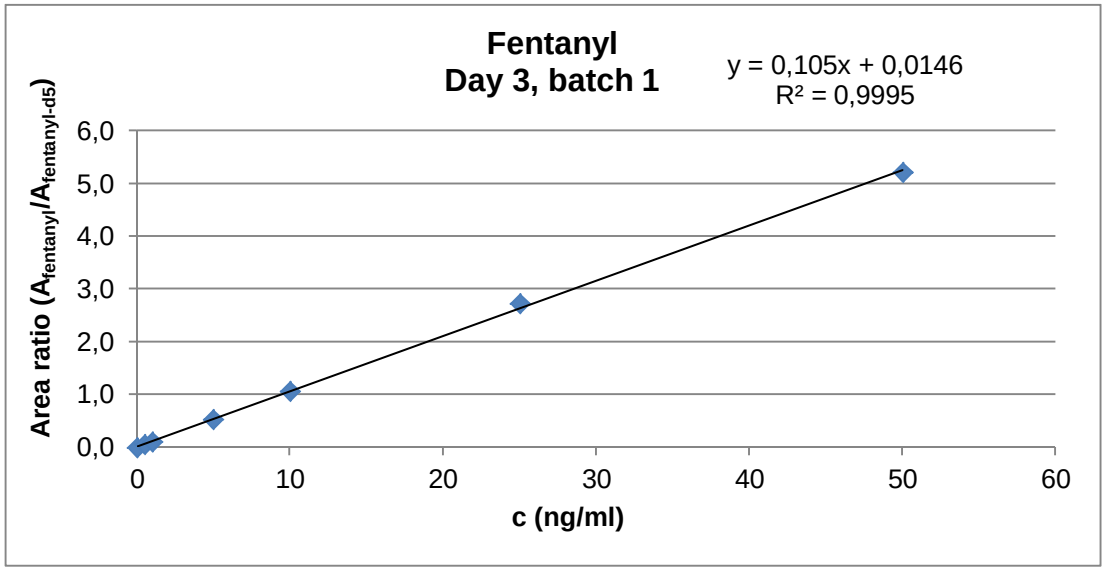

Residual plot
Day 2, batch 1
Residual
0,04
0,02
0,00
-0,02
-0,04
-0,06
-0,08
0
10
20
30
40
50
60
c (ng/ml)

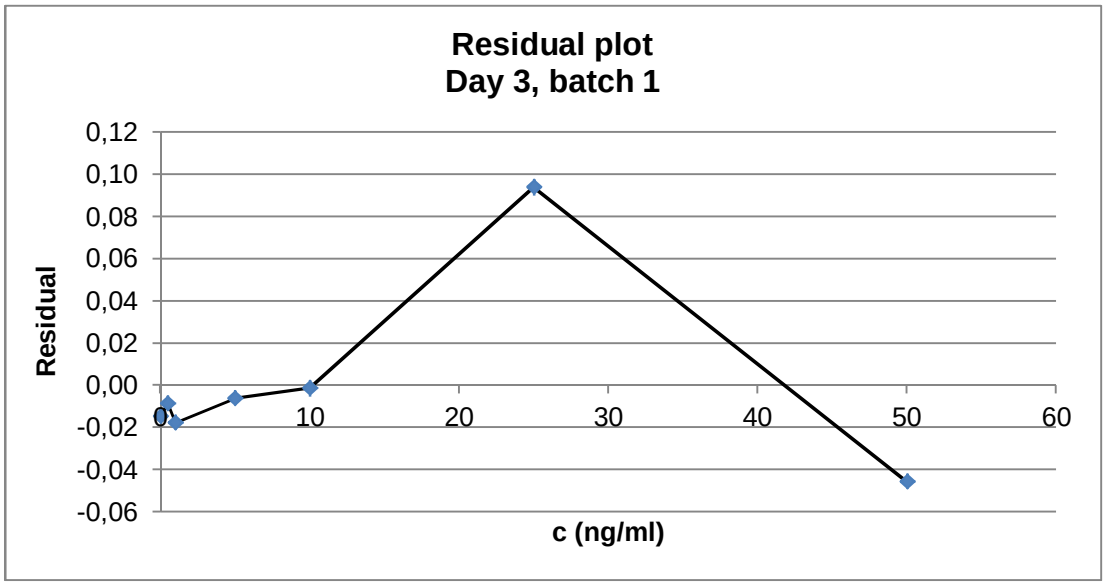

Fentanyl
Day 2, batch 2
y = 0,1001x - 0,01
R² = 0,9999
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)

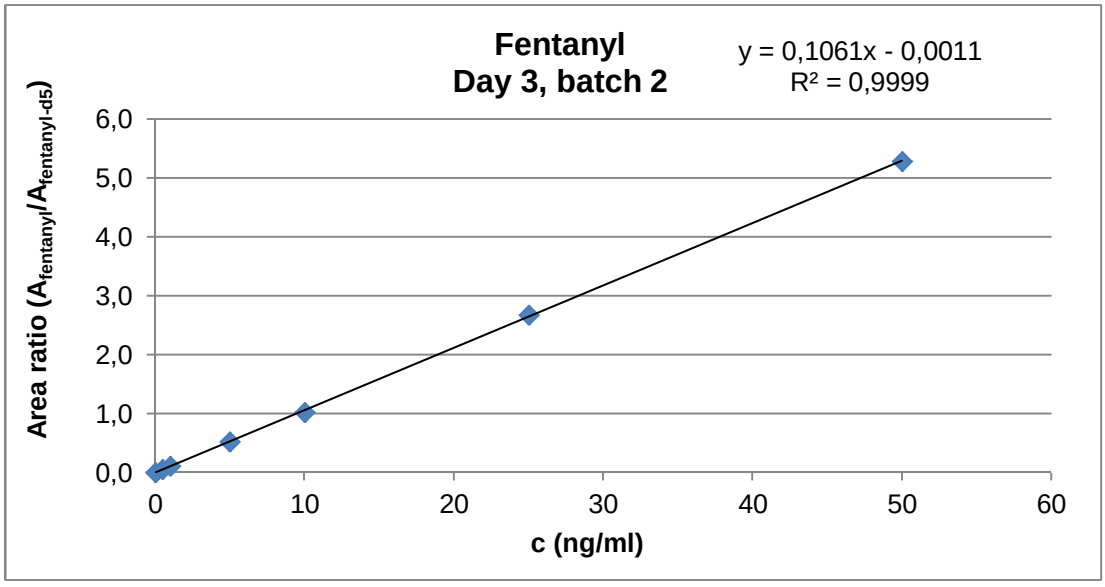

Residual plot
Day 2, batch 2
Residual
0,03
0,02
0,01
0,00
-0,01
-0,02
-0,03
-0,04
-0,05
-0,06
0
10
20
30
40
50
60
c (ng/ml)

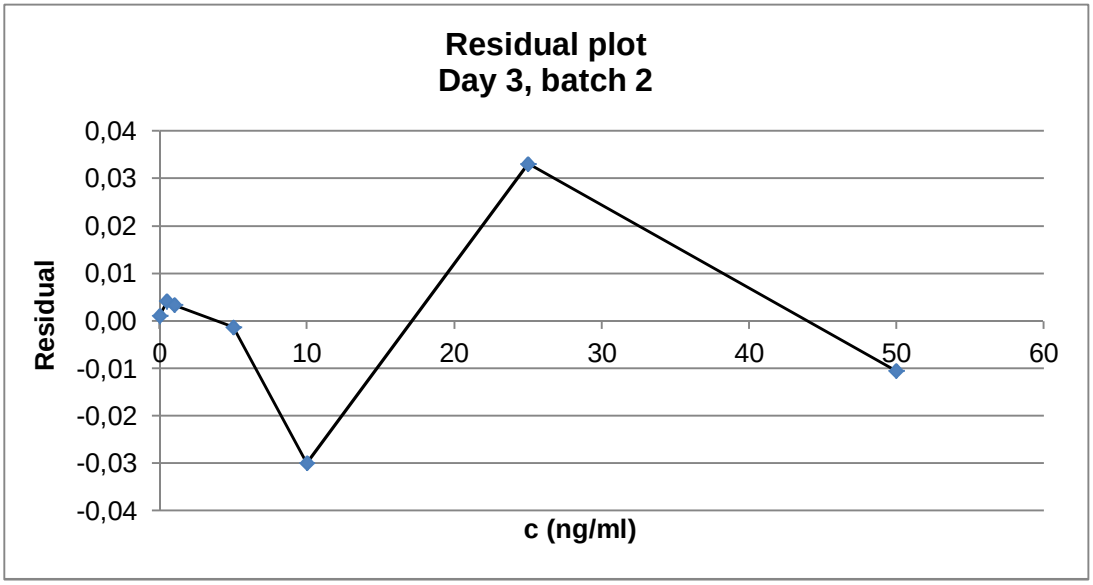

Fentanyl
Day 2, batch 3
y = 0,0992x - 0,0122
R² = 0,9997
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)

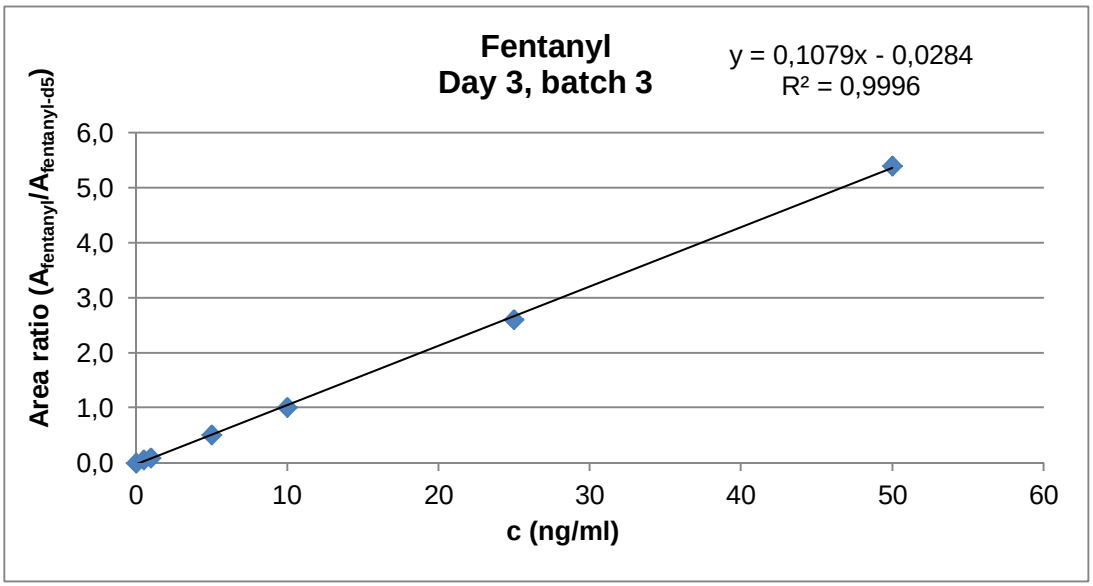

Residual plot
Day 2, batch 3
Residual
0,04
0,02
0,00
-0,02
-0,04
-0,06
-0,08
0
10
20
30
40
50
60
c (ng/ml)

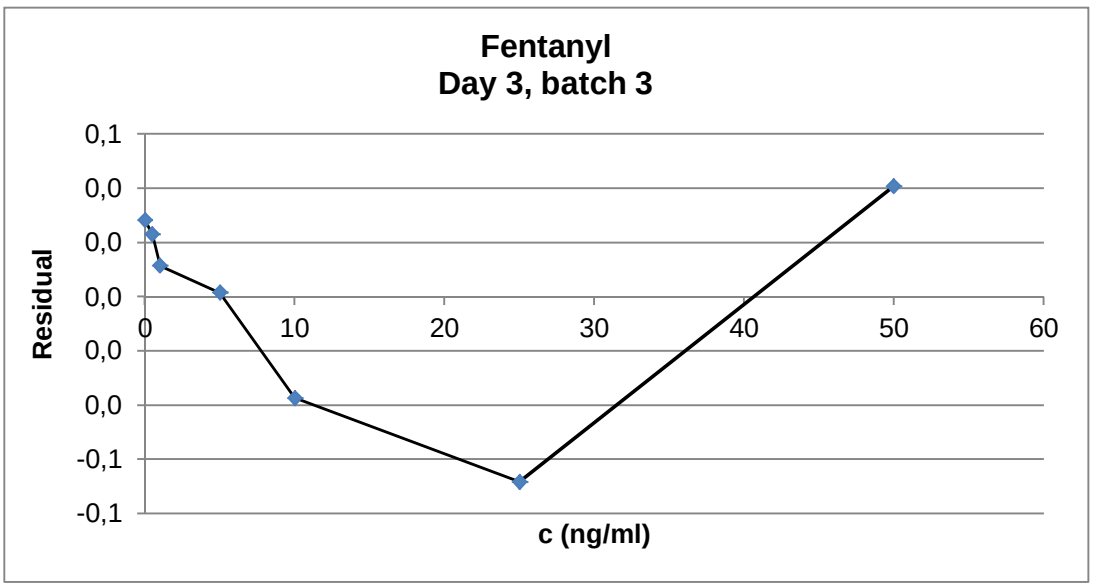

Fentanyl
Day 3, batch 1
y = 0,105x + 0,0146
R² = 0,9995
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)


Residual plot
Day 3, batch 1
Residual
0,12
0,10
0,08
0,06
0,04
0,02
0,00
-0,02
-0,04
-0,06
0
10
20
30
40
50
60
c (ng/ml)


Fentanyl
Day 3, batch 2
y = 0,1061x - 0,0011
R² = 0,9999
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)


Residual plot
Day 3, batch 2
Residual
0,04
0,03
0,02
0,01
0,00
-0,01
-0,02
-0,03
-0,04
0
10
20
30
40
50
60
c (ng/ml)


Fentanyl
Day 3, batch 3
y = 0,1079x - 0,0284
R² = 0,9996
Area ratio (Afentanyl/Afentanyl-d5)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
10
20
30
40
50
60
c (ng/ml)


Fentanyl
Day 3, batch 3
Residual
0,1
0,0
0,0
0,0
0,0
0,0
-0,1
-0,1
0
10
20
30
40
50
60
c (ng/ml)

Appendix 5. Validation results

		LOD and LOQ				
		Slope, m	y-intercept, n	Correlation coefficient (R^2)	LOD (ng/ml)	LOQ (ng/ml)
Day 1	Batch 1	0.1035	-0.0130	0.9999	0.408	1.235
	Batch 2	0.0961	0.0096	0.9998	0.439	1.331
	Batch 3	0.0996	-0.0066	0.9999	0.424	1.284
Day 2	Batch 1	0.0976	-0.0096	0.9997	0.432	1.310
	Batch 2	0.1001	-0.0100	0.9999	0.422	1.278
	Batch 3	0.0992	-0.0122	0.9997	0.425	1.289
Day 3	Batch 1	0.1050	0.0146	0.9995	0.402	1.218
	Batch 2	0.1061	-0.0011	0.9999	0.398	1.206
	Batch 3	0.1079	-0.0284	0.9996	0.391	1.184
Standard deviation of y-intercepts			0.0128	Average LOD and LOQ	0.416	1.259

Recoveries				
Sample ID	c (ng/ml)	Area ratio	c (ng/ml)	Recovery (%)
150904TK18	1	0.088	0.801	80.1
150904TK19	1	0.091	0.826	82.6
150904TK20	1	0.097	0.888	88.8
150904TK21	25	2.426	23.2	92.7
150904TK22	25	2.407	23.0	92.0
150904TK23	25	2.244	21.4	85.7
			Average (1 ng/ml)	83.8
			SD (1 ng/ml)	4.5
			Average (25 ng/ml)	90.1
			SD (25 ng/ml)	3.8

	Observed concentration of standard samples (ng/ml)					
Standard level (ng/ml)	0.5	1	5	10	25	50
Day 1	0.57	0.97	5.02	10.25	25.44	52.03
	0.58	1.09	5.28	9.88	23.68	48.52
	0.56	1.06	5.04	9.63	24.78	50.01
Day 2	0.59	1.07	5.11	9.75	24.05	49.63
	0.60	1.09	5.11	10.04	24.82	50.82
	0.58	1.04	5.08	9.97	24.39	50.43
Day 3	0.60	1.00	5.06	10.04	25.75	49.12
	0.57	1.07	5.01	9.73	25.28	49.81
	0.50	0.90	4.87	9.58	24.52	50.93
Mean	0.57	1.03	5.07	9.88	24.75	50.14
SD	0.03	0.06	0.11	0.22	0.67	1.05
%RSD	4.93	6.07	2.13	2.23	2.70	2.09
Systematic error (ng/ml), bias	0.07	0.03	0.07	-0.12	-0.25	0.14
Systematic error (%), bias%	14.33	3.31	1.33	-1.24	-1.01	0.29

Relative ion abundaces in standard samples at 25 ng/ml				
Sample ID	c (ng/ml)	Area (*m/z* 188)	Area (*m/z* 105)	Relative Ion abundance (%)
150825TK06	25	30027	35101	85.5
150825TK14	25	29187	32015	91.2
150825TK22	25	32623	37302	87.5
150928TK11	25	41349	50678	81.6
150928TK18	25	46489	56452	82.4
150928TK25	25	42431	51606	82.2
150903TK12	25	38096	40645	93.7
150903TK19	25	34315	37902	90.5
150903TK26	25	37639	39864	94.4
150904TK09	25	38466	41694	92.3
150904TK16	25	36738	40362	91.0
		Mean relative ion abundance (%)		88.4

Appendix 6. Analysis of variance

c (fentanyl) = 0.5 ng/ml
Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	3	1.7045725	0.5681908	0.0001506
day 2	3	1.7651746	0.5883915	4.353E-05
day 3	3	1.675172	0.5583907	0.0022872

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001404164	2	0.0007021	0.848831	0.4735624	5.1432528
Within Groups	0.004962697	6	0.0008271			
Total	0.006366861	8				

s_w	0.0288	s_w (%)	5.03
s_b	0.0065	s_b (%)	1.13
s_{tot}	0.0295	s_{tot} (%)	5.16

c(fentanyl) = 1 ng/ml
Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	3	3.1251907	1.0417302	0.0041671
day 2	3	3.198914	1.0663047	0.0005049
day 3	3	2.9740057	0.9913352	0.0066641

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.008763974	2	0.004382	1.1596612	0.375137	5.1432528
Within Groups	0.022672071	6	0.0037787			
Total	0.031436045	8				

s_w	0.061	s_w (%)	5.95
s_b	0.014	s_b (%)	1.37
s_{tot}	0.063	s_{tot} (%)	6.11

c (fentanyl) = 5 ng/ml
Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	3	15.349555	5.1165183	0.020373
day 2	3	15.303055	5.1010184	0.000305
day 3	3	14.945317	4.9817722	0.0097974

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.032616391	2	0.0163082	1.6053858	0.2764176	5.1432528
Within Groups	0.060950566	6	0.0101584			
Total	0.093566957	8				

s_w	0.101	s_w (%)	1.99
s_b	0.045	s_b (%)	0.89
s_{tot}	0.110	s_{tot} (%)	2.18

c (fentanyl) = 10 ng/ml
Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	3	29.760962	9.9203207	0.0957591
day 2	3	29.76671	9.9222366	0.0228458
day 3	3	29.354794	9.7849314	0.0559453

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.037186651	2	0.0185933	0.319564	0.7381092	5.1432528
Within Groups	0.349100523	6	0.0581834			
Total	0.386287174	8				

s_w	0.241	s_w (%)	2.44
s_b	0.115	s_b (%)	1.16
s_{tot}	0.267	s_{tot} (%)	2.71

c (fentanyl) = 25 ng/ml
Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	3	73.903374	24.634458	0.7903887
day 2	3	73.269249	24.423083	0.147505
day 3	3	75.554831	25.184944	0.385823

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.928145828	2	0.4640729	1.0517498	0.4059158	5.1432528
Within Groups	2.647433459	6	0.4412389			
Total	3.575579287	8				

s_w	0.664	s_w (%)	2.68
s_b	0.087	s_b (%)	0.35
s_{tot}	0.670	s_{tot} (%)	2.71

c (fentanyl) = 50 ng/ml
Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	3	150.55662	50.185539	3.1136881
day 2	3	150.8751	50.291699	0.3686536
day 3	3	149.85586	49.951954	0.8345555

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.181258742	2	0.0906294	0.0629823	0.9395725	5.1432528
Within Groups	8.633794443	6	1.4389657			
Total	8.815053185	8				

s_w	1.200	s_w (%)	2.39
s_b	0.670	s_b (%)	1.34
s_{tot}	1.374	s_{tot} (%)	2.74