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Analysis of Psychoactive Compounds in Oral Fluid by Capillary Electrophoresis

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Piret Saar-Reismaa

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Psühhoaktiivsete ühendite analüüs süljes kapillaarelektroforeesi meetodil

PIRET SAAR-REISMAA

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Contents

List of Publications

The list of author's publications, on the basis of which the thesis has been prepared:

I J. Mazina, **P. Saar-Reismaa**, M. Kulp, L. Poryvkina, M. Kaljurand, M. Vaher, Determination of γ-hydroxybutyric acid in saliva by capillary electrophoresis coupled with contactless conductivity and indirect UV absorbance detectors, *Electrophoresis*. 36 (2015) 3042–3049.

II **P. Saar-Reismaa**, M. Vaher, M. Kaljurand, M. Kulp, J. Mazina-Šinkar, Simultaneous determination of γ-hydroxybutyric acid, ibotenic acid and psilocybin in saliva samples by capillary electrophoresis coupled with a contactless conductivity detector, *Analytical Methods*. 9 (2017) 3128–3133.

III **P. Saar-Reismaa**, A. Tretjakova, J. Mazna-Šinkar, M. Vaher, M. Kaljurand, M. Kulp, Rapid and sensitive capillary electrophoresis method for the analysis of *Ecstasy* in an oral fluid, *Talanta*. 197 (2019) 390–396.

IV **P. Saar-Reismaa**, E. Erme, M. Vaher, M. Kulp, M. Kaljurand, J. Mazna-Šinkar, *In Situ* Determination of Illegal Drugs in Oral Fluid by Portable Capillary Electrophoresis with Deep UV Excited Fluorescence Detection, *Analytical Chemistry*. 90 (2018) 6253–6258.

V **P. Saar-Reismaa**, C-A. Brilla, K. Leiman, M. Kaljurand, M. Vaher, M. Kulp, J. Mazina-Šinkar, Use of a newly-developed portable capillary electrophoresis analyser to detect drugs of abuse in oral fluid: A case study, *Talanta*. 211 (2020) 120662.

Author's Contribution to the Publications

The author's contributions to the papers in this thesis include:

I The author was responsible for analysis procedure optimization and most of the experimental work. She performed validation, real OF sample collection, and analysis of the GHB samples. She interpreted the results and participated in the preparation of the manuscript.

II The author was responsible for planning and conducting the experiments. She set up the design of experiments (DOE) plan, validation steps, and performed all experiments. She also interpreted all the data and was the main writer of the manuscript, acting as the first and corresponding author.

III The author was responsible for field testing, sample collection, and some of the CE experiments. She interpreted the OF sample results and assisted with manuscript composition.

IV The author planned the experiments for testing the detector for linearity, noise, and DOA detection. In addition, she participated in field testing and was responsible for all OF testing. She assisted with manuscript composition and is the first author.

V The author planned most of the experiments, participated in field testing, prepared the samples, analysed the samples, and obtained CE results (HPLC-MS results were obtained by Kristiina Leiman). The author performed most of the CE-FD validation, wrote the manuscript, helped with the revisions and is the first author.

Abbreviations

1,4-BD	1,4-Butanediole
a.u.	Arbitrary units
ACN	Acetonitrile
ALC	Allocryptopine
AMP	Amphetamine
Arg	L-Arginine
BGE	Background electrolyte
BZA	Benzylamine
C^4D	Capacitively coupled contactless conductivity detector
CE	Capillary electrophoresis
COC	Cocaine
COET	Coca-ethylene
CTAB	Cetyltrimethylammonium bromide
CV%	Variation coefficient
CWL	Centre wavelength
DA	Diagnostic accuracy
DAD	Diode array detector
DOA	Drugs of abuse
DOE	Design of experiments
DRUID	Driving Under the Influence of Drugs, Alcohol and Medicines
EMCDDA	European Monitoring Centre of Drugs and Drug Addiction
EOF	Electro-osmotic flow
EtOH	Ethanol
EWDTS	European Workplace Drug Testing Society
FD	Fluorescence detector
F_{EM}	Emission filter
FN	False negative
FP	False positive
fwhm	Full width at half maximum
GHB	y-Hydroxybutyric acid
HDMB	Hexadimethrine bromide
HMA	4-Hydroxy-methoxyamphetamine
HMMA	4-Hydroxy-3-methoxymethamphetamine
HPLC-MS	High pressure liquid chromatography mass-spectrometry
i.d.	Inner diameter
IBO	Ibotenic acid
IDL	Instrument detection limit
IQL	Instrument quantification limit
IS	Internal standard
λ	Wavelength

LED	Light emitting diode
L_{ef}	Effective length
LOD	Limit of detection
LOQ	Limit of quantification
L_{tot}	Total length
Mal	Maleic acid
MDA	3,4-Methylenedioxymethamphetamine
MDEA	3,4-Methylenedioxy-N-ethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
ME	Matrix effect
METH	Methamphetamine
N	Peak efficiency
o.d.	Outer diameter
OF	Oral fluid
PMA	Para-methoxyamphetamine
PMMA	Para-methoxymethamphetamine
PMPA	Pinacolyl methyl phosphonic acid
PMT	Photomultiplier tube
POC	Point of care
PY	Psilocybin
r	Correlation coefficient
r^2	Coefficient of determination
ROSITA	Roadside Testing Assessment
Rs	Resolution
S/N	Signal-to-noise ratio
SAMHSA	Substance Abuse and Mental Health Services Administration
SD	Standard deviation
SE	Sensitivity
SP	Diagnostic specificity
Suc	Succinic acid
TEA	Triethanolamine
THC	Tetrahydrocannabinol
TN	True negative
TP	True positive
Tris	Tris(hydroxymethyl)aminomethane
UV/vis	Ultraviolet/visible
WHO	World Health Organization

Introduction

The drug problem is growing rapidly worldwide and exceeds the dimensions of illicit drugs, including medicine, novel psychoactive substances, and other legal substances such as nicotine, coffee, and alcohol. The prevalence of illicit drug consumption, prescription medicine abuse, and recreational experimentation is increasing due to the easy access provided by the internet and the rise of dark web. Profuse amounts of information can be obtained for various products and methods to legally and illegally consume drugs or plants and mushrooms to relieve curiosity or contribute to an already established drug abuse problem.

In 2017, 585 000 deaths were associated with drug use and an estimated 35 million people suffered from drug use disorders requiring treatment. The prevalence of illicit drug use is reported yearly by the World Drug Report compiled by the World Health Organization (WHO). The 2019 report estimated 29 million users for amphetamine (AMP) and methamphetamine (METH); 8.4 to 40 million people for ecstasy (MDMA); and 18.1 million cocaine users (COC), with no decrease forecasted [1]. Depressants such as γ-hydroxybutyric acid (GHB) are not considered to be widely consumed, but in Europe GHB is a top five drug found in emergency presentations [2]. Unfortunately, statistics gathered on psychoactive substances including the magic mushroom compound psilocybin (PY), ibotenic acid (IBO), and medicinal drugs is insufficient for reliable comparisons [3]. Drug use trends are evaluated based on various data collection methods, which includes wastewater and syringe content analysis as well as pill/powder checking at festivals that provide unbiased information, unlike web surveys or one-on-one interviews [1,2,4].

The main effect of psychoactive substances is mind-alteration that can lead to direct health consequences associated with high-risk behaviour. In addition, people injecting drugs are at a high risk for infectious diseases including Hepatitis C and B as well as HIV with the possibility of death due to overdoses and infections [5–7]. Moreover, drug abuse may increase public safety risks as people under the influence of drugs are hazardous due to drugged driving, accidents in safety-critical tasks, or increased violence [7–9]. Accidental poisonings and intentional spiking cases are observed in hospitals and at festivals [10–12], facilitating the need for improved portable testing to prevent harmful outcomes and increase overall safety.

An option for public safety enhancement is random testing for drugs of abuse (DOA). This can be performed by roadside testing by authorities, workplace testing, or in care facilities. Testing high volumes of people is preferably achieved by rapid screening that is simple, fast, and cheap. As impairment is correlated mainly to active intoxication, the samples should reflect the current levels of DOA. Using biological fluids besides blood has increased in popularity as their collection and handling is less invasive and provides reliable results without the need for specially trained personnel or sample transport to specialized facilities. Because urine provides retrospective information, other matrixes like oral fluid (OF) and exhaled breath or sweat are preferred. OF is an established specimen composed of excretions from the salivary glands, gingival crevicular fluid, oro-nasopharyngeal secretions, and cellular debris [13]. OF is representative of the current state of an individual and most DOA appear in their native forms. Moreover, several countries including Germany, France, Belgium, Italy, Finland, and Australia conduct routine roadside rapid testing using OF to evaluate drugged driving [14].

Regardless of the matrix, the most commonly used testers are immunoassay-based due to their easiness of use, low costs, and fast analysis for the determination the presence of a substance by visually interpretable colour indicators. However, the results are qualitative and based on a general type or class of molecule, rather than the actual DOA. The most significant drawback is that immunoassays are associated with a high rate of false-positives due to cross reactions of the antibodies with other medicines, foods, and overall lack of selectivity [15–17]. Although it is used in Canadian roadside testing, the Dräger DrugTest® 5000 faces similar issues in terms of false positives, problems with handling, lack of quantitative results, and other shortcomings [18].

These limitations can be overcome by using analytical techniques for improved selectivity. Separation techniques, including capillary electrophoresis (CE), represent promising alternatives as they provide separation and detection of each compound unhindered by the matrix, but their application is rare due to most of them being non-portable. Unlike many techniques, CE is becoming increasingly popular as the instrumentation is easily miniaturized, and different detection modes can be easily combined. Additionally, CE is considered favourable as it only requires small sample and reagent quantities compared to other separation techniques and provides fast results. Advances in CE have promoted it as a reliable separation technique that is now recommended in DOA testing guidelines [19]. Although, CE sensitivity is hindered by the small analyte amounts, fluorescence can provide additional selectivity and sensitivity for DOA analysis.

Altogether, improvements in chemical analysis for rapid on-site drug testing are highly desirable to provide more detailed information and additional safety to society. This can be achieved by using CE with various detectors and OF samples. Therefore, this dissertation focused on developing and validating analytical procedures to enable onsite detection and quantification of psychoactive substances in OF using existing and newly developed portable CE coupled to conductivity, UV-absorbance, and fluorescence detectors. The majority of the dissertation research was performed under existing projects in our research group to develop portable CE analysers for DOA analysis in OF.

The dissertation is composed of five main sections. First, an overview of the theoretical background of psychoactive compounds, CE, validation, and drug legislation is provided. Chapter 2 explains the main goals of this thesis and Chapter 3 outlines the chemicals and reagents used, as well as the sample preparation techniques, instrumental set-up, and experimental conditions. Chapter 4 presents the results of **Publications I–V** as follows: the detection and validation of GHB by CE using simultaneous UV absorption and C^4D detection and that of GHB, ibotenic acid, and PY by CE-C^4D in OF (**Publications I and II**); the development of analysis procedures for quantification of *ecstasy* and its analogues in OF by CE-LED-FD (**Publication III**); the introduction of a novel portable CE instrument with UVC excitation fluorescence detection of various DOA (**Publication IV**); and validation of the analysis procedure for OF samples with real samples from Weekend Festival Baltics from 2016–2018 are discussed along with the instrumental statistics of the newly developed analyser (**Publication V**). In addition to the published data, the associated research has been presented at international conferences in Portugal, Ireland, France, Latvia, and Estonia.

1 LITERATURE OVERVIEW

1.1 Psychoactive substance abuse

As defined by World Health Organization (WHO) psychoactive substances are "substances that, when taken in or administered into one's system, affect mental processes, e.g. cognition or affect"[20]. The difference between licit and illicit drugs and dependence-producing substances is mainly defined by legislation, making psychoactive substance the most neutral and most accurate definition. Many of these substances have medicinal uses in prescribed dosages, but are considered abused when not used accordingly and are then known as drugs of abuse (DOA).

In 2017 there were 585 000 deaths associated with drugs use and an estimated 35 million people suffer from drug use worldwide, indicating that their use is harmful and treatment is required [1]. Increasingly worrying is the impact of drug abuse on traffic and work-place safety. In 2017 >5% of people over 16 years old drove under the influence of drugs [21] and in Europe samples collected from drivers were positive in almost 2% of all cases [22]. The most commonly found drugs were cannabis and stimulants such as amphetamines and cocaine. Recent studies showed that ~2% of workers tested in Europe were positive for a DOA [23,24]. Therefore, it is of utmost importance to obtain available tools to determine possible threats from being under the influence of DOA.

Psychoactive substances are classified in various overlapping categories, such as naturally occurring or synthetic, stimulants, dependence-inducing, relaxants, and hallucinogens. In addition, these substances can be structurally grouped – amphetamine and derivates, or by their plant origin – coca alkaloids, and even by types of fungi – psychoactive mushroom.

1.1.1 Amphetamines

Amphetamine (AMP) and associated derivates are mainly synthetic central nervous system stimulants associated with the uptake of dopamine, norepinephrine, and serotonin [25,26], providing increased confidence, sociability, and energy [27]. The most common derivates of AMP include methamphetamine (METH), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA; also known as ecstasy), and 3,4-methylenedioxy-N-ethylamphetamine (MDEA), which are all shown in Figure 1.1.

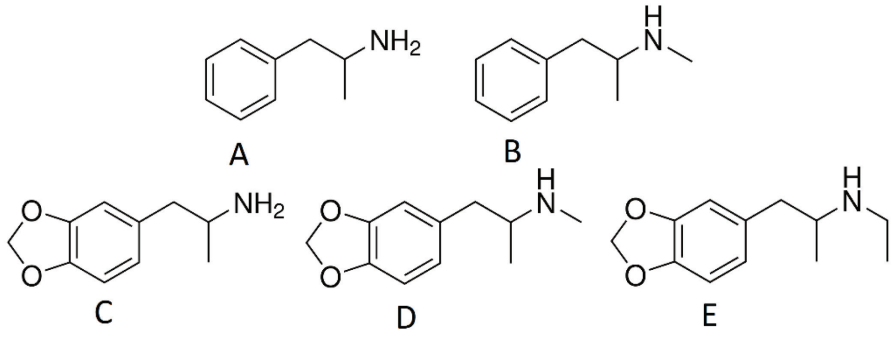

Figure 1.1. Amphetamine and its derivatives. A – AMP, B – METH, C – MDA, D – MDMA, E – MDEA.

AMP was first extracted from *Ephedra vulgaris* by Seldalano in 1887 [27], but the first synthesis was performed by Nagai in 1893 [27]. It was first promoted by Merck as a cold remedy and for narcolepsy [28]. Currently, AMP remains in use to treat attention deficit disorder (ADHD) and narcolepsy [28,29] under the trade name Adderall®. The common side effects are associated with heart and blood vessels, but also include tremors, seizures, hyperactivity, and may cause death [30,31]. The pharmaceutical dosage ranges from 5–60 mg daily [30], but when abused the dosage increases with tolerance, reaching as high as 1 000 mg at time and up to 5 g per day. AMP has been reported to be lethal at a dosage of 1.5 mg/kg [32]. The main metabolites of AMP are 4-hydroxyamphetamine, 4-hydroxynorephedrine, and norephedrine, with 30-40% of AMP excreted unchanged with urine [30]. AMP presents in OF mainly unchanged and can be detected up to 20–50 h after initial ingestion [33]. Real-world OF AMP levels range between 15 and 131 000 ng/mL [34,35] with OF concentrations several times higher than those found in blood [36].

METH was first synthesised in 1893 by Nagayoshi from ephedrine contained in *Ephedra sinica* [37]. As with AMP, it was first used as a non-prescription drug to treat colds, depression, and for weight-loss [38]. Side effects include sweating, hallucinations, aggression, nausea, and moodiness. Similar to AMP, METH causes cardiovascular pathologies as well as liver and kidney damage, which may result in death [27,39]. Medicinal tablets contain 5 or 10 mg of methamphetamine HCl for daily dosages of 20–25 mg [40], while abuse dosages increase with tolerance, reaching levels several hundred-fold greater than the therapeutic dosage at up to 15 000 mg per day [27]. METH is used to treat ADHD and can be used as an aid for short-term weight reduction [41]. The first metabolite is AMP, rendering the differentiation between AMP and METH intake difficult to obtain. METH can be detected in OF 0.5 to 2 h after intake, reaching maximum levels in 2-12 h, with AMP appearing 1–23 h after METH intake [42,43]. Samples collected from drug abusers range from 100–7000 ng/mL [44,45] and OF samples can contain METH up to 72 h after intake [43,46].

MDA was first synthesised by Mannich and Jacobsohn in 1910 [47] and has been used to treat Parkinson's disease and depression, used as an anorectic or even a truth serum [48]. It is a ring-substituted AMP that creates effects of joy, hallucinations, and blood pressure changes [49,50]. Recreational doses range from 75–150 mg [40,51] and the effects of MDA last approximately 8 h [52], with 4-hydroxy-methoxyamphetamine (HMA) as the main metabolite [51]. In addition, MDA is more toxic than MDMA [27,50] and real OF samples from drivers have reached concentrations up to 230 ng/mL [35,53].

The discovery of MDMA is credited to Köllisch in 1912 who described it as a precursor of a Merck patent file [54,55]. Among with MDA, MDMA was first tested for psychological therapies for post-traumatic stress syndrome [56,57]. It creates feelings of euphoria, increased trust, and sensory awareness, but is much less hallucinogenic than MDA [27]. Side-effects include anxiety and insomnia, while higher doses are similar to AMP with cardiovascular pathologies that may lead to overdose-related deaths [27,58]. Recreational and therapeutic testing dosages are the same as MDA at 75 to 150 mg [40,59], but users with increased tolerance may take up to 750 mg a day [27]. MDMA effects begin 15–20 min after intake and last 4–6 h [27,51,60]. The main metabolites of MDMA in OF are MDA and 4-hydroxy-3-methoxymethamphetamine (HMMA) [61], making the abuse of MDA and MDMA difficult to distinguish. MDA concentration levels in OF are ~5% of those of MDMA, while HMMA can be found in trace amounts [61]. Recreational intake for a single admission usually shows levels of 33–3533 ng/mL,

with multiple intakes reaching 7077 ng/mL MDMA [62]. Tested drivers have shown OF concentrations as high as 5000 ng/mL [53].

MDEA was first reported in the 1960s by Shulgin while working on methylenedioxy compounds [63] and is the least common of AMP derivative compared to AMP, METH, MDA, and MDMA [64]. It is associated with feelings of heightened sense, increased tactile sensations, and a strong desire to converse with people [52]. Side-effects include muscle aches, anxiety, and irritability, as well as similar cardiovascular effects as MDA and MDMA in higher dosage [49,65]. In recreational use, doses range from 25 to 3320 ng/mL [62] with MDA as the primary metabolite [40]. MDEA has been detected post-mortem, although no direct linkage with death has been determined [58,66]. The duration of the "high" is only 3–4 h, being the softest and shortest of the 3,4-methylenedioxy analogues of AMP [52,67]. OF concentrations after recreational intake typically range from 25 to 3320 ng/mL [62].

1.1.2 Cocaine, coca-ethylene

Cocaine (COC) (Fig. 2A) is a naturally occurring coca alkaloid that is extracted from the plant *Erythroxylum coca* and *Erythroxylum novogranatense* bush, which were cultivated as early as 3000 BC [27]. At least 14 coca alkaloids, include cocaine, cocamine, hygrines, benzoylecgonine (BZE), and ecgonine are present in the plants. The leaves of the plants are chewed by around three million daily users in South America for increased stamina, reduced hunger, and amelioration of the effects of oxygen deprivation. In 1880 Nieman extracted pure COC form plant leaves [8], which was widely used as an anaesthetic during surgeries, to reduce bleeding [40], and even used briefly in the Coca-Cola recipe [68]. The effects originate from the inhibition of dopamine and noradrenaline reuptake [69], as well as sodium channel blocking [70], making it a useful local topical aesthetic [27].

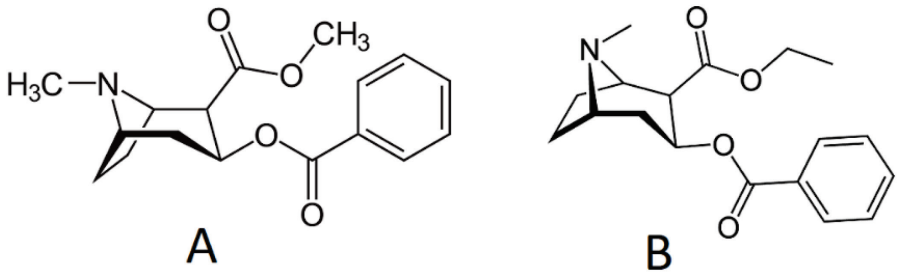

Figure 1.2. Two common coca alkaloids. A – Cocaine, B – coca-ethylene.

Recreationally, COC is used for provoking euphoria, increasing awareness, and reducing sleep requirement, but it causes side effects such as tachycardia increased blood pressure, and can lead to anorexia. The minimal dosage needed for an effect is 25 mg [27], while oral consumption of 1.2 g can lead to death. The onset of effects depends on the route of intake varying from 10 s when smoked to 15 min if taken orally, with the "high" lasting from 5 to 90 min [70]. This effect is often enhanced by simultaneous consumption of ethanol (EtOH), which increases the bioavailability of orally consumed COC by a factor of 3–4 without lengthening the half-life [71,72]. EtOH consumption also inhibits the metabolism of cocaine to BZE and methyl esters, increasing the specific metabolite coca-ethylene (COET) [73] (Fig. 2B). COET is more toxic than COC alone [74] with COC and COET resulting in abnormal locomotor behaviour, presenting with incredible strength, delusions, paranoia, multiple personalities, and overtly psychotic

states. In addition, COC abuse may lead to atherosclerosis, rhabdomyolysis, ischemic strokes, myocardial infarctions, and death [70,75–78]. OF detection times for COC are up to 12 h for a single intake and 24 h for repeated intakes [79,80]. Various studies have reported OF COC levels in drivers of 2000 [35], 70 000 [81], and 2700 ng/mL [53], with COET concentrations as high as 10.5 ng/mL [82,83].

1.1.3 Psychoactive mushroom compounds

Mushroom consumption is an ancient tradition, some of which are food, poisonous, or have psychoactive effects. Psychoactive mushrooms are often referred to as "magic mushrooms" as they induce hallucinogenic effects, feelings of euphoria, and alternative states of mind. Most magic mushrooms belong to the genus *Psilocybe*, with various subfamilies depending on the geographical region. The most common species is *Psilocybe semilanceata*, which is naturally found in Northern Europe, including Estonia [84]. The most commonly sold subfamily is *Psilocybe cubensis* and is widely available on the internet [85]. Collection of these mushrooms is highly legislated in most countries where they grow naturally. Despite the restrictive measures in place, magic mushrooms have been used for centuries in Asian, South American, and Hawaiian cultures [86]. The main psychoactive alkaloids in the mushrooms are psilocybin (PY) and psilocin (PI; Fig. 3 A and B). PY and PI were first isolated by Hofmann et al. in 1959 [27].

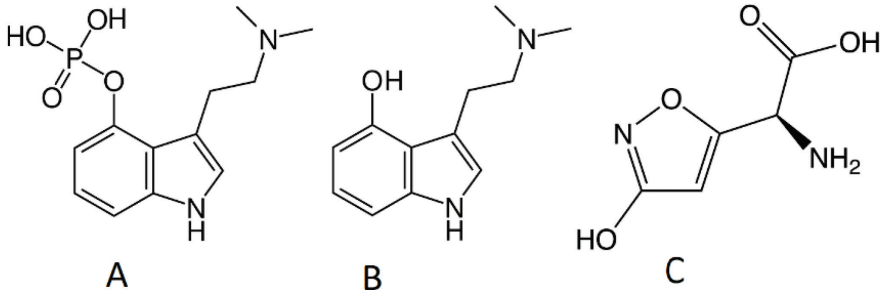

Figure 1.3. Psychoactive compounds naturally occurring in magic mushrooms. A – psilocybin, B – psilocin and C – ibotenic acid.

Although magic mushrooms are mainly considered not toxic [84], some cases of acute renal failure have been reported [11], in addition to documented side-effects of nausea, gas, stomach discomfort, and possible psychotic episodes [87]. Recreational users consume approximately 1 g of dried or 10 g of the fresh mushroom per "trip" with active ingredient dosages of approximately 4–10 mg [88] and the typical effects last between 2 and 6 h [89]. Medicinal usage of magic mushrooms for treatment-resistant depression and cluster-headaches have been suggested [87,90–92]. After consumption of PY, rapid metabolism occurs with conversion to PI within a few minutes for both the oral and IV routes [93]. With administration of 10–20 mg of PY, PI levels in urine and serum can reach as high as 400 ng/mL [93–95]. Although 3–10% of PY typically remains in its original state [84], no PY was detected in the urine or blood at pharmacological dosages [96]. The suggested possible OF detection is up to 8 h after ingestion [97], but no OF concentrations have been reported to date.

Unlike *Psilocybe*, several mushrooms with psychoactive compounds can be gathered freely and legally. The *Amanita* genus is a legal mushroom, with the best-known example of *Amanita muscaria* also known as the red fly agaric. The first mention of *A. muscaria*

effects was reported in 1730 by von Strahlenburg describing the shamans from far north Scandinavia and Russia who used it for rituals [98]. It is considered poisonous due to its muscarine content, but can be used recreationally due to its compensatory ibotenic acid (IBO) content (Fig. 3 C) [10]. IBO is not a scheduled substance, although it exhibits hallucinogenic properties and evokes euphoria 0.5–1.5 h after ingestion. Side effects may include gastrointestinal discomfort, convulsions, agitation, and violent behaviour [99]. In rare cases, severe intoxication can result in a coma with life-threatening respiratory and circulatory disorders [10]. The psychoactive dose for IBO starts at 20 to 60 mg [100], an amount that is usually contained within one mushroom cap [101] and its effects can last for 2–3 h [102]. Most IBO is excreted unchanged resulting in concentrations ranging from 32 to 55 mg/L in urine after ingesting the mushroom [103], but no research has been performed to determine IBO from OF to date.

1.1.4 Gamma-hydroxybutyric acid

γ-Hydroxybutyric acid (GHB) is an endogenous molecule and, along with its precursors 1,4-butanediole (1,4-BD) and γ-butyrolactone (GBL), it is abused as a central nervous system suppressant [104] (structures shown in Figure 4 A–C). Endogenous GHB concentrations are correlated to γ-aminobutyric acid (GABA; Figure 4D). GABA is responsible for the signalling systems in the stomach, pancreas, ovaries, and urinary bladder [105]. Under physiological conditions, GHB originates from GABA which is metabolized to GHB, while exogenously consumed 1,4-BD and GBL are also metabolized to GHB [106].

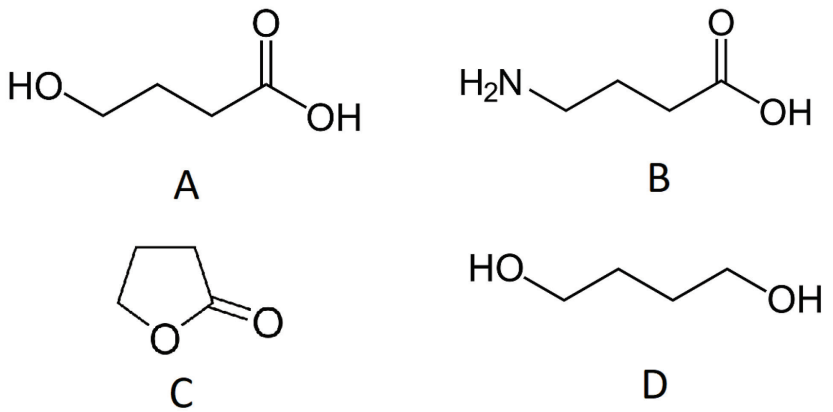

Figure 1.4. The structures of GHB and related molecules. A – GHB, B – GABA, C – GBL, D – 1,4-BD.

In 1874 GHB was first synthesised by Zaytsev by adding NaOH to GBL [107] and GHB salts are commonly referred to as oxybates. Sodium oxybate is used as a prescription medication for narcolepsy and alcoholism treatment in Austria, Italy, USA, France, and Germany [108,109], with other reported uses for spinal anaesthesia and as a hypnotic agent. The medicinal dosage is 50 mg/kg [110,111] and for narcolepsy and fibromyalgia the dosage is 1.5 to 2.25 g orally and every 3–4 h [112,113]. Endogenous levels of these compounds in different bodily fluids and sexes are intervaried, but typically range from 0.5 to 3.33 mg/L in urine, blood, and OF [114–118]. Recreational use of these drugs results in relaxation and mild euphoria [119] as follows: <1 g – mild relaxation; 1–2 g – strong relaxation; 2–4 g – induces sleep for a few hours; 4–8 g – very deep sleep;

>10 g – very deep sleep leading to a coma [120]. Acute GHB poisoning can cause death [110,111,121], but addicts can tolerate doses as high as 100 g per day, as withdrawal induces insomnia and anxiety [122,123]. Elimination of GHB occurs within 4–8 h [104] and can be detected in OF for 150 min as a parent compound [124].

1.2 Detection of drug intoxication

Detection of intoxication is important for safety-critical fields that include driving, working with machinery, and complex cognitive tasks. The impact of being under the influence of drugs may cause accidents that result in death. Therefore, the need to detect intoxication is of the utmost importance. Most primary detection is performed visually by professionals and for example in the USA the most commonly used approach is Standardized Field Sobriety Tests (SFSTs). SFSTs are mainly used to evaluate alcohol intoxication in drivers but can also be used to assess impairment due to other DOAs [125]. SFSTs are reliable and accurate with blood alcohol concentrations of >0.08% [126], while for amphetamines the SFST results were mainly unaffected, but MDMA exhibited significant impairment to overall performance [127]. In Europe, many countries have regulated the important visual signs to distinguish, for example in Estonia:

- personal appearance
- altered response time
- slurred speech
- problems in perception of place, time, and people
- unconsciousness
- memory problems
- co-ordinational errors
- behavioural difficulties
- pupil reactivity indicating drug abuse [128].

Failure of these tests/inspections can result in the requirement to provide evidentiary samples for further evaluation.

In Europe it is estimated that 1–5% of the general driver population uses illicit drugs and 5–10% consume licit drugs that may affect their capabilities [129]. In the USA, a study by National Highway Traffic Safety Administration's (NHTSA) showed that 22% of night-time drivers tested positive for illegal, prescription, or over-the-counter medications, while only 1.5% of night-time drivers tested positive for an alcohol above the legal limit [130]. In addition to roadside testing of drivers, testing is conducted in other areas and in Europe approximately 5% of workplaces test for DOA [131]. A Norwegian study showed that approximately 1% of workers tested positive for illicit drugs [23], while in Italy the rate was 2% [24]. Similar numbers have been found in the USA for pilot testing, showing a rise from 2.3% to 3.8% (1990–2012) positive results for illicit drugs [132]. Interestingly, the testing of the OF of Australian skippers showed that 13% were positive for illicit drugs [133].

The impact of substance abuse is difficult to evaluate as the drug potency, administration route, intake history, testing delays, and test sensitivity limit the comparability to real-life situations [22]. Although various results have been reported for the intake of a single drug, most results from long-term and current users show a negative impact on complex cognitive abilities due to the use of AMP [134], METH [135], MDMA [136], COC [137], and GHB [138], constituting a liability to traffic safety and other overall security-risks [139]. Statistical reports regarding fatal car accidents with drivers

having DOAs in their system shows that up to 60% of drivers in single vehicle crashes had alcohol and/or drugs in their blood samples, compared with 30% of drivers killed in collisions with other vehicles in Nordic countries [140]. This highlights the need for preventive methods to reduce road and other types of accidents. Policies including random testing at workplaces for drugs and alcohol have a significant impact on cost reduction and accidents as consumption rates decrease [141,142].

Most testing is performed in a laboratory using highly sensitive GC-MS or LC-MS instrument for analysis of DOAs in various matrices. GC-MS and LC-MS methods provide detection limits of <1 ng/mL, but often require several preparation steps including derivatization prior to analysis [143–146]. In addition to classical methods based on CE [147,148], ion-mobility spectrometry (IMS) [149,150], near infra-red spectrometry (NIR) [151], Raman [152], and nuclear magnetic resonance (NMR) [153] have been reported for DOA quantification in biological fluids.

1.2.1 Point of care drugs testing in OF

Point of care (POC) testing allows for the evaluation of DOA usage on-site for rapid answers and determination of possible additional intervention. POC instruments should be easy to use, fast, and safe, which also extends to the sampling procedure. As most DOA testing is performed using blood, sample collection becomes an issue in terms of its invasiveness. Using blood requires specialized training and equipment for sample gathering. Recent studies have suggested the use of dried blood spots for analysis, but their use is fairly scant for DOA and is mostly achieved by sending samples to a lab for analysis [154,155].

Alternative matrices have been studied to overcome these complications associated with blood testing. A commonly used matrix is urine, which is fairly easy to collect, but does not reflect the current state of an individual rather previously consumed substances and exhibits a high possibility of tampering. Exhaled breath, as with alcohol testing, has been used for DOA testing, but the concentrations are quite low, requiring additional sample treatment and a sensitivity that can only be currently achieved in a lab setting [156,157]. OF, a combination of saliva and other oro-nasopharyngeal secretions with cellular debris [13], can provide good results in DOA testing as it reflects current intoxication within minutes of consumption with great selectivity and sensitivity for DOAs (91–98%) [40,158]. Furthermore, drugs in OF are non-ionised and unbound to proteins, so the concentrations reflect the free and non-ionized portion in the blood plasma [159], providing an overview of the current intoxication state. OF testing has some minor drawbacks as some drugs, including cannabis, METH, and COC, may induce dry-mouth, resulting in increased sample heterogeneity [160], which can be overcome by dried OF spot testing [161]. In addition to OF collection, the correlation to other biological fluids has been studied with mixed results [162,163]. It should be noted that it is unlikely that there will ever be a 100 per cent correlation among drug tests from different bodily fluids because the results are influenced by sampling timing relative to the last instance of drug intake [164].

The most commonly used POC tests are immunoassays because they are cheap, easy to use, and provide rapid qualitative results. Although quantifying DOAs is possible via blood immunoassays [165], no such tests are commercially available for OF. Immunoassay tests are based on shape-specific antibody-molecule reactions, reacting to broad molecular groups. This leads to a high number of cross-reactivities, for example pseudoephedrine (a cold medicine) for AMP [166], such that manufactures often provide full lists of potential interferent with the devices [167]. Numerous testers are available

for DOA analysis in OF, including the DrugWipe 5+, Dräger DrugTest, Lifepoint Impact, Reditest Oral, Securetec Drugwipe, Sun Biomedical Oraline, Ultimed Salivascreen, and Toxiquick, Oratect. Their major drawbacks include the high proportion of defective testers (as high as every fourth tester), high variation in diagnostic sensitivities (0–97%), and considerable rate of false positive and false negative results (up to 32%) [139,166,168,169]. In addition, the results obtained are for screening purposes and are often qualitative, necessitating additional testing to confirm and quantify the results [170]. Thus, there is a need for a POC instrument that can be used for quantitative analysis of DOAs in OF.

Only a few POC instruments are currently available for DOA testing in OF that are not based on immunoassays. For example, a NIR spectrometer for COC determination in OF with a limit of 10 ng [151] and CE for various illegal drug determinations (discussed in this thesis).

1.2.2 International guidelines for drug abuse testing in OF

International projects have been implemented to regulate the detection and implementation of DOA testing in a variety of fields. These regulations concern sample collection and handling, instruments, and methods for determination and establishment of adequate cut-off limits. Evaluation of alcohol consumption with clear-cut recommendations for legal limits are well established. Similar levels are much harder to define for DOAs, whether licit or illicit drugs, as their correlation between biological fluids and cognitive implementations have not been unequivocally determined.

In Europe, the Driving Under the Influence of Drugs, Alcohol, and Medicines (DRUID) project was implement from 2007–2011 to gather reliable and comparable data on different substance abuse in drivers. The project included 13 countries with 50 000 drivers assayed on the roadside, as well as analysis of hospital records and a case-control study using numerous POC instruments. Approximately 2% of the subjects testing positive for DOA showed much higher stimulant (MDMA, AMP, and COC) levels than the those typically used for scientific research. Therefore, the conclusions drawn from research often do not reflect actual influences in real-life users. Approximate cut-off values were established for both licit and illicit drugs as part of the project, which also highlighted the drawbacks that occur with cut-offs, as some countries have lower limits of quantification in their forensic laboratories. All tested POC OF testing kits lacked instrumental sensitivity, selectivity, and accuracy, indicating the need for further development. The final report recommended that the instrumental sensitivity, specificity, and diagnostic accuracy of a POC should be >80% for real-life usage [139]. In addition, a checklist of clinical signs of impairment is required for DOA detection, including bloodshot eyes, did not give reliable results, implying the need for additional experience and better police officer training.

ROSITA-2 (acronym of RO<u>ad</u><u>SI</u>de <u>T</u>esting <u>A</u>ssessment), evaluated the roadside OF drug tests for the detection of drivers under the influence of drugs as a US/European international study to assess POC devices for OF testing between 2001–2005. The project provided recommendations and testing of the characteristics of 9 devices. Criteria were set for instrumental sensitivity and specificity (>90%) as well as diagnostic accuracy (>95%), which were not met by any investigated device. In real-life situations, several important problems arose while using the devices that included excessively long and complicated procedures, test results that were difficult to read or required instrumental reading, difficult sample collection, weather interference and time constraints. In addition, cut-off limits were suggested to harmonize the results. [171]

The European Workplace Drug Testing Society (EWDTS) provides guidelines on urine, OF, and hair tests for both drugs and alcohol. Although the developed guidelines relate to laboratory testing, POC testing is also acknowledged. As part of this framework, guidelines are provided for sample collection, analysis (and validation), quality assurance, and results interpretation. A thorough paper trail instruction and fatal flaws of custody chains are also specified and described with both screening and confirmation testing maximum cut-off limits provided for various DOAs [19].

The United Nations Office on Drugs and Crime published guidelines for DOA testing in OF, in addition to hair and sweat, with a detailed methodological description for GC-MS with solid phase extraction. The guidelines referenced cut-off limits in OF from the DRUID results [172]. In additional, many country-specific guidelines have been developed, including the Australian Standard (AS 4760) "procedure for specimen collection and the detection and quantitation of drugs in oral fluid"[173]. In addition, the USA Department of Health and Human Services Substance Abuse and Mental Health Services Administration (SAMHSA) has developed mandatory guidelines for federal workplace drug testing programs [174].

In 2007 the Talloires project was undertaken with the aim to develop guidelines for research on drugged driving and could be divided into 3 parts – behavioural, epidemiology, and toxicology. Participants included the National Institute on Drug Abuse, European Commission (EU), European Monitoring Centre of Drugs and Drug Addiction (EMCDDA), French Society of Analytical Toxicology, International Council on Alcohol, Drugs, and Traffic Safety (ICADTS), and International Association of Forensic Toxicology (TIAFT). The goal was to provide an overview of the problems and instructions for comparable research by collecting uniform datasets related to drug detection, resolving various issues and recommending solutions [175]. The cut-off values for DOAs in OF proposed by previously mentioned guidelines/projects are listed in Table 1.1.

Table 1.1. Proposed cut-off values in OF for screening/confirmatory analysis of DOAs where applicable.

Substance	DRUID ng/mL [139]	EWDTS ng/mL [19]	ROSITA-2, ng/mL [171]	SAMSHA, ng/mL [174]	Talloires project, ng/mL [175]	AS 4760, ng/mL [173]
AMP	360	40/15	25	25/15	20	50
METH	410	40/15	25	25/15	20	50
MDA	220	40/15	25	25/15	20	50
MDMA	270	40/15	25	25/15	20	50
MDEA	270	40/15	25	25/15	20	50
COC	170	30/8	8/4	15/8	10	50
COET	-	30/-	-	-	-	50
THC	27	10/2	10/2	4/2	2	25

1.3 Capillary electrophoresis

High performance capillary electrophoresis or just capillary electrophoresis (CE) is a separation technique which, unlike chromatography, is based on the differential migration of species in a conductive medium under an electric field [176]. The first chemist to use CE was Tiselius in the 1930s, who studied serum proteins in a glass U-tube [177]. Subsequently, CE has matured and has gained significant popularity in a variety of

fields including forensic science [178–180], the food industry [181], pharmaceutical control [182,183], metabolomics [184], and environmental studies [185,186].

CE instrumentation is simple, consisting of a capillary, high-voltage supply, detector system, vials for the background electrolyte (BGE), and a control system for the instrument and detector. The electrodes are submerged in the BGE vials which are connected by the BGE-filled capillary and the detector is usually placed on the capillary. The capillary is typically composed of an open fused silica tube with an inner diameter of 25–100 µm and outer diameter 150 or 350 µm, coated with a polyimide coating for flexibility. The sample is most often introduced to the capillary electrokinetically by applying voltage or hydrodynamically by siphoning pressure. The capillary length can be divided into total length (L_{tot}) and effective length (L_{ef}), referring to the length from sample introduction to the detector [177,187,188]

CE has several working modes, the most common being capillary zone electrophoresis (CZE), also known as free capillary zone electrophoresis, the separation is based on the differences of electrophoretic mobilities. In addition to CZE, there is also mode, known as micellar electrokinetic chromatography, where surfactants are added above their critical micelle concertation (CMC) to create a pseudo stationary phase, as well as non-aqueous capillary electrophoresis, capillary gel electrophoresis, capillary isotachophoresis, and capillary isoelectric focusing [188].

1.3.1 Principles of CE

CZE is considered to be the simplest form of CE, where in a BGE filled capillary, charged particles move when a voltage is applied, cations to the cathode and anions to the anode. By applying the voltage, a constant electric field strength (E) is created in the capillary, where ionic species with charge (q) will be driven by a force (F) [188] as follows:

$$F = q * E \,, \tag{1.1}$$

The electric field strength is determined by the voltage applied across the capillary (V) and total length (L_{tot}) of the capillary:

$$E = {}^{V}/_{L_{tot}} \,, \tag{1.2}$$

The moving ion experiences an opposing frictional force (F'), which can be described by Stokes's law for spherical particles and calculated using Eq. (1.3) [188].

$$F' = 6\pi\eta r v_{ep} \,, \tag{1.3}$$

where η is the viscosity of the BGE, r the ionic radius, and v_{ep} is the ion migration velocity. In a constant velocity state F=F' and the migration velocity can be expressed as follows:

$$v_{ep} = \mu_{ep} E \,, \tag{1.4}$$

where μ_{ep} is the electrophoretic mobility of the ion and μ_{ep} is calculated using the ion radius and charge, but is also dependent on BGE viscosity [188].

$$\mu_{ep} = \frac{q}{6\pi\eta r} \,, \tag{1.5}$$

In addition to ion mobility under the electric field, electroosmosis also occurs. Electroosmotic flow (EOF) occurs when an electric field is applied to a solution in contact with a charged solid surface, resulting in a flow of the non-charged liquid. In CZE, a negative charge is induced in the capillary due to the ionization of deprotonated silanol groups (above pH 2) [187]. A double layer is formed, the first being the stagnant Stern layer (near the capillary wall) and the other is the diffuse Gouy-Chapman layer, which is mobile. The positively charged ions in the diffuse layer move towards the cathode, dragging the solvent along and creating EOF. The EOF velocity of can be calculated (υ_{EOF}) using Eq. (1.6):

$$\upsilon_{EOF} = \frac{\varepsilon \zeta E}{4\pi\eta}\,, \tag{1.6}$$

where ε is the dielectric constant of the solution, ζ is the zeta potential, E is the electric field strength, and η is the solution viscosity [176,177,188]. Therefore, the mobility of EOF (μ_{EOF}) can be calculated as follows [187]:

$$\mu_{EOF} = \frac{\upsilon_{EOF}}{E} \tag{1.7}$$

Combining the ionic species electrophoretic mobility μ_{ep} and EOF mobility μ_{EOF}, the apparent mobility μ_a can be expressed as follows:

$$\mu_a = \mu_{ep} \pm \mu_{EOF}\,. \tag{1.8}$$

As EOF is dependent on the surface charge, it is usually much faster than charged molecule electrophoresis and transports both anions and cations along with it, resulting in the separation of both ions [187]. As EOF is an implemental force in CE, the nature of the capillary inner wall is of paramount importance. Depending on the charge of the inner wall, the EOF direction can be changed and the velocity decreased dramatically or even prevented by addition of organic solvents, inorganic salts, cationic surfactants, cellulose derivates, divalent amines, and other polymers to the BGE. Adding cationic surfactants such as alkylammonium salts of bromide including tetradecyltrimethylammonium bromide (TTAB), hexadecyltrimethylammonium bromide or cetyltrimethylammonium bromide (CTAB), and hexamethonium bromide (HMB), can reduce or reverse EOF. Positively charged alkylammonium anions can assemble on the capillary wall as a monolayer when the CMC is not reached, while above the CMC they can form a uniform coating (DDAB) or spherical aggregates (CTAB) [188–190]. In addition to reducing EOF, dynamic coating can be used to prevent protein adsorption on the capillary wall, providing a more stable EOF, stabilizing the analytical run, and improving reproducibility. Hexadimethrine bromide (HDMB), also known as polybrene, and poly(diallyl dimethyl ammonium chloride) (PDADMAC) are commonly used as such coatings [191,192].

Equivalent to chromatography, separation can be evaluated by assessing the number of theoretical plates and resolution between analytes of interest. Because the double layer in CE is extremely small, it results in a laminar flow profile, providing high peak efficiencies and good resolution. Peak efficiency usually ranges from 10^5 to 10^7 [193], which is high compared to pressure driven methods such as HPLC (10^3–10^4) [176]. Peak efficiency, given in theoretical plates N, can be calculated as follows [194]:

$$N = 5.54\left(\frac{t}{w_{½}}\right)^2, \tag{1.9}$$

where t is the peak migration time and $w_{½}$ is the width of the peak at half height. The resolution (R_s) between peaks can be calculated using the migration times of the peaks (t_1, t_2) and their baseline widths (w_1, w_2) as follows [194]:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}, \tag{1.10}$$

1.3.2 Detection methods for CE

Detection methods for CE are similar to other separation techniques and include ultraviolet-visible (UV/vis) absorbance, mass-spectrometry (MS), fluorescence (FD), conductivity, and chemical detection methods. The detector should be chosen based on the properties of the analyte of interest. Simultaneous detection using several sequentially placed detectors has also been reported [195].

Capacitively coupled contactless conductivity detectors (C^4D) are commonly used for CE as they are universal for all types of analytes, usually require no sample pre-treatment, and are compatible with many BGEs. The main principle is the measurement of a current that is directly proportional to the solution conductivity within the capillary. This is achieved by placing two well-fitted tubular electrodes on the capillary that transmit a signal from the excitation to the pick-up electrode. The signal passes at a high frequency (kHz to MHz) to reduce the capacitive reactance of the capillary wall, enabling the measurement of solution resistance between the electrodes [189]. Selectivity can be determined by the separation, with detection limits reaching as high as 10^{-16} M [177,196]. The main drawbacks of C^4D is the significant temperature dependence of the signal and low selectivity [189].

UV/vis absorbance detectors are used in most commercially available CE instruments due to their wide applications and easy use. Diode array detectors (DAD) are preferred over conventional UV/vis absorbance detectors as they provide peak purity and some structural information. DAD detectors measure the absorbance of the solute according to Beer's law, which is proportional to the solute concentration. A deuterium lamp is used to measure from 180 to 600 nm and the polyimide coating is removed to produce a detector cell with a typical pathlength of 25–100 µm, restricting the detection limit. For non-absorbing analytes, indirect absorbance is achieved by adding an absorbing compound to the BGE. The most common absorbance detectors provide detection limits of approximately 10^{-7} M [176,177,188]. LEDs have also been used for absorbance detection [197,198].

Fluorescence detectors are useful for CE as they impart selectivity and sensitivity even with small sample quantities. Fluorescence occurs when a single excited electron relaxes to the ground state by emitting photons. The excitation wavelength is shorter than the emitted wavelength as some energy is converted into vibrational energy. Fluorescence can be native to a molecule, usually arising from an aromatic system, or produced via derivatization. Excitation sources include lasers, LEDs, and lamps. Lasers are the most well-known light sources in CE fluorescence detection because their optical power and coherent nature are well-suited for CE but are not commercially available in the UVC range (200–280 nm). LEDs are favoured due to their an extended lifetime, narrow emission band, high portability, and low parasitic emission light. [177,188] Studies

regarding the use of LEDs at 235 [198,199] and 255 nm [200] have been reported but are not currently commercially available. A solution to UVC excitation is provided by using traditional light sources – lamps, which can be mercury, mercury-xenon, xenon, xenon flash, or deuterium based. These lamps require additional filtering to provide a narrow band excitation but exhibit shorter life spans and are more complex optical systems for CE. Important detector parameters include the excitation and emission wavelengths that can be interchangeable depending on the instrument, spectral radiance of the light source, and optical scheme for the focusing to the capillary. Although the optical passing pathlength is the same as in UV/vis absorbance, fluorescence provides detection up to 10^{-18} M [177] or even 10^{-21} M with laser-induced fluorescence [201].

1.4 Validation

To ensure the fitness-for-purpose of the analytical procedure, it should be thoroughly validated. The validation step is crucial to better understand the actual limitations and possibilities of the procedure. Validation protocols regarding quality assurance are commonly found for every field, organization, and lab. The choice of appropriate objectives for validation should be based on specified guidelines applicable to the field of the analytical procedure that fulfil a regulation. Usually validation protocols for analytical methods include the evaluation of the following parameters: selectivity/substance identification, limit of detection (LOD), limit of quantification (LOQ), linearity of calibration, precision (reproducibility and repeatability of the measurements), trueness (recovery, matrix effects), overall accuracy, analyte stability, and robustness [202–204]. The determination of these parameters can be achieved in a step-wise manner by hand or using a number of statistical programs, functions, and instruments. A major aim of this thesis was to evaluate and validate new bioanalytical methodologies. The terminology and definitions used herein are based on the European Medicine Agency (EMA) Bioanalytical Method Validation guideline [205]. An overview of the main terminology and related definitions is provided in Appendix 2.

1.4.1 Data pre-treatment

The preparation of raw data is crucial for obtaining reliable results. As many in-house-built systems are not as robust and stable as commercial systems, standardized steps to prepare data for comparative results between instruments and analyses must be followed.

In CE, the migration times of analytes are often unpredictable due to unstable EOF caused by environment fluxes (temperature, pH, flushing, and capillary inner wall changes) or uncertainty introduced from the user. For example, sample injection performed by hand can result in small variances in injection amount or system starting time after sample introduction [206,207]. As analyte recognition is often achieved by migration time analysis, it is important to ensure equivocally understandable results, which can be achieved by using relative migration times or correcting migration times. An option for such corrections was suggested by Zhang et al. [206] and uses a “golden standard” electropherogram from which one or two internal standard migration times are used to calculate the corrected migration times for following electropherograms.

In this method, first a correlation coefficient (γ) is calculated:

$$\gamma = \frac{\frac{1}{\hat{t}_I} - \frac{1}{\hat{t}_p}}{\frac{1}{t_I} - \frac{1}{t_p}}, \tag{1.11}$$

where t_I, t_p are migration times of first and second IS in the standard electropherogram and $\hat{t}_I$, $\hat{t}_p$ are migration times of the first and second IS in the electropherogram to be corrected.

The obtained correlation coefficient is subsequently used to calculate the new migration time (t_x) using Eq. (1.12):

$$t_x = \left[\frac{1}{t_I} - \frac{1}{\gamma}\left(\frac{1}{\hat{t}_I} - \frac{1}{\hat{t}_x}\right)\right]^{-1}, \tag{1.12}$$

where γ is the correction coefficient; t_I and t_p are the migration times of the first and second IS in the standard electropherogram, respectively; $\hat{t}_I$ and $\hat{t}_p$are the migration times of the first and second IS the electropherogram to be corrected, respectively; t_x is the corrected migration time for the corrected electropherogram; and $\hat{t}_x$ is the migration time of the electropherogram under correction.

This approach provides stable migration times for the internal standards and reduces the variability of relative migration times, allowing for easier visual comparison.

1.4.2 Design of experiments for optimization and robustness testing

Design of experiment (DOE) is an organized method for determining the relationships between factors and responses, wherein factors are quantities that affect the response and the response is the measured/observed subject. The goal is to mathematically describe the entire system with a minimal amount of experiments that are well-planned in a systematic manner. In analytical chemistry, namely CE methodology, common factors for optimization are the BGE composition, BGE pH, capillary length, applied voltage, and injection time. The measured responses include the resolution, efficiency, peak area, and migration or analysis time. Experimental designs can be mainly divided into two applications – screening and optimization. During screening, the goal is to study a wide range of input factors to determine their effects on the measured responses. Optimization designs are then used to determine the levels for factors where the best results are obtained (i.e. maximum efficiency and best resolution) [208,209].

The first step in this process is to determine factors that affect the method, which can be achieved by univariate procedures or multivariate screening, where univariate is a one-variable-at-time (OVAT) approach and multivariate uses two or more. OVAT is commonly used as it is simpler, although as the interactions between factors are not evaluated and incomplete information is obtained. In addition, the time expenditure of this process is considerable and chemical consumption can be costly as usually the number of variables affecting separation and CE results is large, even more so for in-house built instrumentation with few automated parts. Therefore, the use of multivariate DOE is a good alternative to the OVAT approach, as it can evaluate interactions between factors, reduce the amount of required experiments, and provide more global data [210].

Box-Behnken is an experimental design from 1960s that is used for surface response methodology (RSM). The design fits a quadratic model with an estimation variance depending only on the distance from the centre – a rotatable design. A benefit of this

method is that it requires a small number of experiments, where the number of experiments (N_E) is related to the number of evaluated factors (f) as follows:

$$N_E = (2f(\,f - 2) + 1\,, \tag{1.13}$$

where 1 is the central point [211]. Only three levels are required for each factor, which are varied according to a design set-up for each experiment. All experiments, including the nominal experiment, are performed in a single run and selected responses are measured, from which prediction formulas can be generated. Thus, an RSM set-up provides no design points where all factors are set to extreme values simultaneously or outside the set range, as is common with central composite designs [212]. RSM provides a visual representation of the relationships between variables and their responses based on the calculated prediction formulas. Typically, a 3D model is used where two variables and a response are plotted in one graph that can also be described in 2D. An advantage of the 2D plot is the possibility of adding further responses using other colours/lines for increased information regarding relationships. The Box-Behnken design can also be used for robustness testing to screen for factors that significantly impact the obtained results. In such cases, the procedure is identical, but the factor values are set to a much smaller variability according to the levels of real-life changes in the factor values. After setting boundary conditions for the responses, the prediction formula can be used to determine an area wherein the response falls within the set limits (Example shown in Appendix 4, Fig. A4.1 white area, R ≥2) and the probabilities of each factor influence on the response can be calculated [212]. Robustness can also be evaluated using a highly fractionated Plackett-Burman experimental design. As no high-order interactions are evaluated, unlike in Box-Behnken, the Plackett-Burman is used to evaluate the main effects of factors from a small number of experiments. The statistical change of a response caused by a factor can be evaluated to determine if the methodology is robust in response to that factor [213]. The number of experiments and factors tested can be described by Equation 1.14, where N_E is the number of experiments, n is a positive natural number, and f the number of evaluated factors [204].

$$N_E = 4n, f = 4n - 1 \tag{1.14}$$

Therefore, for 3 or 7 factors, only 4 and 8 experiments are needed, respectively, when 4n-1 factors are under evaluation, for example 8 or 5, dummy factors or additional factors must be added. A dummy factor is a factor that has no impact on the result, such as performing the experiments only on even or odd-numbered hours. Each factor is subsequently changed to one of the two levels according to a design pattern for each experiment and the effects associated with each factor are calculated. The standard deviation of the measurements at a nominal value should be evaluated separately or based on dummy factors if available. The significance of the factor can then be evaluated by comparing the effect of a factor to the corresponding standard deviation (representing normal experimental error of the method) using a Student's *t* test. [204]

1.4.3 Performance characteristics of the analysis procedure

Optimized methodology validation typically includes determination of the major performance characteristics, which include the limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, and recovery during sample preparation. The accuracy and precision are usually determined by repeated runs within

or between days using the same sample or preparing an equivalent sample for testing. The obtained results are presented as a standard deviation (SD) or relative standard deviation (RSD) presented in the form of a variation coefficient (CV%). Unlike accuracy and precision, linearity determination and measurement limits have a wider variety of possibilities. Options for determining those parameters are rooted in the use of statistical measurements resulting from the instrumental noise levels, detector signal output stability, and sensitivity [187,203].

For quantitative analysis, a function describing the concentration dependence and instrument response is fundamental. Linear relationships are preferred, where the equation can be written as follows:

$$y = a + bx, \tag{1.15}$$

where y is the instrumental signal, x the analyte concentration, b is the slope of the line, and a is y-intercept. This assumes that all x values are error-free and that all errors are contained in the y value. The resulting equation is referred to as a calibration curve and is based on the least-squared method for model fit. As the preferred relationship is linear, the associated evaluation can be achieved using various methods. The most commonly used method is the correlation coefficient (r) calculated using Eq. (1.16) [214].

$$r = \frac{\sum_i\{(x_i - \bar{x})(y_i - \bar{y})\}}{\{[\sum_i(x_i - \bar{x})^2][\sum_i(y_i - \bar{y})^2]\}^{½}} \tag{1.16}$$

where x_i and $\bar{x}$ are the *i*th concentration and mean concentration, respectively, and y_i and $\bar{y}$ are the *i*th instrument signal and mean instrument signal, respectively. The values range from -1 ≤ r ≥ 1, with 0 indicating no correlation and ±1 a perfect (positive or negative) correlation. The main drawback of this approach is the linearity presumption as it does not prove linearity, but evaluates the data compared to a linear line [214]. In addition, a visual inspection of the residual dispersion around the curve can be used to evaluate linearity if no pattern can be detected [203]. Moreover, a method based on analysis of variance (ANOVA) can be used to quantify the actual linearity adherence. The method considers two main discrepancies of the measured y values, the random measurement error and the actual lack of fit to a linear model (e.g. non-linearity). If k solutions of each concentration (i) are measured n_i times, and each response is y_{ij} with a mean signal response at *i*th concentration of $\bar{y}_i$, the square sum of the measurement error (MS_{ME}) can be calculated as follows:

$$MS_{ME} = \sum_{i=1}^{k}\sum_{j=1}^{n_i}\left(y_{ij} - \bar{y}_i\right)^2. \tag{1.17}$$

Evaluation of the lack of fit error to the linear model uses the squared sum of lack of fit (MS_{LOF}) calculated using Eq. (1.18), where the $\hat{y}_i$ – y-value is predicted by the regression line.

$$MS_{LOF} = \sum_{i=1}^{k} n_i(\hat{y}_i - \bar{y}_i)^2 \tag{1.18}$$

The *F* statistic value can be obtained by dividing MS_{LOF} with MS_{ME} and the associated probability calculated for a one-sided distribution *F* with k-2 (MS_{LOF}) and N- k (MS_{ME}) degrees of freedom, where N is the overall number of measurements performed. Depending on the significance, the linearity can be evaluated. For 95% probability, P >0.05 allows acceptance of the null hypothesis, indicating that the relationship is linear. [204]

Evaluation and calculation of LOD and LOQ can be achieved using several approaches. The LOD is the smallest concentration that can be detected by a method in a sample matrix and the instrumental detection limit (IDL) is the smallest concentration of analyte that can be detected by the instrument in a standard solution. The LOQ is the smallest concentration that is quantifiable with a known certainty in a sample, while the instrumental quantification limit (IQL) involves quantification of a standard solution [205,214]. The simplest method to determine IDL, LOD, IQL, and LOQ is based on signal-to-noise (S/N) ratios. This approach requires the analyte to produce a signal, in CE it is a peak, which is clearly identifiable from instrumental noise. The noise must be easily measured along with the peak intensities and the calculations are quite straightforward, involving multiplying the S/N ratios as follows:

$$IDL, LOD = 3 * S/N, \tag{1.19}$$

$$IQL, LOQ = k * S/N, \tag{1.20}$$

where k is 10 [214] or 5 [215] depending on the source cited. The S/N ratio of 3 for IDL and LOD provides a 99% probability of the analyte presence in the sample. Although a simple approach, several circumstances must be considered, including the noise measurement and whether the full- or the half-with of the signal is used, the homogeneity of noise, appearances of spikes, and peak shapes – as asymmetrical peaks or low effectiveness reduce these values significantly. This approach is suggested by The International Conference on Harmonization (ICH) and International Organization for Standardization (ISO) guidelines, resulting in its widespread adoption in chromatography and CE [214,216]. Additional approaches can be used that are based on pre-determined linear regressions or standard deviations.

1.4.4 Diagnostic performance of the procedure

Numerous validation protocols require that the results obtained by the novel method are validated against a well-known method to ensure its diagnostic accuracy, which is known as cross-validation. The compared results can both be qualitative and/or quantitative.

Clinical analyses include instrumental statistics, when the results obtained using the new method are compared to a “gold standard” (GS) method. All positive and negative results are compared to the GS method, while the results obtained by the GS are regarded as true. Four kinds of results can be obtained:

- True positive (TP) – the method under evaluation provided a positive result, confirmed by the GS
- True negative (TN) – the method under evaluation provided a negative result, confirmed by the GS
- False positive (FP) – the method under evaluation provided a positive result, which was disproven by the GS, indicating that the sample was actually negative,

- False negative (FN) – the method under evaluation provided a negative result, which was disproven by the GS, indicating that the sample was actually positive

Using the TP, TN, FP, and FN results, diagnostic performance parameters can be calculated, including sensitivity (SE), diagnostic specificity (SP), and diagnostic accuracy (DA) [217,218]. SE describes the capability of the novel method to correctly determine the positive cases, suggesting that if the number of FNs is low, and only a few cases would be missed. SE is, evaluated using the following formula:

$$SE = \frac{TP}{TP + FN} * 100\%, \tag{1.21}$$

The ability to correctly detect negative samples is SP, and a high value is important for preventing unnecessary protocols for innocent people. The SP can be calculated as follows:

$$SP = \frac{TN}{TN + FP} * 100\%, \tag{1.22}$$

DA is used to assess the ability of the novel method to distinguish between positive and negative cases, and can be described as follows:

$$DA = \frac{TP + TN}{TP + TN + FP + FN} * 100\%, \tag{1.23}$$

In an ideal case, both SP and SE are 1, but often one parameter is improved at the expense of the other. Using this approach, additional information can be gathered to determine if a system/methodology is valid and suitable for real-life use [219,220].

Quantitative results can be compared using a correlation coefficient (as described in Chapter 1.4.3) to measure the correlation of two methods, where the x-axis contains the result one method and the y-axis the second method results. In addition, the use of z-scores is recommended to estimate the coincidence of the two values obtained by two different methods. The same samples should be measured using both methods, with corresponding values for method 1 (x_1) and method 2 (x_2). Using previously determined standard uncertainties for both methods ($u_1\ and\ u_2$), the z-score for each sample can be calculated as follows [214,221]:

$$z = \frac{(x_1 - x_2)}{\sqrt{u_1^2 + u_2^2}}, \tag{1.24}$$

Under the presumption that both measured values correspond to the normal distribution, the z-value should be ±1.96 if the probability is set to 95% ($\alpha < 0.05$). If $z \leq \pm 2$, the results are regarded as satisfactory and obtained results using the two methods are in accordance with each other [204,214].

2 AIMS OF THE STUDY

The present thesis aimed to develop and validate analytical procedures to enable onsite detection and quantification of psychoactive substances in OF by using existing and newly developed portable CE coupled to different detection modes.

The more specific aims for the work are as follows:

- Development of a fast and reliable CE-C^4D/UV-vis absorbance analysis procedure for the detection of GHB in OF;
- Development and validation of a portable and rapid CE-C^4D analysis procedure for the determination of GHB and psychoactive mushroom compounds – namely ibotenic acid and psilocybin in OF;
- Development of a simple methodology for the detection of ecstasy in OF using an in-house built CE-LED-FD instrument;
- Capability evaluation of a newly developed portable CE instrument with UV fluorescence detector (CE-FD), equipped with a Xe lamp as an excitation source in the UVC region for analysis of DOAs;
- Development and validation of the procedure for the analysis of seven DOAs, namely AMP, METH, MDA, MDMA, MDEA, MDEA, COC, and COET, in OF by CE-FD;
- Application of CE-FD for on-site determination of DOAs in real OF samples obtained from suspected persons and confirmation of the results by HPLC-MS analysis;
- Evaluation of the diagnostic performance of the newly developed procedures for determination of CE-FD reliability.

3 EXPERIMENTAL

3.1 Reagents and samples

3.1.1 Chemicals and reagents (Publications I–V)

All chemicals used were of analytical grade.

Acetonitrile (ACN), allocryptopine (ALC), benzylamine*HCl (BZA), cetrimonium bromide (CTAB), citric acid, formic acid (FA), glutamic acid, hexadimethrine bromide (HDMB; ≥94%), ibotenic acid (IBO), KNO_3, lactic acid, L-arginine (Arg), maleic acid (Mal), methanol (MeOH), $MgSO_4$, Na_2HPO_4, NaOH, NaOH, NaSCN, $NaSO_3$, pinacolyl methylphosphonic acid (PMPA), succinic acid (Suc), tartaric acid, triethanolamine (TEA), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Aldrich (USA). Ortho-phosphoric acid (H_3PO_4; 85wt%) was obtained from Fluka (Germany).

3,4-Methylenedioxyamphetamine (MDA; 1 mg/mL in MeOH), 3,4-methylenedioxyethylamphetamine (MDEA; 1 mg/mL in MeOH), 3,4-methylenedioxy-methamphetamine (MDMA; 1 mg/mL in MeOH), 3-methyl-fentanyl (1 mg/mL in MeOH), 3-monoacetylmorphine (3-MAM), 4-hydroxy-3-methoxyamphetamine (HMA; 1 mg/mL in MeOH), 4-hydroxy-3-methoxymethamphetamine (HMMA;1 mg/mL in MeOH), 4-OH-amphetamine (1 mg/mL in MeOH), benzoylecgonine (1 mg/mL in MeOH), cannabidiol (CBD; 1 mg/mL in MeOH), cocaethylene (COET; 1 mg/mL in ACN), cocaine (COC; 1 mg/mL in MeOH), cocaine-d3 (COC-d3; 1 mg/mL in MeOH), codeine (1 mg/mL in MeOH), d,l-3,4-methylenedioxymethamphetamine-d3 (MDMA-d3; 1 mg/mL in MeOH), d,l-amphetamine (AMP; 1 mg/mL in MeOH, 10 mg free base powder), d,l-amphetamine-d3 (AMP-d3; 1 mg/mL in MeOH), d,l-fentanyl (1 mg/mL in MeOH), d,l-methamphetamine (METH; 1 mg/mL in MeOH, 10 mg free base powder), ecgonine (1 mg/mL in MeOH), ephedrine (1 mg/mL in MeOH), heroin (1 mg/mL in MeOH), hydromorphone (1 mg/mL in MeOH), lysergic acid diethylamide (LSD; 1 mg/mL in MeOH), methadone (1 mg/mL in MeOH), morphine (1 mg/mL in MeOH), norephedrine (1 mg/mL in MeOH), para-methoxyamphetamine (PMA; 1 mg/mL in MeOH), para-methoxymethamphetamine (PMMA; 1 mg/mL in MeOH), psilocin (1 mg/mL in MeOH), psilocybin (1 mg/mL in MeOH), sodium γ-hydroxybutyrate (GHB; 1 mg/mL in MeOH, 1 mg free base), and tetrahydrocannabinol (THC; 10 mg/mL in EtOH) were purchased from Lipomed (Switzerland). In addition, 4-OH-methamphetamine (1 mg/mL in MeOH), named Pholedrine®, was purchased from Toronto Research Chemicals, Canada.

Milli-Q water used for all experiments and was obtained using a Millipore Milli-Q system (USA).

3.1.2 OF samples (Publications I–V)

In **Publications I and II** OF sample were collected by spitting. In **Publications I and II**, the OF was precipitated by adding ACN at different ratios. After precipitation, the samples were centrifuged for 10 min at 3000 rpm using a mini-centrifuge (Sarstedt AG&Co, Germany) and the supernatant was used in CE. In **Publications III–V** OF samples were collected using Salivette® (Sarstedt, Germany) collectors using previously described protocols [222]. Briefly, the collectors with OF were centrifuged at 8000 rpm (EBA 200S, Hettich, Germany) for 2 min and 1 mL of ACN added, after which the collector was centrifuged again and the second centrifugate subjected to analysis. Fortified OF samples were prepared by pipetting standard solutions into the OF collected by spitting and then processing the samples as described. The samples were spiked with different IS standards depending on the analysis procedure.

3.1.3 Real GHB OF samples after wine drinking (Publications I and II)

Two red wines were purchased from local Estonian stores, namely "Le Grand Noir" 85% Cabernet and 15% Shiraz, 13 vol% EtOH (France 2013), and "Vina Maipo Vitral" 100% Cabernet Sauvignon, 13.5 vol% EtOH, Valle del Maipo (Chile 2011). Wine dosing was performed in 100 mL increments and 10 min intervals (3–4 min drinking, 2 min pause before OF collection and a small pause afterwards). Samples were collected from 6 volunteers over a total time of ~30 min, each consuming 400 mL of wine in total. The OF samples were subsequently processed as previously described.

3.1.4 Mushroom extracts (Publication II)

Preparation of the mushroom extracts was performed according to the procedures described in the literature [223]. Briefly, 0.3 g of air-dried mushrooms *Amanita muscaria* and *Psilocybe semilanceata* were ground in a mortar and 3 mL of MeOH was added to the powder. The solution was sonicated for 15 min and the first extract was transferred. Subsequently, 2 mL of MeOH was added to the mushroom and sonicated for 10 min. The first and second extracts were combined and centrifuged for 5 min at 3000 rpm with a mini centrifuge (Sarstedt, Germany). The *Amanita muscaria* extract was concentrated by a factor of 25 using a rotary evaporator (Laborota 4000, Heidolph, Germany) under reduced pressure.

3.1.5 Pharmaceuticals (Publication V)

The pharmaceuticals used in **Publication V** were purchased from a local drug store. All the pills were weighed and crushed in a mortar and the obtained powder was mixed with 1000 µL EtOH and ultrasonicated in an ultrasonic bath (Bandelin electronic, Germany) at 30 °C for 20 min and centrifuged for 10 min at 3000 rpm in a mini-centrifuge (Sarstedt AG&Co, Germany). The supernatant was filtered using a 0.20 µm cellulose filter (Sartorius Stedim Biotech, Germany). The solutions were subsequently diluted to 500 mg/L in EtOH or up to their maximum solubility. All used pharmaceuticals and their active ingredients are listed in Appendix 3.

3.1.6 Legal and ethical considerations

All psychoactive substances and mushroom material were purchased under the Estonian Agency of Medicine license IN-3-8.1/5520-2. The OF samples were collected with permission from the Tallinn University of Technology ethical committee and all volunteers provided expressed consent. All OF samples were identified by numeric code and no personal information was obtained from the volunteers.

3.2 Experimental setup

3.2.1 CE with UV/vis absorbance-C^4D detection (Publications I and II)

All experiments were performed using CE instruments that were mostly in-house built along with an Agilent 3D CE instrument (Agilent Technologies, Waldbronn, Germany). **Publications I and II** used an in-house built CE equipped with a C^4D [224] (Chemistry and Biotechnology Department, TalTech, Estonia) detector, combined into the Agilent CE instrument, as described in **Publication I**. The instruments used in **Publications I and II** are shown in Figure 3.1, where A is the commercial Agilent instrument with a DAD detector and B is the in-house built CE with a C^4D detector.

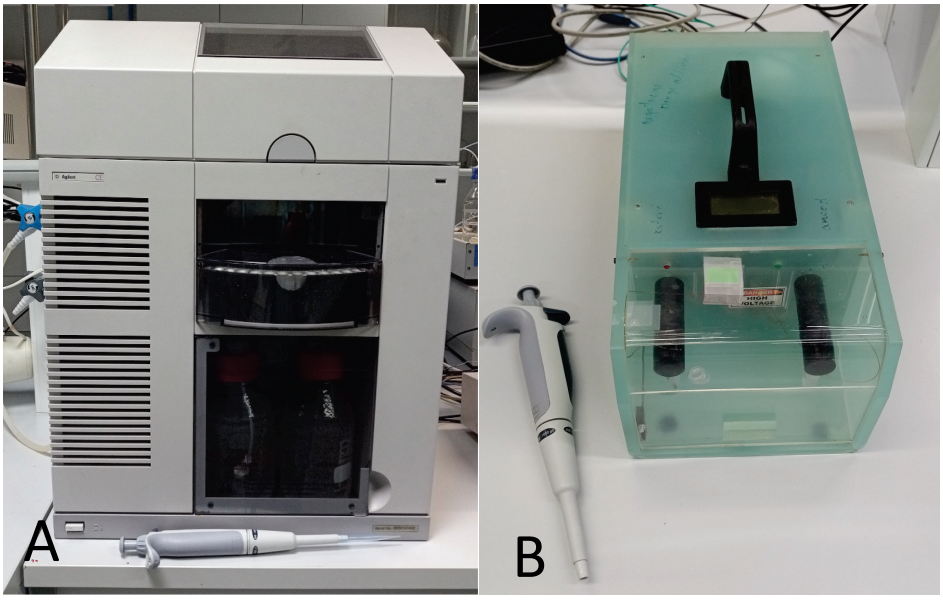

Figure 3.1. Instruments used in Publications I and II. A – The commercial Agilent instrument; B – in-house built CE-C^{4}D instrument.

In **Publication II**, the conductivity cell in the in-house built equipment was incorporated into the Agilent CE cassette, with the injection and high voltage of the Agilent instrument used. The conductivity signal was registered using in-house built software for CE-C^{4}D and the absorbance signal monitored by the Agilent CE instrument at 210 nm. In **Publication II** only CE-C^{4}D was used and the conductivity signal was monitored.

The capillaries used in both publications were uncoated fused silica capillaries (Agilent Technologies, USA) with an i.d. = 50 µm with varying lengths. In **Publication I**, the BGE was composed of 15% ACN, 8.5 mM Mal, 17 mM Arg, and 255 µM CTAB at pH = 7.65 and a hydrodynamic injection was performed for 3 s at 35 mbar. In **Publication II**, the BGE was composed of 17.9 mM Arg, 9.6 mM Suc, and 0.0019% (w/v) HDMB at pH 7.3 and an electrokinetic injection at -19 kV was employed for 3 s.

3.2.2 CE with LED fluorescence detection (Publication III)

The CE apparatus used in **Publication III** was an in-house built instrument equipped with an LED-fluorescence detector. The LED-FD was designed and constructed by Laser Diagnostic Instruments AS (Tallinn, Estonia) and is shown in Figure 3.2. The excitation source was a UV-LED (Roithner Lasertechnik, Austria) at λ_{ex} = 280 nm and an emission filter at λ_{em} = 326 nm (Andover Corporation, USA) was used. Further details regarding the instrument can be found in the literature [222,225].

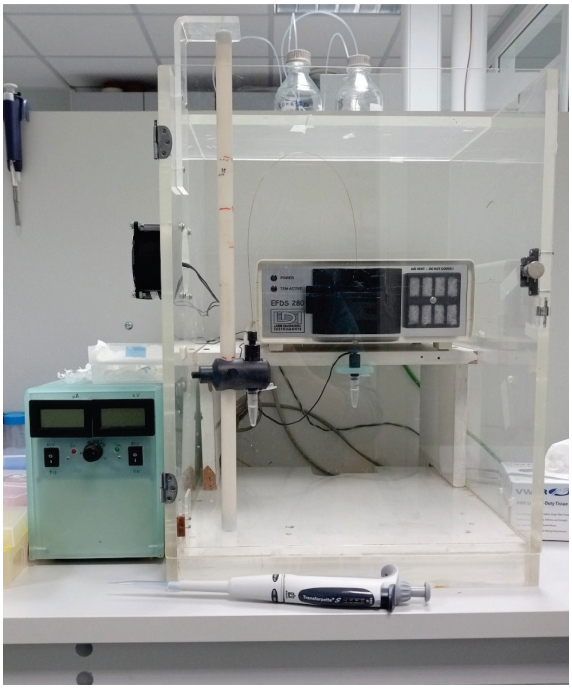

Figure 3.2. The CE-LED-FD apparatus used herein.

The BGE consisted of 40 mM H_3PO_4 with 10 mM TEA at pH 2.5. An uncoated fused silica capillary with an i.d. of 75 µm and o.d. 365 µm (Polymicro Technologies, USA) with L_{tot} = 62 cm and L_{ef} = 48 cm was utilized for all analyses. The voltage was +17 kV and the sample was introduced to the capillary via hydrodynamic flow from 15 cm for 30 s.

3.2.3 Portable CE with UVC fluorescence detection (Publications IV and V)

The instrument used in **Publications IV and V** was constructed in co-operation with the company Omec OÜ (Tartu, Estonia) and the TalTech Department of Chemistry and Biotechnology (Tallinn, Estonia). The fluorescence detector used a Xe flash lamp module (Hamamatsu, Japan) with an excitation channel with three optical filters for blocking the parasitic long wavelength radiation, yielding a final excitation spectra with a λ_{max} = 240 nm. The instrument contained five interchangeable emission filters: (1) 285/18 nm (F_{EM1}); (2) 302/10 nm (F_{EM2}); (3) 315/20 nm (F_{EM3}); (4) 337/10 nm (F_{EM4}); (5) broadband 300–600 nm (F_{EM5}). The intellectual property is protected by utility model U201700032 [226]. The portable CE apparatus was constructed around the fluorescence detector and consisted of a high-voltage power supply (EMCO 250 DXn, USA) and electrodes (Agilent, USA).

Figure 3.3. The new portable UVC excitation fluorescence CE instrument.

In **Publications IV and V** uncoated fused silica capillaries with i.d. 75 µm and o.d. 365 µm (Polymicro Technologies, USA) were used with varying lengths. Two BGEs were used for different substance groups (Group 1: AMP, METH, MDA, MDMA, MDEA, PMA, PMMA, COC, and COET) consisting of 42.5 mM H_3PO_4, 30 mM Tris at pH 2.5 (**Publication IV**) or 6 mM Tris, 38 mM H_3PO_4 at pH 3.3 (**Publication V**) using F_{EM1} and F_{EM3}. The other BGE used for THC and CBD analysis was composed of 2.5 mM NaOH in MeOH:ACN (1:1) at pH 12, as described by Kulp et al. [222] and F_{EM2} was used. All injections were performed hydrodynamically at a height difference of 20 cm for 10 s and a -19 kV voltage was applied.

3.3 Software

Conductivity data was obtained using in-house software (TalTech, Tallinn), as was the software for the CE-LED-FD and CE-FD instruments. Agilent Chemstation software (Agilent Technologies, Germany) was used for signal acquirement and data processing for indirect UV absorption measurements. Data processing for conductivity and fluorescence data was performed using MatLab R2011b and R2019 (The MathWorks, Inc. USA) and in Microsoft Excel (Microsoft, USA) and the Box-Behnken designs were created using JMP (SAS, USA).

4 RESULTS AND DISCUSSION

The results presented in this thesis are based on **Publication I–V**. All publications describe the analysis procedure development, optimization, and validation for different psychoactive substances in OF using CE using various detection methods.

The first section describes the analysis of GHB and psychoactive compounds from mushroom in OF using CE with conductivity and absorbance detection (**Publications I–II**). The second paragraph describes DOA analysis by LED excitation CE in OF (**Publication III**). The third and final section presents a novel CE analyser with native fluorescence detection with UVC excitation source for DOA quantification in OF (**Publication IV**). The validation and application of the CE-FD for real on-site collected OF samples for DOA detection is discussed and the diagnostic performance parameters and correlation to a HPLC-MS method were evaluated (**Publication V**).

4.1 GHB and psychoactive mushroom compounds detection (Publications I and II)

GHB is a recreationally used central nervous system depressant and hypnotic, exhibiting similar effects as alcohol. Throughout Europe, GHB use is a well-documented issue, as it is associated with date-rape when spiked into drinks. GHB use can lead to disturbances in rational behaviour or even death. Similar to GHB, the prevalence of psychoactive compound use remains stable as the intake of mushroom containing IBO or PY occurs both recreationally and accidentally. Moreover, routine toxicological analysis of blood and urine cannot detect GHB, its precursors, as well as IBO and PY intake reliably due to the time constraints, leading to the need for novel analytical procedures for accurate detection.

GHB occurs naturally in biological fluids, so the proposed cut-off level of 10 mg/L has been suggested for the differentiation of GHB of exo- and endogenous origin. As the half-life of GHB is 20–50 min, sample collection, preparation, and analysis must be achieved rapidly. Moreover, the intake of magic mushrooms containing PY with half-life of 50 min and psychoactive mushroom containing IBO can lead to intoxication or death. CE-C^4D was chosen as a suitable method for detection of small polar molecules and complex sample matrices at neutral pH. GHB has a pKa value of 4.7 [95], while IBO and PY contain acidic and basic groups, with IBO exhibiting pKa values of 3.0, 5.0, and 8.2 [227] and the pKa values of PY are 1.3, 6.5, and 10.4 [228]. This ensures that all analytes at least partly negatively charged at neutral pH. The use of OF instead of blood or urine allowed for simple sample collection and rapid information regarding the ingested substances.

CE-C^4D in parallel with UV absorption detection (CE-UV-C^4D) was used for GHB analysis for fast and reliable detection in the OF matrix. The analysis was expanded for simultaneous detection of GHB with IBO and PY using a CE-C^4D in **Publication II**. DOE was used to optimize the analysis and robustness by Box-Behnken design. In addition, samples were obtained and analysed for GHB after wine drinking and for IBO and PY determination in the OF samples fortified with mushroom extract. All obtained data was pre-processed using a method proposed by Zhang and Chen [206] and further discussed in Section 1.4.1.

4.1.1 Optimization and robustness testing

The BGE used for simultaneous UV absorbance and conductivity detection for GHB was adjusted from the methodology first proposed by Hauser et al. [229], which was used for GHB analysis in urine and plasma by CE-C^4D. The original BGE was composed of 20 mM Arg, 10 mM Mal and 30 µM CTAB at pH 7.35, with an analysis time of ~1000 s. GHB testing in OF required the procedure to be adapted to OF as a sample matrix with a LOD of 10 mg/L, as well as a notably faster analysis time, under 10 min, for possible on-site testing.

This goal was achieved using a negative voltage of -19 kV and a CTAB concentration of 300 µM, which reversed the EOF, achieving an analysis time of <10 min. In addition, an organic modifier was added to the BGE to improve baseline stability, furthermore the capillary length, injection time, as well as Arg and Mal concentration were optimized. The final analysis conditions of procedure I is provided in Table 4.1. As the rapid on-site testing was based on OF, the sample preparation was crucial for providing selectivity for C^4D detection, while remaining simple and reaching completion in <5 min. Thus, precipitation was used and an illustrative electropherogram of OF with added GHB and pinacolylmethylphosphoric acid - PMPA (IS) is shown in Figure 4.1.A. UV absorbance detection was performed by indirect detection, as Mal produces a high absorption in the BGE at 210 nm, providing simultaneous detection of GHB and IS. The system was composed of the C^4D detector from an in-house built CE-C^4D instrument coupled to an Agilent instrument, with high voltage, injection, and UV absorbance results obtained using the Agilent and C^4D signal registered by in-house built software on a secondary computer.

The robustness of analysis procedure I was analysed using a Box-Behnken design to evaluate key points during the methodology usage in field settings. Robustness was only evaluated using CE-C^4D at GHB 10 mg/L as the absorbance did not reach the proposed cut-off value. A randomized design was created using JMP® software to evaluate the GHB resolution and peak efficiency of Arg, Mal, and CTAB concentrations with six replicates of each order – for a total of 78 experiments. The results showed that N_{GHB} is extremely sensitive to the BGE composition, resulting in a five-fold difference from $0.28*10^5$ to $1.46*10^5$, mostly affected by CTAB concentration as well as Mal-CTAB and Mal-Arg interactions (p-value <0.050). Thus, extreme attention is required for BGE preparation.

Because the BGE with Arg, Mal, and CTAB has a rather unstable system eigenpeak (Fig 4.2) and a high sensitivity to even small changes in the BGE, a more stable analysis procedure was required. To improve the stability and decrease the analysis time, the BGE composition was changed accordingly. First, Mal was replaced by Suc for faster and more sensitive analysis, CTAB and ACN were replaced by HDMB, a positively charged polymer, providing a stable capillary coating. This resulted in a remarkable reduction in analysis time and effective separation of three psychoactive compounds, as seen in Figure 4.1, where A and B correspond to analysis procedures I and II, respectively. Two rounds of optimization were performed for II procedure, where the first covered a wider range of parameters of interest to determine their respective influences. Based on the results of the first set of optimizations, the number of parameters was reduced to include only the most significant factors and narrower intervals. The first set of parameters consisted of capillary length, injection time, pH, as well as concentrations of Arg, Suc, and HDMB. The first set showed that the chosen pH range was excessively wide for the PY and IBO detection, the ideal injection time was 3 s, and the optimal capillary length is L_{ef} = 40 cm. The second set was based on Box-Behnken DOE with evaluation of GHB, IBO, and PY resolution, as well as the N for each factor. The results were determined using surface plots and a contour profiler (representative examples are provided in Appendix 4).

The optimal BGE was 17.9 mM Arg, 9.6 mM Suc, and 0.0019% (w/v) HDMB at pH 7.3, where R_s values were 1.55 for U1–GHB, 1.53 for GHB–IBO, 1.50 for IBO–U2, and 2.33 for U3–PY.

The final optimized and validated procedure I for GHB analysis involved simultaneous UV absorbance and C^4D detection, while the parameters of procedure II for GHB, IBO, and PY analysis by CE-C^4D are listed in Table 4.1. The two optimized analysis procedures provided rapid results using OF for the determination of GHB, IBO, and PY in <10 min, with simple sample processing by precipitation by adding ACN and IS.

Table 4.1. Optimized analysis procedures for GHB and GHB, IBO, and PY.

Parameter	CE-UV-C^4D [I]	CE-C^4D [II]
BGE	17 mM Arg 8.5 mM Mal 300 µM CTAB 15% ACN	17.9 mM Arg 9.6 mM Suc 0.0019% (w/v) HDMB
pH	7.65	7.3
IS	PMPA 100 µM (18 mg/L)	PMPA 100 µM (18 mg/L)
Sample injection	10 s, 35 mbar	3 s, -19 kV
Capillary length	L_{tot} = 65 cm, L_{ef} = 56.5 cm (UV), L_{ef} = 45 cm (C^4D)	L_{tot} = 55 cm, L_{ef} = 40 cm
Voltage	-19 kV	-19 kV
Sample	1:3 OF:ACN	1:2 OF:ACN
Analysis time	8 min	4.5 min
Analytes	GHB	GHB, IBO, PY

The improvement from procedure I to II can be visually established, as Figure 4.1 depicts the C^4D results, where A and B correspond to analysis procedures I and II, showing a significant difference in analysis time and sensitivity.

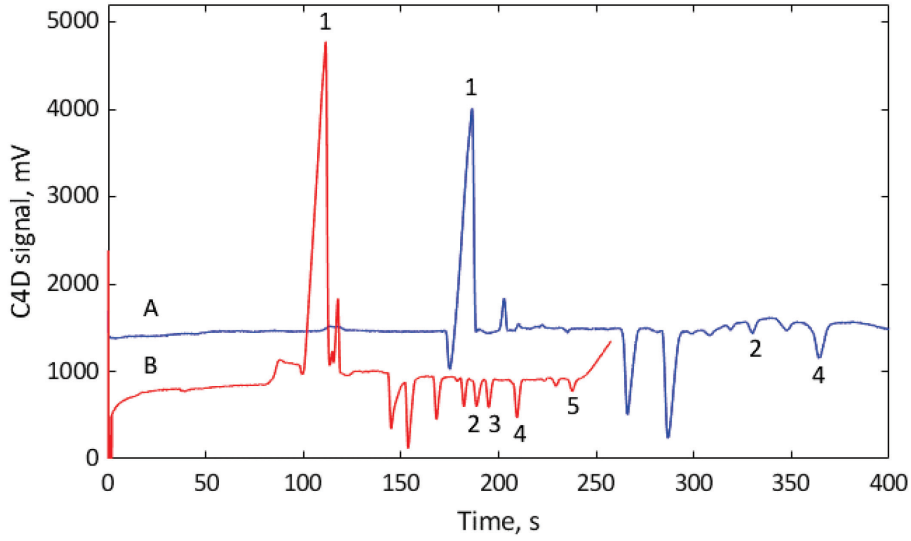

Figure 4.1 –The newly developed CE-C^4D analysis procedures of DOAs in OF. A – Procedure I from Publication I; B – Procedure II from Publication II. 1 – Cl^-, 2 – GHB, 3 – IBO, 4 – PMPA (IS), 5 – PY.

4.1.2 Validation of the proposed methodologies

The optimized methodologies were validated according to the EMA bioanalytical method validation guideline [205]. Linear ranges and associated determination coefficient (r^2) along with IDLs and IQLs were determined with LODs and LOQs after sample preparation methods were established. Within-run and between-run precision were evaluated in addition to the recovery, which also described accuracy. Validation of procedure I was performed in two matrixes – Milli-Q water and OF, as OF mainly consists of water, while the procedure II was validated in OF.

In addition, the methodology selectivity was evaluated by testing six compositions of endogenous matrix compounds from healthy volunteers who consumed only water before testing. Admittedly, the use of OF provides additional information regarding the anions present, which can be used as biomarkers for disease. Figure 4.2 shows all the anions identified from the OF samples by procedure I as well as the added GHB and PMPA. For example, high levels of nitrite and phosphate in OF have been associated with chronic renal failure [230,231] and tempo mandibular disorders result in twice the levels of glutamate compared to healthy individuals [232]. The selectivity was tested for both methodologies using OF obtained from smokers as well as from volunteers after working out, drinking tea, consuming juice, and suffering from upper respiratory tract infections. A total of 10 different scenarios were tested that are reflective of various aspects of everyday life without any observed interfering peaks.

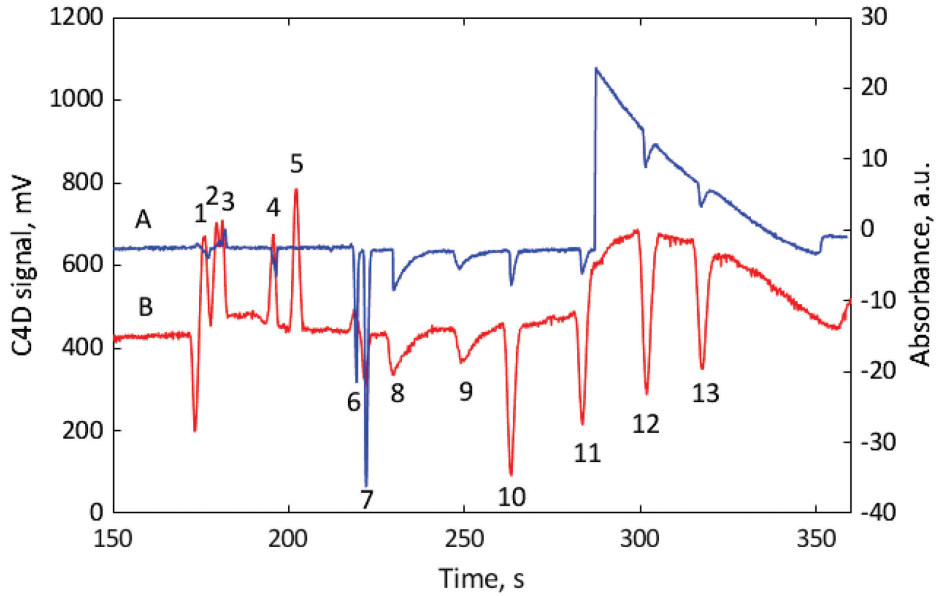

Figure 4.2 – All anions detected by procedure I in Milli-Q water: A – UV absorbance detection and B – conductivity, where 1 – 5 mg/L chloride; 2 – 10 mg/L nitrite; 3 – 10 mg/L nitrate; 4 – 10 mg/L sulphate and 5 mg/L sulphite; 5 – 40 mg/L thiocyanate; 6 – 120 mg/L tartrate; 7 – 80 mg/L succinate; 8 – 120 mg/L citrate; 9 – 20 mg/L hydrogen phosphate; 10 – 20 mg/L lactate; 11 – 20 mg/L GHB; 12 – 20 mg/L glutamate GHB; 13 – PMPA (IS) 100 µM [I]

The detection limits of procedure I were evaluated by S/N ratio analysis, with results similar for both water and OF ranging from 0.5 to 2 mg/L by conductivity and from 3.7 to 17.0 mg/L by indirect UV absorbance. Although the absorbance detection limits were

remarkably higher, no endogenous GHB were detected and could be useful as an additional confirmation at higher concentrations. The linearities of the methodologies were evaluated for both detection modes with all r^2 values of >0.98. Both within- and between-run precision was evaluated with results listed in Table 4.2. All results were <15%, meeting the guideline requirements. The recovery was evaluated at four concentrations with good results, as listed in Table 4.2, where lower concentrations showed higher recoveries. This is likely due to the endogenous GHB concentration, providing additional analyte, which was further supported by the matrix effect evaluation. The matrix effect (ME%) between water and OF for low concentrations was +53% and +24% for conductivity and UV absorbance, respectively, showing a relatively high matrix effect, while it was only ±2% at higher concentrations. Therefore, GHB concentrations from 2.5 to 25 mg/L were evaluated by a calibration curve constructed via standard addition or a correction coefficient based on natural GHB level in OF.

Table 4.2. Validation characteristics for CE-C^4D-UV absorbance. [I]

Matrix	IDL (mg/L)	IQL (mg/L)	LOD (mg/L)	LOQ (mg/L)	Precision, %		Recovery, %
					Within-run	Between-run	
Conductivity detection							
Water	0.62	2.1	2.5	8.3	0.9 – 2.2	1.5 – 6.6	95 – 119
OF	0.49	1.6	2.0	6.5	1.4 – 2.6	1.6 – 9.0	82 – 104
UV Absorbance – indirect detection (λ_{ABS}=210 nm)							
Water	3.7	12.4	14.8	49.7	1.1 – 4.8	2.2 – 14.6	85 – 113
OF	5.1	17.0	20.4	68.0	2.1 – 9.3	5.6 – 10.1	79 – 109

The same parameters were evaluated in for analysis procedure in **Publication II** for GHB, IBO, and PY in OF. The IDL and IQL were determined and for the LOD and LOQ, the sample dilution factor was included with LODs ranging from 1.5 to 3.6 mg/L and LOQs from 5.0 to 12.0 mg/L. Calibrations were constructed for all three analytes up to 30 or 200 mg/L with all r^2 values of >0.95. Within- and between-run precisions were determined and the values are listed in Table 4.3 with detection limits and recovery values. Recovery of the method was also determined for all analytes – GHB 94 – 130%; IBO 94-109%; and PY 94-111%. All results agreed with the EMA guidelines. The sample stability was not evaluated, as previous studies have shown that PY is degraded by light [233] and would provide unreliable results.

Table 4.3 Validation characteristics of CE-C^4D. [II]

	IDL (mg/L)	IQL (mg/L)	LOD (mg/L)	LOQ (mg/L)	Precision, %		Recovery %
					Within-run	Between-run	
GHB	0.5	1.7	1.5	5.0	0.7 – 3.3	3.9 – 11.4	91 – 103
IBO	0.7	2.3	2.1	7.0	1.9 – 3.2	4.3 – 8.5	96 – 105
PY	1.2	4.0	3.6	12.0	3.9 – 8.6	6.8 – 10.3	94 – 109

Both analysis procedures were in accordance with the guidelines and showed excellent promise for real OF sample analysis. They also showed good C^4D limits in OF enabling to distinguish between naturally occurring GHB levels and exogenous intake. Indirect UV absorbance detection in procedure I could be used for the analysis of real

intake or poisoning, where GHB levels can reach as high as 257 mg/L [234]. Procedure II showed improved stability and lower LOD and LOQ values for GHB. Moreover, it was possible to detect IBO and PY from OF and reduced the overall analysis time by approximately 4 min.

4.1.3 Analysis of OF samples for GHB and psychoactive mushroom compounds

The optimized and validated analysis procedures were tested using simulated samples. As GHB is an endogenous compound, even blank OF samples may contain some GHB depending on individual physiology. As GHB naturally occurs in various fermented drinks, including in wine ranging from 4.1 to 21.4 mg/L [235], drinking red wine was used to test real OF samples with increased GHB levels. Four glasses of wine, 100 mL each, were ingested within 30 min and samples were taken in between each glass. Two different wines were tested, "Le Grand Noir" (13 vol% EtOH, France 2013) was consumed by one volunteer, showing a small LOD level peak after the third glass. Vitral "Cabernet Sauvignon" (13.5 vol% EtOH, Chile, 2011) was also tested with two volunteers providing samples after each of the four glasses. The OF samples from the first volunteer after drinking wine two are shown in Figure 4.3, where a visible GHB peak was observed after the second glass of wine (Fig 4.3 red line). The samples were analysed using indirect UV absorption simultaneously, but the LOD levels in indirect absorption were noticeably higher and even in the C^4D analyses, the GHB levels were under the LOQ and no GHB was observed in any OF samples. Similar results were obtained with the second volunteer for the second wine, where no distinguishable peak enlargement was observed after drinking the first type of wine.

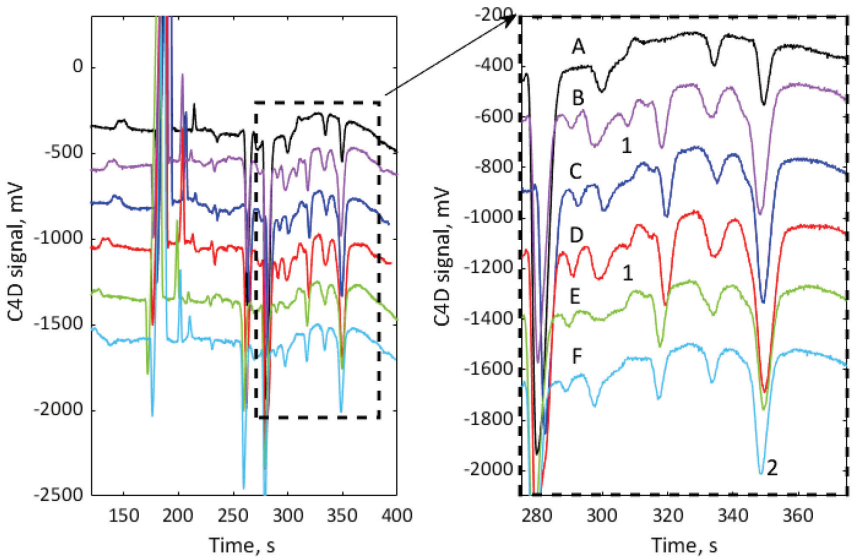

Figure 4.3. Electropherograms of volunteer 1 who consumed wine 2. A – OF before wine; B – OF after 1 glass of wine; C – OF after 1 glass with spiked GHB; D – OF after two glasses; E – OF after three glasses; and F – OF after four glasses. 1 – GHB, 2 – PMPA (IS). [I]

To evaluate procedure II for GHB, IBO, and PY, the GHB samples were produced as described previously, by drinking red wine in 100 mL increments. The OF samples showed a GHB peak increasing after 2nd and 3rd glass, as demonstrated in Figure 4.4 D. IBO and

PY in the real OF samples could not be obtained, but to demonstrate the potential of the analysis procedure, simulated samples were prepared. Air-dried *Amanita muscaria*, containing IBO, and *Psilocybe semilanceata* containing PY were prepared in MeOH extracts, as described in Section 3.1.4. The OF was fortified with a mushroom extract for IBO and a second OF sample was enriched with PY, and subsequently prepared as usual and analysed. The OF containing the *Amanita muscaria* extract (Fig 4.4 E) showed a peak increase for the IBO peak, which was not observed in the blank OF. The sample containing *Psilocybe semilanceata* extract (Fig 4.4 C) showed a PY peak, corresponding to a concentration of 20.7 mg/L.

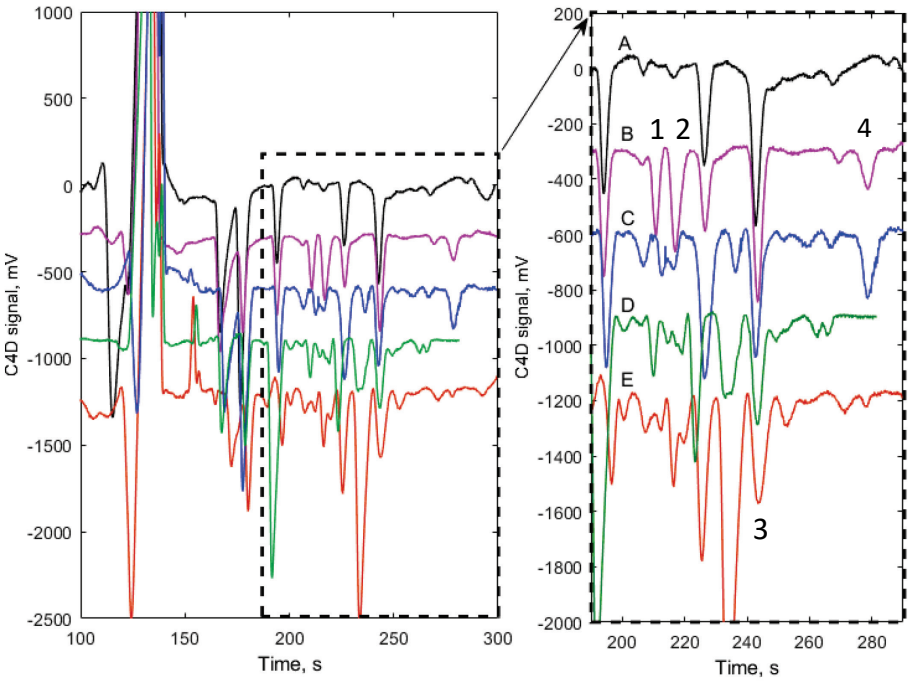

Figure 4.4. OF samples after wine and fortified with mushroom extracts. A – blank OF + IS; B – OF + GHB, IBO, and PY standards; C – OF + Psilocybe semilanceata extract; D – OF after drinking wine; and E – OF + Amanita muscaria concentrated extract. 1 – GHB, 2 – IBO, 3 – PMPA (IS), 4 – PY. [II]

Results from the OF samples after wine drinking and with the addition of mushroom extracts showed that the proposed analyses are useful for real-life situations. Endo- and exogenous GHB can be distinguished using a portable CE-C^4D and poisonings or recreational usage can be rapidly distinguished. IBO and PY can be also determined in <5 min from OF fortified with mushroom extracts, showing perspective for real-life samples. Further analysis of the samples from people who have been unwillingly exposed to GHB or recreationally taken GHB should be performed to determine if these methodologies are suitable for GHB detection at the cut-off limit, as with individuals who consume psychoactive mushrooms.

4.2 Detection of DOA using CE-LED-FD (Publication III)

The rise of synthetic AMP derivates in Europe, namely MDMA – *ecstasy*, is a growing epidemic as in 2017 an estimated 6.6 million tablets were confiscated. MDMA has multiple derivates, including MDA and MDEA, that can be consumed to obtain the same mind alternating effects. Therefore, there is a growing need to determinate if a person is under the influence of these substances as part of possible accident investigations. As the concentration of the DOAs is on the order of ng/mL, the sensitivity of the analytical methodology is essential. The use of CE for OF analysis can fulfil requirements for rapid on-site testing but may lack the required detection sensitivity. LEDs can be used to achieve long lifespan, specific wavelength selection, miniaturization. Using a LED-based detector allows for a portable instrument in combination with the required sensitivity and selectivity that can be achieved by fluorescence.

A CE-LED-FD instrument equipped with a 280 nm LED as an excitation source was used with an emission filter at 326 nm. This excitation and emission chosen were based on the fluorescence spectra, showing that MDA, MDMA, and MDEA exhibited emission at approximately 320 nm with the same quantum yield when excited at 230 or 280 nm [236], but no 230 nm LEDs were available. As the molecular weights of MDA, MDMA, and MDEA are 179.2, 193.3 and 297.3 g/mol, respectively, with pKa values ranging from 9.7 to 10.2, their separation by CE is straightforward. Under an acidic separation environment, the secondary amines become positively charged, migrating towards the cathode. The use of 40 mM H_3PO_4 and 10 mM TEA provides a stable acidic environment at pH 2.5 with sufficient buffering capacity. Using OF as the sample matrix provides the opportunity to analyse active intoxication because all the synthetic analogues are present as parent compounds in OF after intake. In addition, simultaneous analysis of MDA/MDMA/MDEA can provide additional information regarding the intake time as MDA is the first metabolite found in OF for both MDMA and MDEA.

The proposed methodology was validated according to the EMA guidelines. The selectivity was evaluated by using six blank OF samples without any interfering peaks (Figure 4.5). The LOD and LOQ were determined by fortifying 1 mL of the OF samples, with LODs ranging from 3.3 to 3.8 ng/mL and LOQs from 5.5 to 6.4 ng/mL. Two calibration curves were constructed in pure ACN and by standard addition to OF. In both cases, the area of the analyte peak was divided by the area of the internal standard, ALC. The linear ranges were from 10 to 150 ng/mL (r^2 >0.99) with regression errors of <20%, showing good linearities for all analytes. The extraction recovery was evaluated based on the calibration curve slopes, showing that all recoveries were <50%. The low recovery was mainly due to the poor analyte adsorption to the Salivette collector, but even with a 10-fold dilution, the LOQ of the OF samples would be ~60 ng/mL, which is below the recommended DRUID 220–270 ng/mL cut-off values. The methodology reliability was evaluated by calculating the precision and accuracy at three different concentrations. The precision was 2.3–5.0% for all analytes and accuracy was 98–109 %. The detailed results are listed in Table 4.4 and no carry-over was observed in negative samples after the 100 μg/mL sample analysis.

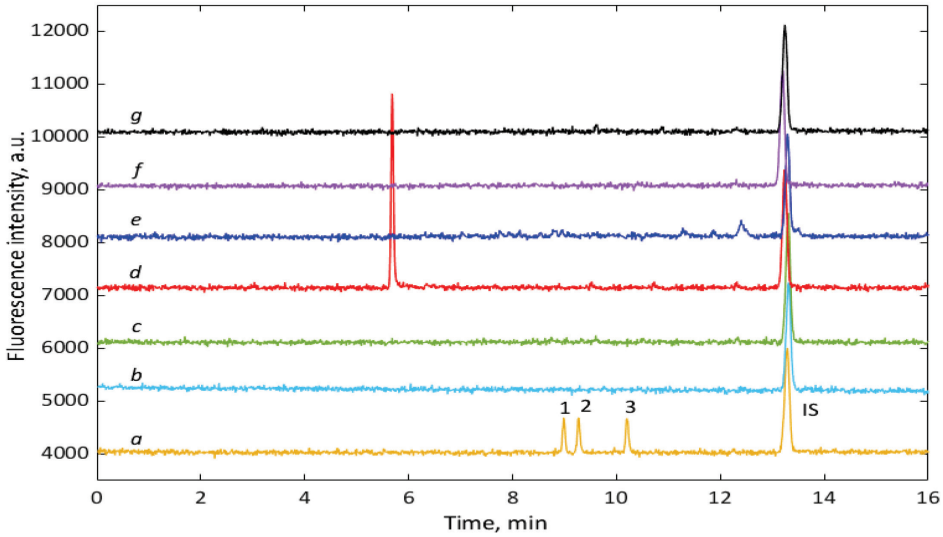

Figure 4.5. CE separations of the MDA, MDMA and MDEA standards (a) and analyses of independent OF samples from the six drug-free volunteers (b-g). Analytes: 1 –MDA (10 ng/ mL), 2 – MDMA (10 ng/mL), 3 – MDEA (10 ng/mL), IS – ALC (35 ng/mL). [III]

4.2.1 Plackett-Burman robustness study

The analysis procedure robustness was evaluated using a multivariate approach, namely the Plackett-Burman design. A total of 10 real factors and one dummy factor were chosen to evaluate the effect of small changes in the method. The selected factors were injection time, voltage, rinsing time of the BGE, Milli-Q, NaOH, H_3PO_4 concentration, TEA concentration, capillary length, injection height, and temperature as they all may affect the EOF speed, species mobility, capillary equilibria, resolution, and theoretical plates. All factors were analysed at low or high values, according to the experimental plan. The ratio of analyte area was divided by the internal standard area and taken as the response.

After all experiments, the effect of each factor was calculated for MDA, MDMA, and MDEA. The limit value for a statistically significant effect was assessed using a statistical t-test [237]. All $t_{calculated}$ values are listed in Table 4.5, where values with a negative sign indicate that the changing the associated factor from a low to high value exhibited a negative impact on the response and vice versa. Four factors significantly impacted the measurement results, the injection time, H_3PO_4 concentration, capillary length, and temperature. To better understand the impact of the changes, the non-significance levels were calculated for the identified factors. The most sensitive of the factors was temperature changes, which had a non-significance level of only 0.8 to 1.1 °C, indicating that a change of as little as 0.8 °C significantly impacted the results. The levels for non-significant injection time, H_3PO_4 concentration, and capillary length variances are also provided in Table 4.5. With variation of these factors within the non-significance intervals, no significant effects were observed.

The robustness of the analysis procedure was clearly an important consideration for on-site testing set-up. Automation of the sample injection and thermoregulation would greatly improve the stability and outcome of the analysis. In addition, the Plackett Burman design provided clear results from only 12 experiments for understanding the main disadvantages and advantages of the methodology robustness.

Table 4.4. CE-LED-FD validation results. [III]

Analyte	Regression I		Regression II		Rec ± U (%)	LOD, ng/mL	LOQ, ng/mL	Precision, RSD%			Accuracy, %		
	r^2	RE (a.u./%)	r^2	RE (a.u./%)				20 ng/mL	80 ng/mL	150 ng/mL	20 ng/mL	80 ng/mL	150 ng/mL
MDA	0.995	0.039/4.8	0.993	0.017/4.7	40 ± 4	3.3	5.5	3.2	2.3	2.9	98	109	106
MDMA	0.993	0.047/5.9	0.991	0.022/6.0	43 ± 3	3.8	3.8	4.8	3.4	3.5	102	103	105
MDEA	0.994	0.065/6.5	0.992	0.024/5.3	35 ± 3	3.6	6.1	2.4	5.0	4.3	104	107	105

Table 4.5. Robustness analysis for CE-LED-FD analysis by Plackett-Burman design [III]

Factor		Level		$t_{calculated}$			Non-significance level		
		Low	High	MDA	MDMA	MDEA	MDA	MDMA	MDEA
A	Injection time, s	27	31	-4.77*	-3.67*	-5.01*	± 2.0	± 2.7	± 1.9
B	High voltage, kV	15	19	0.55	2.02	2.06			
C	Rinse time (BGE), s	108	132	-0.97	1.64	0.23			
D	Rinse time (Milli-Q), s	108	132	0.27	-0.41	2.34			
E	Rinse time (NaOH), s	108	132	-0.40	-0.58	-0.01			
F	[H_3PO_4], mM	18	22	3.85*	5.67*	4.20*	± 1.7	± 1.1	± 1.5
G	[TEA], mM	5.4	6.6	2.39	1.34	2.71			
H	Capillary length, cm	60	64	-4.71*	-5.29*	-3.89*	± 1.4	± 1.2	± 1.7
I	Injection height, cm	13.5	16.5	2.81	2.16	0.68			
J	Temperature, °C	21	25	5.91*	8.77*	7.45*	± 1.1	± 0.8	± 0.9
K	Dummy			-2.00	-2.24	-2.16			
$t_{crit(0.01;9)}$ 3.25									

*significant at P = 0.01.

4.3 Detection of DOAs using CE with UVC fluorescence (Publications IV and V)

Detection of three DOAs by fluorescence was discussed in **Publication III**, but the sensitivity using excitation at 280 nm was not satisfactory for many common DOAs. For COC and COET the LOQs were approximately 600 and 800 ng/mL, respectively [238]. Therefore, **Publication IV** demonstrated the construction and possible application of a miniature Xe lamp as an excitation source for a UVC range excitation fluorescence detector CE instrument. Using excitation of <260 nm enables the detection of a wider range of DOAs with improved sensitivity and a miniature lamp would improve the instrument portability. The selected DOAs were amphetamine type stimulants: AMP, METH, MDA, MDMA, MDEA, PMA, PMMA; COC and its metabolite COET; as well as cannabis as THC and CBD. This selection covered the most common classes of DOAs.

The fluorescence detector was constructed in collaboration with OÜ OMEC (Tartu, Estonia). The detector used a xenon flash lamp with an optical system providing UVC range excitation with a maximum of 240 nm. The excitation band passing the set of filters is shown in Figure 4.6. as F_{ex}. The choice of filters was based on the excitation end emission characteristics of the analytes of interest, manufacturer information, and performance of the layout was confirmed experimentally. The excitation and emission spectra (Figures 4.6 and 4.7) were measured using a NarTest NTX2000 Drug Analyzer (NarTest AS, Estonia) using previously reported specifications [236]. All fluorescence data is displayed in normalized arbitrary units.

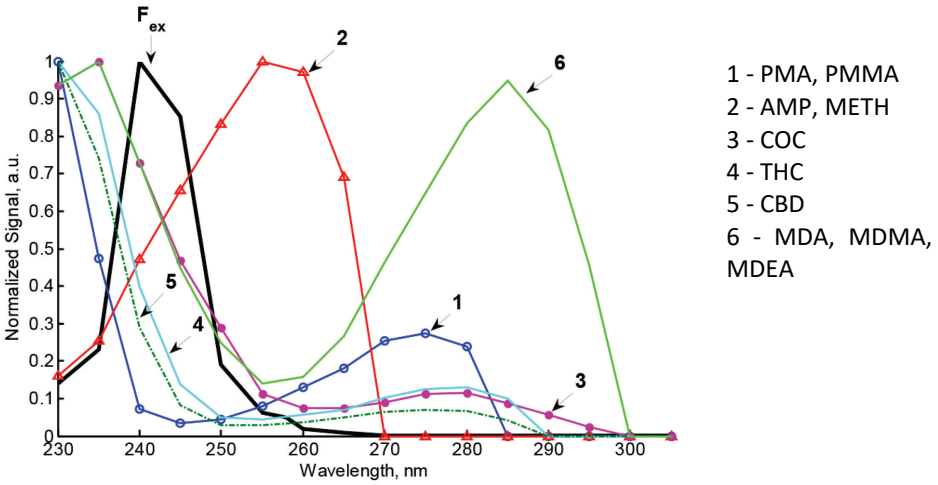

Figure 4.6. Excitation spectra of DOAs and the excitation filter set in the CE-FD instrument. [IV]

The detector was equipped with five interchangeable emission filters and depending on the filter, the analysis selectivity could be shifted towards a specific illicit drug. Consequently, F_{EM1} provided the best selectivity and sensitivity for AMP and METH emission spectra, while F_{EM2} was best for THC and CBD, while F_{EM3} was optimal for MDA, MDMA, MDEA, and COC (Figure 4.7). The most universal filter was demonstrated to be F_{EM3}.

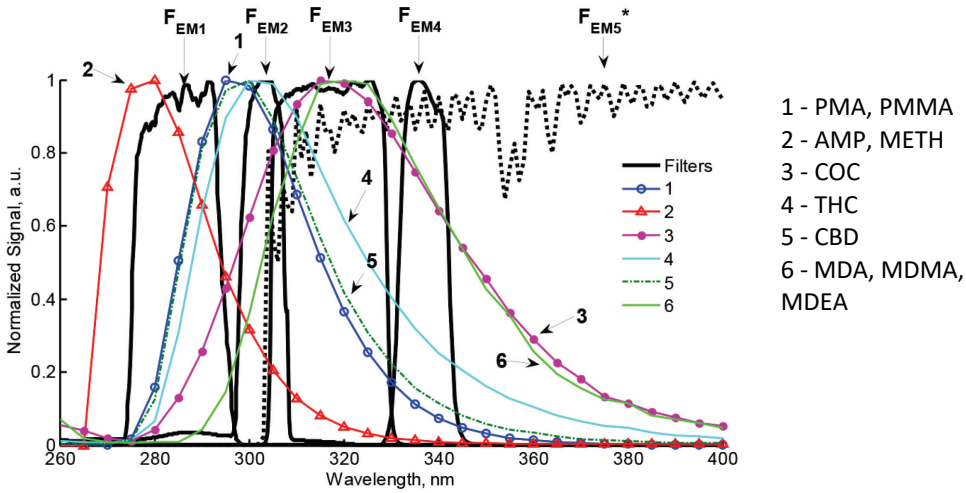

Figure 4.7 Emission spectra of DOAs and emission filters (F_{EM1}, F_{EM2}, F_{EM3}, F_{EM4}, and $*F_{EM5}$ up to 600 nm). [IV]

The portable CE apparatus was constructed around a fluorescence detector. The portable instrument weighed approximately 3 kg with dimensions of 20 x 10 x 30 cm and is pictured in Figure 3.3.

4.3.1 Detector performance characteristics

CE-FD detector performance was evaluated in terms of linearity, noise, selectivity, and specificity. The detector linearity was evaluated according to the ASTM E578 – 07 (2013) standard [239]. The quinine solution was replaced with MDMA in ACN because MDMA shows a high stability towards exciting radiation, large quantum yield even in dilute solutions, and a minimal overlap of fluorescence and abortion peaks. MDMA solutions were prepared in ACN at final concentrations ranging from 0.25 to 100 000 µg/mL and measured in triplicate in a static mode with no voltage or separation. The third emission filter F_{EM3} (λ = 315/20 nm) was used for signal detection as it was the most sensitive to MDMA emission. Fluorescence correction was achieved by subtracting the ACN baseline intensity.

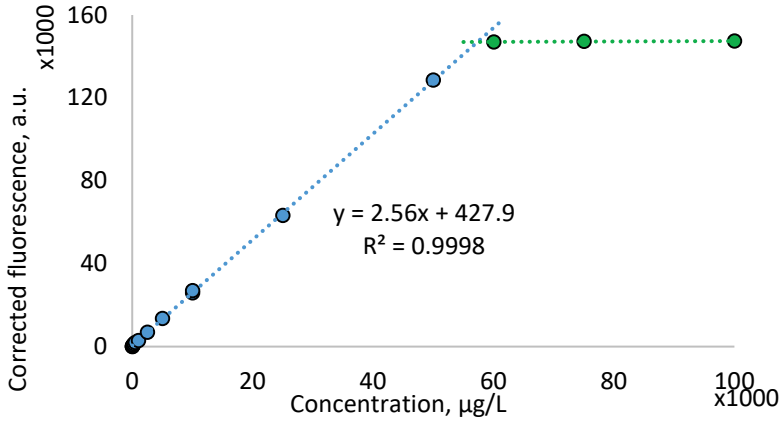

Figure 4.8 Detector linearity estimation using MDMA in ACN at filter F_{EM3} and PMT at 500 V. [IV]

Furthermore, noise levels were evaluated in static and dynamic modes with electrophoretic separation, with F_{EM3} as the most universal filter for the detection of illicit substances. The noise was estimated using the root-mean-square (RMS) of a peak-to-peak value, which was divided by 5.16 (at the 99% confidence level) [240]. Static measurements were obtained over 10 min for a new uncoated fused silica capillary (i.d. 75 µm) with a burnt detection window filled with air (noise CV 1.1%) and with ACN (noise CV 0.6%). The dynamic noise CV% was evaluated for different PMT voltages ranging from 500 to 800 V at intervals of 100 V. The noise CV% was evaluated for 2–5 min for each level (500, 600, 700, and 800 V), showing CV% values of 0.95%, 0.70%, 0.90%, and 0.80%, respectively. A PMT voltage of 500 V allowed for the measurement of samples at both low and high analyte concentrations without oversaturation and was subsequent used.

The specificity of the CE-FD analyser is based on the selective excitation/emission filtering and electrophoretic separation conditions. Only certain DOAs exhibit native fluorescence at 230–255 nm excitation and different emissions depending on the F_{EM} used. The selectivity of CE-FD different emission filters was evaluated using a sample containing 66 µg/mL AMP and METH; 3.3 ng/mL MDA, MDMA, and MDEA; 83 ng/mL COC; and 100 ng/mL COET in ACN and analysed at F_{EM1}, F_{EM2}, and F_{EM3} (Figure 4.9). The F_{EM3} analysis corresponds to the IQL levels of the above-mentioned substances at the specified parameters.

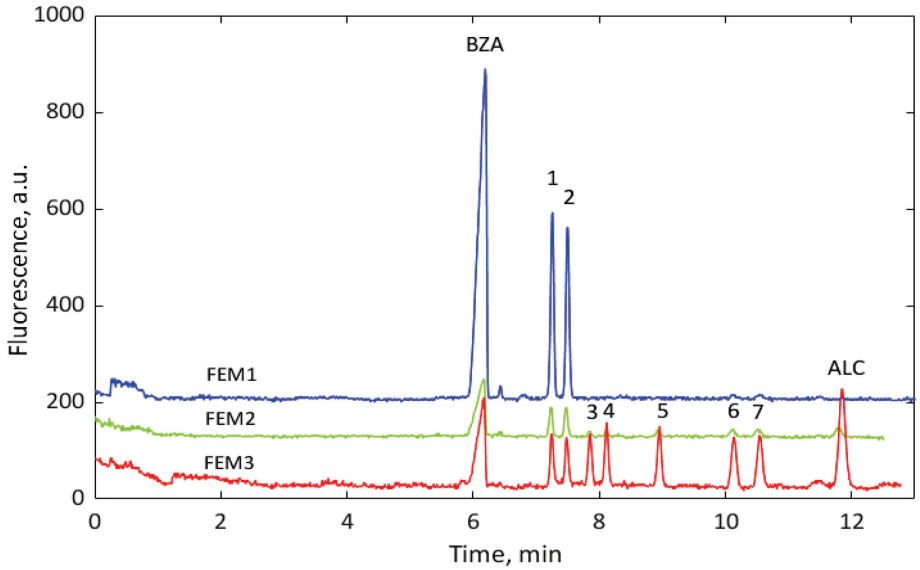

Figure 4.9. Electropherogram of DOAs in ACN determined at three emission filters. BZA 300 µg/mL, 1 – AMP 66 µg/mL, 2 – METH 66 µg/mL, 3 – MDA 3.3 ng/mL, 4 – MDMA 3.3 ng/mL, 5 – MDEA 3.3 ng/mL, 6 – COC 83 ng/mL, 7 – COET 100 ng/mL, ALC – 33 ng/mL.

In addition to AMP, METH, MDA, MDMA, MDEA, COC, and COET, PMA and PMMA were separated using the BGE containing Tris and H_3PO_4. Moreover, a NACE-based BGE reported by Kulp *et al*. [225] was used to detect THC and CBD in a pre-processed OF matrix at F_{EM2} (λ = 302 nm). The IDL and IQL were measured using the S/N approach for all substances and are listed in Table 4.6. The IDL was determined at a 3:1 S/N ratio and IQL at 10:1 ratio.

Table 4.6. Instrumental detection and quantification limits for DOAs for CE-FD. [IV]

Substance	F_{EM}, nm	IDL, ng/mL	IQL, ng/mL
AMP	285	3000	10 000
	315	9500	31 670
METH	285	3000	10 000
	315	9500	31 670
MDA	315	0.5	1.6
MDMA	315	0.5	1.6
MDEA	315	0.5	1.6
COC	315	13	42
COET	315	15	50
PMA	315	10	33
PMMA	315	10	33
THC	302	25	83
CBD	302	25	83

Thus, the CE-FD instrument showed good linearity, allowing for the analysis of real-life samples where concentrations can vary over a wide range. Furthermore, the detector showed remarkably low noise for both dynamic and static measurements, providing excellent IDL and IQL levels for various DOAs if interchangeable emission filters were used. The number of DOAs detected can be expanded by testing additional psychoactive substances, synthetic cannabinoids, and other natively fluorescing molecules.

4.3.2 DOA analysis and validation in OF

As the CE-FD instrument showed great promise for detecting DOAs in ACN, its reliability and usability were examined for OF matrixes. In **Publication V**, the analysis procedure was validated for the DOAs AMP, METH, MDA, MDMA, MDEA, COC, and COET.

The main sample preparation procedure was adopted from previous work (**Publications III and IV**) with changes to the addition of the IS solution and its constituents. A second IS, namely benzylamine (BZA), was used for improved determination and identification along with the previously used ALC. In addition, the IS was not added from a stock solution to the sample, but by adding the sample to pre-made Eppendorfs containing the IS. The use of the air-dried mixture of IS allowed the user to pre-make tubes for on-site testing, reducing the need for pipetting small amounts of solution on-site and simplifying the sample preparation procedure.

Methodology validation in the OF matrix was performed according to the EMA guidelines for bioanalytical method validation [205] and consisted of the following performance characteristics: selectivity, LOD, LOQ, linearity of calibration, matrix effects (MEs), extraction recovery (Rec), analyte stability, precision, accuracy, and carry-over effects. The methodology was re-optimized with a final BGE containing 38 mM Tris, 36 mM H_3PO_4 at pH = 3.3 and a capillary length of l_{ef} = 35 cm, L_{tot} = 53 cm. These conditions provided efficient peak separation (Rs >1.2) for all analytes of interest at even high concentrations in 8 min. The BGE was stable for six months when stored at room temperature (+23 °C).

The analysis selectivity was evaluated using OF from various volunteers. Endogenous matrix influence was assessed using samples from 35 DOA-free volunteers fortified with BZA and ALC. Most samples were free from any interferents (Figure 4.10, A–D). Two notable cases were identified as metoprolol (Fig 4.10 E), a hypertension medicine, and

tyramine (Figure 4.10 D). Tyramine is an endogenous amine that is a metabolite of tyrosine and it is found in fermented foods and drinks [241]. Excessive tyramine concentrations are associated with hypertension and migraines [242], and commonly found in high concentrations in smokers [243].

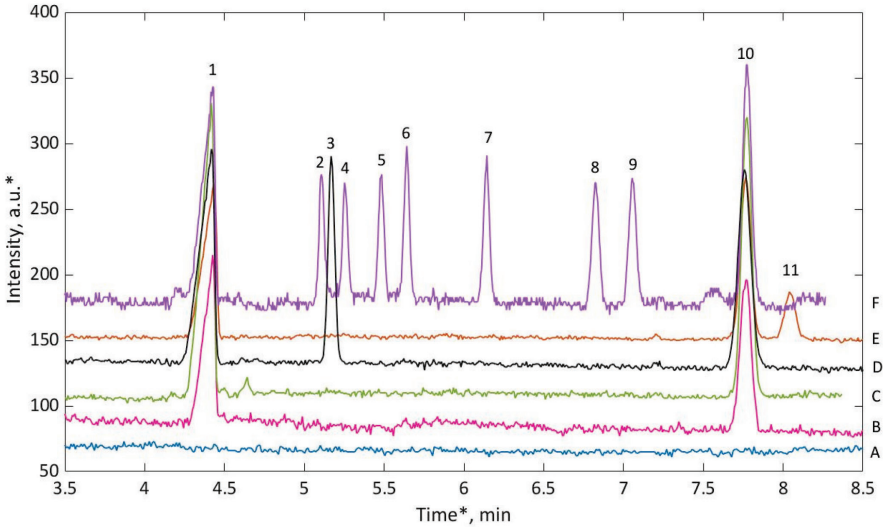

Figure 4.10 – Electropherograms of different OF samples. A – Blank OF, B – Blank OF with IS, C – OF from a snuff user with IS, D – OF from a smoker with IS, E – OF from volunteer, F – OF with all analytes of interest. Peaks: 1 – BZA 300 µg/mL, 2 – AMP 133 µg/mL, 3 – tyramine, 4 – METH 133 µg/mL, 5 – MDA 20 ng/mL, 6 – MDMA 20 ng/mL, 7 – MDEA 20 ng/mL, 8 – COC 95 ng/mL, 9 – COET 90 ng/mL, 10 – ALC 35 ng/mL, 11 – metoprolol. [V]

In addition, the selectivity was also evaluated by fortifying the PPOF matrix with 58 potential interfering substances, mostly metabolites of the DOA of interest (8), additional DOAs (14), pharmaceuticals (33), and other compounds (3). Only 2 possible compounds were identified that could hinder the analysis, codeine and citalopram. A full list of the tested substances is provided in Appendix 5, Table A3.

Calibration curves were constructed in three matrixes – ACN, pre-processed OF (PPOF), and OF. The peak area ratio pots (analyte to ALC) as a function of the concentrations of the standards in the three matrices exhibited acceptable linearities for the analytes of interest with r^2 values of >0.99 and regression errors of <10 %. Linearity was confirmed and quantified by statistical lack-of-fit ANOVA testing where all estimated probabilities (P) were >0.05. All values for all regression lines are provided in Table 4.8.

The LODs and LOQs were determined for the Salivette® collector by the S/N approach, where LOD was 3 times the S/N ratio and LOQ 5 S/N, respectively. The LODs for MDA, MDMA, and MDEA were 6 ng/mL, satisfying the requirements of all international programs. The LODs for COC and COET were approximately 30 ng/mL, falling below the DRUID project recommendations, the EWDTS set limits, and AS 4760. AMP and METH LODs were on order of µg/mL and above the required limits, mainly due to the F_{EX} and F_{EM} used and the poor recoveries. This can be improved by changing the optical system or increasing the recoveries of the substances. All LODs with the required detection limits are listed in Table 4.7.

Table 4.7. Detection limits of CE-FD and the proposed cut-off values in OF (ng/mL). [V]

Substance	CE-FD	DRUID [139]	EWDTS [19]	ROSITA -2 [171]	SAMSHA [174]	Talloires project [175]	AS 4760 [173]
AMP	40*	360	40	25	25	20	50
METH	40*	410	40	25	25	20	50
MDA	6	220	40	25	25	20	50
MDMA	6	270	40	25	25	20	50
MDEA	6	270	40	25	25	20	50
COC	28	170	30	8	15	10	50
COET	27	-	30	-	-	-	50

* µg/mL

All recoveries using the Salivette collector and sample treatment for the selected DOAs were <50%, ranging from 11% to 35%, as shown in Table 4.9. The low recoveries were mainly caused by sample processing, as the first centrifugation retained 0.28 ± 0.04 g (CV% 14.1%, n=39) of OF in the pad, and did not depend on the original OF collected or pipetted. The weak adsorption of the analytes to the cotton pad resulted in a loss of the analytes caused by discharging of the first centrifugate. This could be improved with the use of different collectors such as the StatSure Saliva Sampler™ (StatSure Diagnostic Systems, USA), Quantisal® (Immunalysis Corporation, USA), or Intercept® (OraSure Technologies, Inc., USA) [244]. The use of recovery buffers in the mentioned collectors would increase the sample dilution factor and corresponding LOD and LOQ values. As Salivette® showed stable OF retention, provided a cleaning step for OF samples, and exhibited the best cost-effectiveness, it was preferred above others.

According to EMA requirements for the precision, accuracy, and uncertainty of the procedure, these parameters should not exceed 15, 15, and 30%, respectively. The determined values for precision, accuracy, and uncertainty at two concentrations are listed in Table 4.8. The precision ranged between 1 and 11% and the accuracy varied from 5.1 to 9.1%, fulfilling the guideline requirements. In addition, the obtained uncertainty values satisfied the EMA requirements as they were consistently <30%.

The sample stability was evaluated at two concentrations at room temperature (23 °C) for 12 days, in the refrigerator (5 °C), and in the freezer (-20 °C) for 12 weeks. The freezer samples were used to determine the possible freezing and thawing cycle stability. Because of the freezing, additional peptides were denatured and affected the separation, so the recommended sample storage temperature was 5 °C. Carry-over was evaluated to assess if high concentrations within random samples could influence the subsequent analysis. A high concentration (10 µg/mL each) of DOA in OF was analysed, followed by a DOA-free sample. All DOAs were below the LOD in the blank analysis, indicating no carry-over.

The resulting thoroughly validated analysis procedure for DOAs in OF was in compliance with the requirements set by the EMA. The use of Salivette devices showed low but stable recoveries, resulting in LODs and LOQs meeting several international guideline requirements for most DOAs. However, further developments for AMP and METH analysis should be achieved to provide additional sensitivity. Nevertheless, the newly developed CE-FD methodology is suitable for testing on real samples.

Table 4.8. Calibration curve statistics and limits for detection and quantification for CE-FD in OF. [V]

Analyte	Regression (I)			Regression (II)			Regression (III)			LOD, ng/mL	LOQ, ng/mL
	RE (a.u., %)	r^2	P	RE (a.u., %)	r^2	P	RE (a.u., %)	r^2	P		
AMP	0.03/0.5	0.9957	0.754	0.07/1.2	0.9989	0.690	0.050/0.1	0.9966	0.901	40 000	66 000
METH	0.02/0.5	0.9967	0.677	0.05/1.0	0.9992	0.711	0.040/0.2	0.9969	0.863	40 000	66 000
MDA	1.05/1.6	0.9993	0.764	0.13/2.0	0.9997	0.817	0.119/1.1	0.9995	0.928	6	10
MDMA	1.38/1.8	0.9990	0.487	0.32/1.4	0.9998	0.996	0.307/2.2	0.9991	0.928	6	10
MDEA	0.91/1.1	0.9997	0.586	0.42/1.5	0.9996	0.828	0.197/1.8	0.9992	0.905	6	10
COC	0.42/3.6	0.9967	0.993	0.10/0.9	0.9997	0.646	0.026/1.2	0.9998	0.840	28	47
COET	0.23/1.9	0.9991	0.936	0.22/1.5	0.9991	0.625	0.068/2.2	0.9992	0.882	27	45

Table 4.9. Performance parameters in OF. [V]

Analyte	Conc., ng/mL	Within-run precision, (CV %)	Between-run precision, (CV %)	Rec% (CV%)	ME% (CV%)	Accuracy, (RMS bias, %, n=10)	Uncertainty, k=2
AMP*	200 000	1.0	8.5	11 (7.0)	113 (2.6)	5.2	19.9
	400 000	0.7	8.6	11 (3.7)	119 (2.4)	5.0	19.8
METH*	200 000	1.9	7.3	13 (4.2)	111 (1.0)	9.1	23.4
	400 000	1.0	5.2	13 (3.7)	113 (2.7)	7.8	18.7
MDA	100	0.7	5.5	35 (12.0)	106 (2.2)	5.1	15.1
	1000	2.8	9.1	17 (5.3)	99 (2.5)	7.0	22.9
MDMA	100	1.2	7.2	31 (12.5)	98 (1.9)	7.4	20.6
	1000	2.9	9.1	19 (3.8)	98 (1.3)	7.9	24.1
MDEA	100	2.4	8.0	28 (15.2)	94 (2.1)	6.6	20.8
	1000	1.6	5.6	16 (3.3)	103 (1.6)	7.6	18.9
COC	300	3.1	9.2	25 (3.8)	104 (3.3)	8.6	25.2
	2000	3.5	9.0	19 (3.2)	103 (1.1)	8.2	24.3
COET	300	2.5	5.8	25 (2.8)	110 (2.7)	7.6	19.1
	2000	3.1	11.1	22 (2.5)	117 (1.3)	8.5	27.9

4.3.3 Weekend Festival Baltics OF samples

Real samples from people suspected of being under the influence of drugs were collected from 2016 to 2018 during the Weekend Festival Baltic in co-operation with the Estonian Police. A total of 110 samples were collected – 36 in 2016, 37 in 2017, and 37 in 2018. The Weekend Festival Baltic is an outdoor rave-type party associated with high levels of drug and alcohol consumption.

All samples were gathered on a voluntary basis and tested on-site to obtain results for AMP, METH, MDA, MDMA, MDEA, COC, and COET. Because the LOD levels for AMP and METH were 40 000 ng/mL and real values of AMP and METH in OF range from 230 to 10 000 ng/mL [34,43], their detection was unfortunately not achieved. Therefore, AMP and METH were not considered in the comparative study. Figure 4.11 shows the representative electropherograms of representative samples, where all samples were compared to a standard containing all analytes of interest (Fig 4.11 A). A small amount of every OF sample was spiked with standards to verify compound identification (Fig 4.11 C). MDMA samples (Fig 4.11 D) usually exhibited a smaller peak for MDA and, if COC was consumed with alcohol, a COET peak was observed (Fig 4.11 E). In 40 of the obtained festival samples, tyramine was observed (Fig 4.11 B), which is likely due to alcohol consumption and smoking, as smoking may result in a 400-fold higher concentration of tyramine in the OF sample [243].

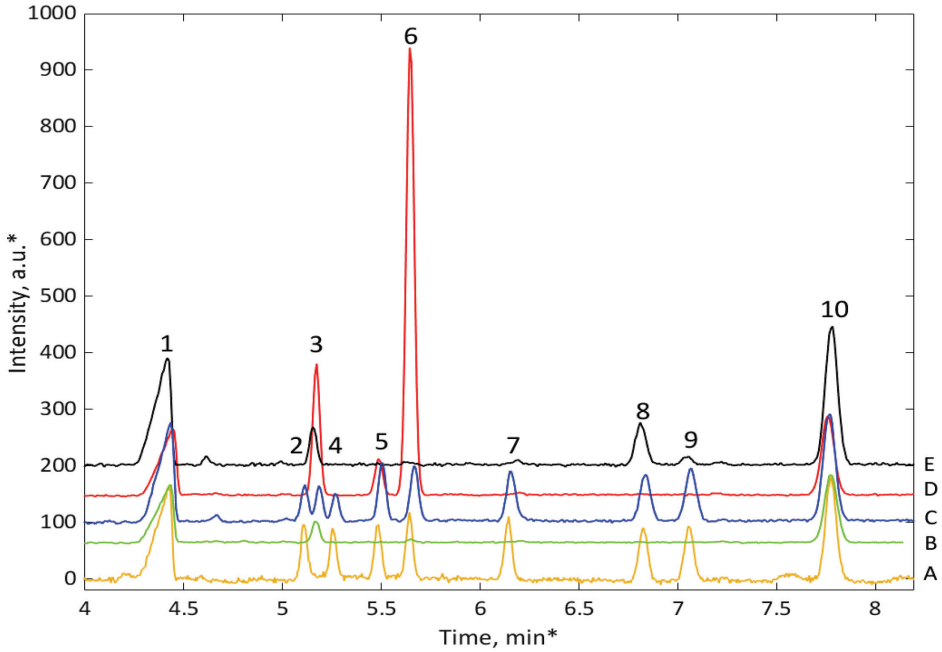

Figure 4.11. Representative examples of samples obtained from participants in the Weekend Festival Baltics, 2018. A – Standards in OF (2x LOQ levels), B – W18-012, C – W18-012 with spiked standards, D – W18-009, E – W18-024. Analytes of interest: 1 – BZA, 2 – AMP, 3 – tyramine, 4 – METH, 5 – MDA, 6 – MDMA, 7 – MDEA, 8 – COC, 9 – COET, 10 – ALC. [V]

Detailed results obtained from all Weekend Music Festival samples from 2016–2018 are provided in the Supplementary Data of **Publication V**. Table 4.10 shows the summary data of all results obtained per year and per substance with the corresponding concentrations.

The most common drug was MDMA, which was present in 62% of the collected samples. Following MDMA, the next most common substance was MDA, which was likely derived from the metabolism of MDMA in OF, as well as being an active DOA itself. The prevalence of COET indicates alcohol consumption mixed with drug intake. The prevalence of COC in positive samples dropped from 48% in 2016 to 11% in 2018 and no real OF samples contained MDEA throughout the years.

Table 4.10. Weekend music festival results from 2016 to 2018. [V]

Substance	2016	2017	2018	2016-2018
MDA:				
Positive (n)	21	18	16	55
Median ± MAD*, ng/mL	302 ± 312	213 ± 141	266 ± 264	259 ± 231
Min – Max, ng/mL	10 – 1469	39 – 778	10 – 1807	10 – 1807
MDMA:				
Positive (n)	23	25	20	68
Median ± MAD*, ng/mL	2894 ± 2246	4451 ± 4385	4602 ± 5042	3967 ± 3855
Min – Max, ng/mL	13 – 9773	43 – 31400	43 – 34767	13 – 34766
COC:				
Positive (n)	15	4	3	22
Median ± MAD*, ng/mL	766 ± 4253	223 ± 85	669 ± 1600	654 ± 689
Min – Max, ng/mL	95 – 4253	128 – 327	81 – 1469	81 – 4253
COET:				
Positive (n)	10	2	1	13
Median ± MAD*, ng/mL	117 ± 38	162 ± 26	27 ≤ x ≤ 45	124 ± 41
Min – Max, ng/mL	69 – 268	136 – 188	27 ≤ x ≤ 45	69 – 268
Total number of samples	36	37	37	110
Total number of positives (at least one analyte of interest)	30 (83.3%)	27 (73%)	25 (67.6%)	81 (73.6%)
Total number of true negatives	6 (16.7%)	10 (27%)	12 (32.4%)	29 (26.3%)

** MAD or median absolute deviation, p=99.7%*

The CE-FD results were compared to HPLC-MS results obtained under the conditions described in **Publication V**. Both quantitative and qualitative results were obtained and compared for MDA, MDMA, COC, and COET. The quantitative results for MDA show a correlation of $r^2 = 0.9937$; MDMA $r^2 = 0.9983$; and COC $r^2 = 0.9935$. Furthermore, the results of the two methods were compared by calculating corresponding z-scores for all results obtained for the samples collected in 2018. The MDA z-scores ranged from 0.1–1.4 (n=13); MDMA 0.1–1.9 (n=17); and 0.2–0.8 for COC (n=3). As all z-scores were <2, indicating no statistical difference between the results obtained by CE-FD and HPLC-MS.

The comparative analysis confirmed the reliability of the CE-FD portable instrument, demonstrating that the DOA analyser provides accurate detection and quantification of DOAs in OF.

4.3.4 Diagnostic performance of the newly developed CE-FD instrument

Using the results obtained by comparing the CE-FD and HPLC-MS results, instrumental statistics were evaluated. In addition to the Weekend Festival Baltic samples, the Estonian Police provided 18 OF samples from suspected drugged drivers in 2017 (results shown in the Supplementary Data of **Publication V**). All on-site instruments should show a great level of SE, SP, and DA for reliable detection of intoxication from real samples. According to DRUID, the values should be >80% for all parameters, and the ROSITA project set levels for the SP and SE of >90% and for the DA, >95%.

A total of 188 OF samples were analysed, containing both negative and positive samples. The numbers of true positives and true negatives were evaluated by comparing the on-site results with HPLC-MS. In the 9 cases of positive AMP and METH by HPLC-MS, the LODs were much lower than CE-FD, and the detected cases were not identified by CE-FD. However, if the same samples contained MDMA/COC detected by CE-FD, the sample was classified a positive. All diagnostic statistics were evaluated for samples with at least 20 TP and TN, therefore no SE, SP, or DA values were available for AMP, METH, MDEA, and COET, with the related results shown in Table 4.11.

Table 4.11. Diagnostic statistics for the CE-FD DOA analyser. [V]

Substance/ Parameter	AMP	METH	MDA	MDMA	MDEA	COC	COET	Min 1 DOA
TP*, (n)	6	6	55	77	0	25	13	92
TN*, (n)	182	182	133	111	188	163	175	96
Positive**, (n)	0	0	56	77	0	25	13	91
Negative**,(n)	188	188	132	111	188	163	175	97
FP, (n)	0	0	1	0	0	0	0	1
FN, (n)	6	6	0	0	0	0	0	0
FP rate, %	0	0	0.8	0	0	0	0	1.0
FN rate, %	-	-	0	0	-	0	-	0.0
SE, %	-	-	100	100	-	100	-	100.0
SP, %	100	100	99.2	100	100	100	100	99.0
DA, %	-	-	99.6	100	-	100	-	99.5

* ≥LOD, detected by reference method,

** suggested by CE-FD DOA

Achieving all parameters >99% shows extreme potential for the use of CE-FD for on-site analysis of DOAs. The high SE and SP values show that the analyser is capable of correctly detecting negative and positive samples, while the DA confirms that the analysis procedure is capable of distinguishing between these cases with excellent accuracy. Even though no MDEA and few COET were detected, this can be improved by continuous co-operation with Estonian Police and additional samples. Further improvements for AMP and METH detection are being developed. Nevertheless, these results indicate that the CE-FD for DOA analysis in OF is a promising future tool for psychoactive substance testing.

5 CONCLUSIONS

The aim of this dissertation was to develop and validate analytical procedures to enable onsite detection and quantification of psychoactive substances in OF using existing and novel portable CE instruments coupled to conductivity, UV-absorbance, and fluorescence detectors. In addition, a novel portable CE apparatus was constructed in collaboration with Omec OÜ with a UVC excitation fluorescence detector for determination and quantification of DOAs in OF for on-site applications.

The results of this thesis demonstrated the capability of the newly developed portable CE for analysis of various psychoactive substances in OF and the major conclusions can be summarized as follows:

- CE with UV absorption and C^4D detection was fast and reliable for analysis of GHB and psychoactive mushroom compounds (IBO and PY) in OF
    - An analytical procedure was developed and validated for analysis of GHB in OF by simultaneous UV absorption and C^4D detection
    - The procedure was expanded to include GHB, IBO, and PY in OF using a portable CE-C^4D
    - Box-Behnken designs were used to optimize and determine analytical methodology robustness
    - Simple sample preparation and an analysis time of <5 min was achieved
    - The detection limits allowed to distinguish between endo- and exogenous GHB concentrations
    - OF samples were collected after drinking red wine, showing increased GHB concentrations
    - Mushroom extracts spiked into OF were used as simulated samples and proved the capability for detection of IBO and PY in the OF matrix
    - The newly developed analysis procedures can be used to determine anionic biomarkers in OF
- LED-based excitation provided detection of ecstasy and its analogues in OF by native fluorescence
    - MDA, MDMA, and MDEA were analysed in 15 min with LODs under the DRUID suggested cut-off limits
    - OF collection using the Salivette® collector provided simple sample collection and clean-up
    - A thorough validation of the developed procedure was performed according to the EMA guidelines
    - Analytical procedure robustness was evaluated by using a Placket-Burman design, establishing the extreme sensitivity to small temperature changes as well as injection time, H_3PO_4 concertation, and capillary length of the developed system
    - Excitation at 280 nm does not provide sufficient versatility for the detection of multiple groups of DOAs
- As a development of previous research, a novel CE-FD instrument was constructed in co-operation with Omec OÜ and evaluated for the detection of different DOAs
    - A miniature Xe lamp provided the possibility to construct a portable CE with a detector peak excitation wavelength of 240 nm

    - The linearity and noise of the detector were determined, indicating sufficient capacity for samples with a wide range of analyte concentrations, suitable for real-life use
    - Five interchangeable filters were used to further increase selectivity and sensitivity for specific compounds
    - IDLs for AMP, METH, MDA, MDMA, MDEA, PMA, PMMA, COC, COET, THC, and CBD were determined.
    - The sensitivity of AMP and METH should be improved
- The novel portable CE-FD analyser was validated for 7 DOAs in OF
    - An analytical procedure was validated according to EMA guidelines with adequate results
    - The determined detection and quantification limits for MDA, MDMA, MDEA, COC, and COET met the most of the proposed cut-off levels
    - Methodology selectivity was thoroughly studied, showing only two possible interfering substances
- On-site testing of the CE-FD instrument and the newly developed procedure in co-operation with the Estonian Police and Border Guard was conducted at Weekend Festival Baltics in 2016–2018
    - A total of 110 OF samples from people suspected of being under the influence were collected during the Weekend Festival Baltic
    - All samples were analysed on-site for AMP, METH, MDA, MDMA, MDEA, COC, and COET, with no AMP, METH, or MDEA detected
    - MDMA was found in 68 cases (62%), MDA in 55 (50%), COC in 22 (20%), and COET in 13 (12%)
    - The results obtained with CE-FD were compared to HPLC-MS with an overall correlation of r^2 = 0.998 and all z-scores were <±2 for the quantitative results
    - Diagnostic performance was determined for MDA, MDMA, and COC, where SE was 100% in all cases, SP ranged from 99.2 to 100%, and the DA was 99.6 to 100%.

The future perspectives of this research include the improvement of CE-FD sensitivity for AMP and METH detection, as well as further development of procedures for analysis of novel psychoactive substances. Moreover, the OF sample processing could be improved and automated, as well as be applicable to additional matrixes for DOA detection. The possible use of CE-C^4D for biomarker analysis is an interesting additional benefit.

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Abstract
Analysis of Psychoactive Compounds in Oral Fluid by Capillary Electrophoresis

Psychoactive substances produce a mind changing effect and cover a wide range of compounds including endogenously occurring γ-hydroxybutyric acid (GHB), mushrooms containing ibotenic acid (IBO) and psilocybin (PY), plant-based compounds such as cocaine (COC), and synthetic substances such as amphetamine (AMP), methamphetamine (METH), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymeth-amphetamine (MDMA), and 3,4-methylenedioxy-N-ethylamphetamine (MDEA). The use of these compounds may be therapeutic in some forms and quantities as prescribed medicine, but can also be abused, leading to health and safety risks.

Drugs of abuse (DOAs) have gained popularity with the spread of modern infrastructure and in 2017 there were 29 million users of amphetamines and prescription stimulants, 21 million ecstasy users, and 18 million cocaine users worldwide according to the World Health Organization Drug Report, 2019. This type of drug use has adverse health consequences that put users at higher risks of Hepatitis C, HIV, and other infectious disease. Moreover, the influence of drugs may be a public hazard if it results in drugged driving, violence, and accidents in safety-critical industries.

Undoubtedly, the prevention of accidents and increased public safety is desirable. Therefore, it is important to detect if a person is currently under the influence of drug on-site and rapidly without the need for complex sample collection and long transportation and analysis times. The sample should be representative of the current impairment, which can be achieved by using oral fluid (OF) due to its non-invasive collection and good reflection of current intoxication. Currently the most widely used methods for on-site OF testing are the immunoassay kits, which contain an antibody that reacts to a certain class of molecule, providing a qualitative result. Unfortunately, the selectivity of these methods is poor as cross reactions produce many false positives, leading to further testing that requires additional time. Use of a separation technique, namely capillary electrophoresis (CE), provides a selective alternative for analysis. All DOAs can be separated, detected, and quantified individually, and the CE apparatus is easily miniaturized, providing portable instruments with a variety of detection modes and possible automation.

This study developed and validated novel analysis procedures to enable on-site determination of psychoactive compounds in OF. First, GHB analysis was optimized and validated using simultaneous C^4D-UV absorbance detection. The proposed methodology was further advanced for possible GHB, IBO, and PY determination by CE-C^4D. Both analysis procedures were tested using OF samples with increased GHB concentrations from wine drinking and mushroom extract spiking. Analysis of ecstasy and two related derivates was achieved using CE with a LED-based fluorescence detector. The methodology was validated and proven to be suitable for real OF samples. Unfortunately, the excitation wavelength did not provide suitable selectivity to detect additional DOA groups.

Finally, a novel CE fluorescence detector (FD) instrument based on a miniature xenon lamp that provided UVC range excitation with maximum λ_{ex} = 240 nm was constructed in co-operation with OÜ Omec (Estonia). The instrument was validated and possible DOAs for further analysis were established. The instrument provided detection for AMP, METH,

MDA, MDMA, MDEA, PMA, PMMA, COC, COET, and cannabis. The OF validation was conducted for AMP, METH, MDA, MDMA, MDEA, COC and COET. In co-operation with the Estonian Police and Border Guard Board, real samples from Weekend Festival Baltics were collected and analysed on-site from people suspected of being under the influence of drugs. Between 2016 and 2018, 110 real OF samples were collected and tested for AMP, METH, MDA, MDMA, MDEA, COC, and COET. Unfortunately, the detection limits for AMP and METH were too high for detection and no positive samples for MDEA were determined. A total of 81 positive samples were analysed and the results obtained from CE-FD were compared to classical high-pressure liquid chromatography mass-spectrometry results, showing a good correlation of r^2 = 0.998 for the quantitative results. All z-scores were ≤ ±2 as well. Moreover, the instrumental statistics proved that the novel analyser was adequate for on-site testing of DOA in OF.

In conclusion, this research indicates that there is a need for future development of novel analysers and methodologies for the determination of psychoactive substances. The results obtained are promising for the further development of CE-FD instrumentation to achieve improved sensitivity for AMP and METH and for analysis of additional substances.

Lühikokkuvõte
Psühhoaktiivsete ühendite analüüs süljes kapillaarelektroforeesi meetodil

Psühhoaktiivsete ühendite tarvitamine mõjutab inimeste üldist võimet tajuda ennast ning ümbritsevat maailma. Selliste ainete hulgas on lai valik erinevatest klassidest ühendeid, nagu endogeenselt esinev γ-hüdroksübutüürhape (GHB), erinevates seeneliikides leiduvad iboteenhape (IBO) või psilotsübiin (PY), taimne kokaiin (COC), aga ka sünteetilised ained amfetamiin (AMP), metamfetamiin (METH), 3,4-metüleendioksüamfetamiin (MDA), 3,4-metüleendioksümetamfetamiin (MDMA) ja 3,4-metüleendioksü-N-etüülamfetamiin (MDEA). Nende kasutamine retseptiravimitena või meelelahutuslikel eesmärkidel omab teatud terapeutilist mõju, kuid väärkasutamise korral juhuslikes doosides võivad nad kaasa tuua terviseprobleeme ning ohtlikke olukordi.

Maailma Terviseorganisatsiooni 2019. aasta uimastiaruande kohaselt on psühhotroopsete ühendite väärkasutus oma populaarsuse saavutanud tänapäevase infrastruktuuri vahendusel. 2017. aastal oli kogu maailmas 29 miljonit mitmesuguste ergutite (retseptiravimid ja amfetamiin) kasutajat, 21 miljonit *ecstasy* kasutajat ja 18 miljonit kokaiini tarvitajat. Selle tulemuseks oli inimeste tervisliku seisundi halvenemine, aga ka haiguste nagu C-hepatiit ja HIV levik. Lisaks võib psühhotroopsete ainete tarvitamine põhjustada täiendavaid ohuallikaid avalikkusele: sõiduki narkojoobes juhtimine, vägivald ja õnnetused täpsust nõudvatel tööaladel.

Kahtlemata on ühiskonna turvatunde huvides vajalik ennetada ohtlikke olukordi. Seetõttu on oluline tuvastada, kas inimene on hetkel psühhoaktiivsete ainete mõju all. Kiireks reageerimiseks on vaja määrata narkojoovet sündmuskohas kiiresti, ilma keeruka proovivõtmise ning pika transpordi- ja analüüsiajata. Kasutatav proov peaks kajastama inimese joovet selle võtmise hetkel. Käesolevas töös kasutati sellise analüüsi jaoks süljeproovi, mille võtmine, erinevalt vereanalüüsidest, ei ole invasiivne ning mis annab ülevaate narkojoobest mõõtmise hetkel, mida ei võimalda uriinianalüüs. Käesoleval ajal kasutatakse testimiseks kõige sagedamini immunoteste, mille puhul antikehad reageerivad teatud tüüpi molekulidega, andes kvalitatiivse tulemuse. Kahjuks on selliste meetodite selektiivsus halb, kuna tuvastatakse ainult ühendite rühma, mitte konkreetseid molekule. Tekkivad ristreaktsioonid annavad palju valepositiivseid tulemusi, nõudes täiendavaid analüüse, mis põhjustavad täiendavat raha- ja ajakulu. Kapillaarelektroforeesi (CE) kasutamine on sobiv alternatiiv parandamaks selektiivsust, kuna sel juhul kõik psühhotroopsed ühendid lahutatakse, tuvastatakse ja kvantifitseeritakse individuaalselt. Lisaks on CE seadmed hõlpsasti miniaturiseeritavad kaasaskantavateks instrumentideks, mida on võimalik kasutada koos erinevate detektoritega ning vajadusel automatiseerida.

Käesoleva töö eesmärk oli välja töötada ja valideerida uusi metoodikaid psühhoaktiivsete ühendite kohapealseks määramiseks süljest kapillaarelektroforeesi meetodil nii olemasolevatel kui ka uutel väljatöötatud instrumentidel. Esiteks optimeeriti ja valideeriti GHB analüüs, kasutades samaaegselt C^4D ja UV neeldumise detektoreid. Loodud metoodikat arendati tuvastamaks süljest CE-C^4D abil samaaegselt GHB, IBO ja PY. Metoodikate optimeerimisel ja robustsuse hindamisel kasutati katsete planeerimise meetodeid, et saada täpsemad tulemused väiksemate katsete arvuga. Mõlemat analüüsiprotseduuri testiti ka süljeproovidega, milles oli veinijoomisest tingituna suurenenud GHB kontsentratsioon või oli lisatud seenekstrakte IBO ja PYga. Järgnevalt töötati välja ning valideeriti metoodika MDMA ehk *ecstasy* ja selle kahe derivaadi (MDA,

MDEA) analüüsiks LED ergastusega fluorestsentsdetektoriga CE meetodil. Sülje proovide kogumiseks ja puhastamiseks kasutati Salivette® koguteid, mis tagasid kiire ja lihtsa proovi eeltöötluse. Metoodika valideeriti vastavalt Euroopa Meditsiiniagentuuri bioanalüütiliste metoodikate valideerimise eeskirjale ja tõestati selle sobivus reaalsete süljeproovide analüüsiks. Paraku ei võimaldanud kasutatud ergastuslainepikkus teiste psühhotroopsete ainete tuvastamist.

Koostöös OÜ-ga Omec (Eesti) arendati uudne CE instrument, mille fluorestsentsdetektor (FD) põhines miniatuursel ksenoonlambil, andes maksimaalse ergastuslainepikkusega 240 nm. Hinnati instrumendi töökindlust ning detektori lineaarsust ja müra. Seade võimaldas tuvastada ühendeid AMP, METH, MDA, MDMA, MDEA, para-metoksüamfetamiin (PMA), para-metoksümetamfetamiin (PMMA), COC, kokaetüeen (COET), kannabidiool (CBD) ja tetrahüdokannabinool (THC). Edasine analüüsimetoodika arendamine hõlmas ainult enimlevinud psühhotroopseid ühendeid ja süljemaatriksis valideerimine viidi läbi ainult AMP, METH, MDA, MDMA, MDEA, COC ja COET jaoks. Saadud määramispiirid süljes vastasid rahvusvaheliselt tunnustatud piirväärtustele, välja arvatud AMP ja METH korral.

Koostöös Politsei- ja Piirivalveametiga koguti üritusel *Weekend Festival Baltic* reaalseid proove inimestelt, keda kahtlustati narkojoobes. Ajavahemikul 2016 kuni 2018 koguti ja analüüsiti kohapeal 110st proovist AMP, METH, MDA, MDMA, MDEA, COC ja COETi sisaldus. AMPi ja METHi avastamispiirid süljes olid kahjuks võimaliku tuvastamise jaoks liiga kõrged ning MDEA-positiivseid proove ei leitud. Kokku leiti 81 proovis vähemalt üks narkootiline ühend. CE-FD metoodikaga saadud tulemusi võrreldi klassikalise kõrgsurvevedelikkromatograafia-massispektromeetria tulemustega, mis andis kvantitatiivsete tulemuste korrelatsiooni r^2 = 0,998. Lisaks määrati instrumendi tehnilised parameetrid hindamaks analüüsi selektiivust, spetsiifilisust ning analüütilist täpsust. Kõik saadud tulemused jäid vahemikku 99 - 100% ja tõestasid, et loodud uudne analüsaator on töökindel ning täpne narkootiliste ühendite määramiseks süljes sündmuskohal.

Kokkuvõtteks demonstreerivad saadud tulemused, et töös välja töötatud CE metoodikad on nii juhtivus, UV-neelduvus- kui ka fluorestsentsdetektoreid kasutades väga sobivad psühhoaktiivsete ühendite määramiseks süljes. Lisaks on välja arendatud portatiivne CE-FD instrument, mis on sobiv narkojoobe tuvastamiseks sündmuskohal, andes kvantitatiivseid tulemusi tarvitatud ühendite kohta 15 min jooksul. Saadud tulemused on paljulubavad CE-FD instrumendi edasiarendamiseks, saavutamaks paremat tundlikkust AMP ja METH jaoks. Samuti on võimalik täiendavalt lisada uusi analüüsimetoodikaid lisanduvate psühhotroopsete ainete tuvastamiseks.

Appendix 1

Publication I

J. Mazina, **P. Saar-Reismaa**, M. Kulp, L. Poryvkina, M. Kaljurand, M.Vaher, Determination of γ-hydroxybutyric acid in saliva by capillary electrophoresis coupled with contactless conductivity and indirect UV absorbance detectors, Electrophoresis. 36 (2015) 3042–3049.

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Research Article

Determination of γ-hydroxybutyric acid in saliva by capillary electrophoresis coupled with contactless conductivity and indirect UV absorbance detectors

The aim of the current study was to optimise and validate the methodology for determination of γ-hydroxybutyric acid (GHB) in saliva by CE combined with a contactless conductivity detector (C^4D) and indirect UV absorbance detection (λ_{ABS} = 210 nm). The optimized BGE, consisting of 8.5 mM maleic acid, 17 mM arginine, 255 μM cetyltrimethylammonium bromide (CTAB), and 15% acetonitrile, was evaluated for the separation of GHB in saliva within 6 min. The performance characteristics of the CE-C^4D-indirect UV methodology was validated. The instrument detection and quantification limits were 0.49 and 1.6 mg/L for C^4D, and 5.1 mg/L and 17.0 mg/L for indirect UV, respectively. The linearity was obtained over the range from 2.5 to 400 mg/L for C^4D and from 12.5 to 400 mg/L for indirect UV. The interday precisions were within 2.3–5.7% and intraday precisions were within 1.6–9.0% for C^4D as well as 2.1–9.3%, 5.6–10.1% for indirect UV in spiked saliva, respectively. The recoveries were within 87.2–104.4%. The matrix effects were +53.2% for small concentrations up to 25 mg/L for C^4D and +23.6% for concentrations up to 75 for mg/L for indirect UV detection. No matrix effects were observed for higher concentration levels. In conclusion, CE-C4D-indirect UV can offer a rapid, accurate, sensitive, and definitive method for the determination of GHB abuse in saliva samples as a forensic screening tool.

Keywords:
CE / Contactless conductivity detection / γ-Hydroxybutyric acid / Indirect UV absorbance detection / Saliva
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1 Introduction

A global epidemic of drug abuse and addiction can be compared to the "Plague," encountering millions of victims every year. One of the well-known and popular drugs of abuse among teens and young adults is γ-hydroxybutyric acid (GHB). GHB can be used as a recreational drug or as a drug facilitating sexual assault, robbery, money extortion, and other crimes. Drug-facilitated crime implies that a victim is unable to resist assault due to unconsciousness caused by the influence of substances. GHB is also known as a central nervous system depressant and hypnotic. It occurs naturally in the human body at low levels (saliva 0.15–3.33 mg/L [1], urine 0.64–4.20 mg/L [2], serum 0.62–3.24 mg/L [2]) as a metabolite of GABA (γ-aminobutyric acid) [3]. As an illegal drug, GHB is often abused at bars, "raves" and clubs. It can be consumed orally as a solid white powder or added to different alcoholic beverages. The latter is the predominant method of consumption. GHB analog such as γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) can also be used instead of GHB. The analogs are converted to GHB in the human body (1,4-BD to GBL and GBL then to GHB). Therefore, the detection of

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Abbreviations: ABS, absorbance; **C^4D**, contactless conductivity detector; **GHB**, γ-hydroxybutyric acid; **IDL**, instrument detection limit; **IQL**, instrument quantification limit; **IS**, internal standard; **LLOQ**, lower LOQ; **ME**, matrix effect; **MLOQ**, medium LOQ; **PMPA**, pinacolyl methylphosphonic acid; **ULOQ**, upper LOQ; **URT**, upper respiratory tract; **UV**, ultraviolet

Colour Online: See the article online to view Figs. 1 and 2 in colour.

GHB is of utmost importance and reflects illegal drug abuse. Although, the different and, even more severe, toxic effects of GHB analogs might be taken into account.

The GHB dose drastically varies from 0.5 g for a slight and up to 4 g for strong or severe one. In the case of regular drink volumes, 100–150 mL, the GHB concentration can be between 3 and 40 g/L in liquids. It is demonstrated that the effects of GHB last for 3–6 h, starting 15–30 min after intake. Nevertheless, GHB is rapidly eliminated from the body (half-life of 10–53 min) [4] and, therefore, the detection window is rather short, that is, approximately 3 h in oral fluids, 5 h in blood samples and less than 12 h in urine samples [4, 5]. Thus, samples should be collected as quickly as possible after intake. It is especially critical for the detection of GHB abuse using oral samples and blood.

Despite the illicit consumption of chemically synthesized compounds, GHB and GBL are fermentation by-products that occur naturally in different beverages such as soya as well as white and red wines. In contrast to the illicit consumption doses, the concentration levels of GHB and GBL are very low. The highest content found in red wines is from 4.1 to 21.4 mg/L [6]. Therefore, wine drinking can increase, to a minor extent, the endogenous GHB content in biological samples. But, the levels of GHB after wine drinking are not comparable to the illicit drug doses, which are several orders higher.

Furthermore, GHB is a well-known prescription drug for the treatment of narcolepsy, alcoholic abuse and catalepsy [7]. Moreover, GHB is also used by body builders as a muscle-growth enhancer [8]. Therefore, the accurate endogenous and exogenous GHB level interpretation in biological samples is critical for point-of-care and drug-abuse testing. The following cut-off limits are proposed to distinguish endogenous from exogenous GHB in clinical samples: 4–10 mg/L in urine [9] and 2–5 mg/L in blood [2].

The above facts make the analysis of GHB challenging in biological samples. Undoubtedly, the accurate and sensitive analysis of the small polar organic molecule with weak UV-absorbing properties is a complex feature for toxicological sample investigation. The main methods for the determination of GHB in biological samples are GC-FID, GC-MS, LC-MS-MS [10], and HPLC-MS/MS [3]. These methods are generally time consuming and require complex sample preparation including derivatization. However, there are a limited number of studies investigating the application of CE for the determination of either endogenous or exogenous GHB levels in biological samples. Most publications using CE as a separation method deal with urine, serum, or blood samples [11], but neither of them utilize saliva samples. Biological samples have mainly been investigated by CE coupled with indirect UV absorbance detection or MS [12], and few of them by contactless conductivity detector (C^4D). Nevertheless, the only reported research by Hauser et al. [13] has implied CE-C^4D for the detection of GHB, but only in urine and blood serum samples. Therefore, the current study implements CE-C^4D with sequential indirect UV detection for the first time in the determination of GHB in saliva samples.

Different hyphenated techniques with different methodologies are proposed for the determination and quantification of GHB in urine [13, 14], blood [14], and saliva [1, 15]. Certainly, one of the suitable methods for the detection of weakly or nonabsorbing small organic and inorganic molecules is CE coupled with a C^4D. The main advantage of CE-C^4D is the miniaturization possibility of the instrument and, therefore, the possibility to conduct measurements on the scene of the crime. CE-C^4D was successfully applied for the detection of simple organic and inorganic compounds in oral fluids, blood serum, urine, exhaled breath condensate, and saliva by Kubáň et al. [16, 17]. Moreover, saliva is a perspective sample matrix containing thousands of compounds that can be suggested as markers for health disorders. Therefore, known markers can be used to monitor metabolism processes in organisms [18]. Despite the marker exploration, saliva is an excellent source for the determination drug of abuse. Saliva has the merit of being easy to collect.

The purpose of the present study was to demonstrate the use of saliva as a sample in the screening of GHB abuse by CE. The feasibility study of the sequential dual detection system using conductivity and indirect UV for biological sample analysis was performed and evaluated. Both detection modes, with the optimized methodology, were validated according to the European Guideline on bioanalytical method validation (EMA/275542/2014) [19].

2 Materials and methods

2.1 Standards and chemicals

All chemicals were analytical grade. Sodium γ-hydroxybutyrate (GHB) powder and a solution of 1 mg/mL GHB salt in methanol were purchased from Lipomed AG (Germany). ACN (HPLC grade), maleic acid, L-arginine (Arg), CTAB, pinacolyl methylphosphonic acid (PMPA), calcium chloride, potassium nitrate, potassium nitrite, magnesium sulfate, sodium sulfite, sodium thiocyanate, disodium hydrogen phosphate, sodium hydroxide, and succinic, tartaric, citric, lactic acid, and glutamic acid were obtained from Sigma-Aldrich (Germany). Ultrapure water (Milli-Q) (resistivity ≥18 MΩcm) was obtained using a Milli-Q integral water purification system (Merck KGaA, Germany). Saliva samples were collected from six volunteers (both male and female, age ranging from 25 to 70). Two red wines ''Le Grand Noir" (85% Cabernet/15% of Shiraz, 13% vol, France, 2013) and "Vina Maipo Vitral" (Cabernet Sauvignon, 13.5% vol, Valle del Maipo, Chile, 2011) were purchased at a local store in Estonia.

2.2 Separation procedure

The in-house built portable CE device equipped with a C^4D [20] (Chemistry Department, TUT, Estonia) and an Agilent 3D CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a DAD were combined in this study.

The conductivity detection cell was incorporated into the Agilent 3D CE capillary cassette. The sample injection, CE analysis was conducted inside Agilent 3D CE instrument. The conductivity signal was registered by in-house built hardware for CE-C^4D, absorbance signal by Agilent 3D CE instrument hardware, respectively. An uncoated fused silica capillary (Agilent Technologies, USA) with a total length of 65 cm, effective length of 45 cm to the C^4D and 56.5 cm to the DAD (id 50 μm and od 360 μm) was used to separate the analyte of interest. The applied voltage was –19 kV. The C^4D frequency was set to 150 kHz. The indirect UV absorbance was recorded at $\lambda_{ABS} = 210$ nm.

The proposed methodology for GHB analysis in urine and serum samples by Hauser et al. [13] was modified and optimized for saliva samples and the current system. The stock BGE solution consisted of 10 mM maleic acid, 20 mM Arg, and 300 μM CTAB adjusted to pH 7.35. As an organic modifier, 15% acetonitrile was added to the stock BGE, with slight adjustment of the final pH to 7.65 and decreasing the BGE components content to 8.5 mM maleic acid, 17 mM Arg, and 255 μM CTAB. The final BGE was ultrasonicated (Bandelin electronic, Germany) for 10 min, degassed by 10% at 30°C and then filtered through a 20 μm cellulose filter (Sartorius Stedim Biotech, Germany). Prior to CE analysis, the capillary was activated and washed every day with 1 mM NaOH for 10 min, Milli-Q water for 10 min and BGE for 15 min. The injection was conducted hydrodynamically for 10 s at 35 mbar. Between the experiments, the capillary was rinsed for 3 min with BGE. Moreover, the capillary was washed with methanol for 10 min, Milli-Q water for 10 min and BGE for 15 min, at least after 20 analyses in order to prevent adsorption on the capillary surface.

2.3 Sample collection and preparation

The GHB salt was dissolved in an acetonitrile/Milli-Q solution (1:1) (5 g/L) and further dilutions were prepared in acetonitrile. Saliva samples were collected prior to analyses and stored in a freezer (–18°C) until used (but not longer than 6 months). A minimum of 400 μL of saliva sample was collected in a 1.5 mL Eppendorf tube. Then, 2 μL of 10 mM PMPA (final 100 μM) in water as internal standard (IS) and 148.5 μL of acetonitrile added to 49.5 μL of the saliva sample. The sample was thoroughly mixed and the supernatant was separated by centrifugation for 10 min at a maximum speed of 6000 rpm in a mini-centrifuge for 1.5 mL Eppendorf tubes (Sarstedt AG&Co, Germany). The supernatant was transferred to a new Eppendorf tube and used for further analysis by CE.

2.4 CE data preprocessing

The reproducibility in terms of the migration time is of importance in CE in order to identify the analyte of interest. Therefore, electropherograms were corrected using the method proposed by Zhang and Chen prior to the analysis of the CE data [21]. Two internal substance components, such as exogenous IS (PMPA) and endogenous IS (chloride ion), were used for correction coefficient evaluation for each electropherogram against the standard, using the following equation:

$$\gamma = \frac{\frac{1}{\widehat{t_I}} - \frac{1}{\widehat{t_P}}}{\frac{1}{t_I} - \frac{1}{t_P}}. \quad (1)$$

The new migration time (t_x) was found using the calculated correction coefficient γ:

$$t_x = \left[\frac{1}{t_I} - \frac{1}{\gamma}\left(\frac{1}{\widehat{t_I}} - \frac{1}{\widehat{t_x}}\right)\right]^{-1}, \quad (2)$$

where γ is the correction coefficient, t_I and t_P are migration times of the exogenous and endogenous ISs, respectively, $\widehat{t_I}$and $\widehat{t_P}$ are migration times of PMPA and chloride ions, respectively, in the electropherogram under correction;t_x is the corrected migration time for corrected electropherogram and $\widehat{t_x}$is the migration time of the electropherogram under correction.

2.5 Validation

Validation of the CE methodology was carried out in concordance to the European Guideline on bioanalytical method validation (EMA/275542/2014) published by the European Medicine Agency in 2009 and adapted in 2012 [19]. This guideline sets the minimum performance parameters for small (MW $<$ 1000 Da), medium (1000 $<$ MW $<$ 10 000 Da), and large molecules (MW $>$ 10 000 Da) that are to be evaluated and set their acceptance criteria. As the GHB (*Mr* = 104.1 g/mol) is referred to as a small molecule, the following CE performance parameters were evaluated: selectivity, carry-over effect, lower LOQ (LLOQ), calibration curve, accuracy, precision, matrix effect (ME), and efficacy/extraction recovery.

2.5.1 Design of experiments

The design of experiments for the robustness study was developed using the Box–Behnken experimental design [22]. Small variations in the BGE composition were investigated in the robustness study using response surface regression analysis [23]. The changes represent typical errors that can occur in daily analyses. The following parameter ranges were used to conduct a Box–Behnken design at three levels: maleic acid 10 ± 1 mM, arginine 20 ± 2 mM, and CTAB 300 ± 25 μM. The levels were coded as –1 for the minimum, 0 for nominal and 1 for the maximum change. The BGE pH remained the same and was always adjusted to 7.35 prior to analysis, using the appropriate amount of aqueous sodium hydroxide solution. Six independent replicates were conducted for each design order.

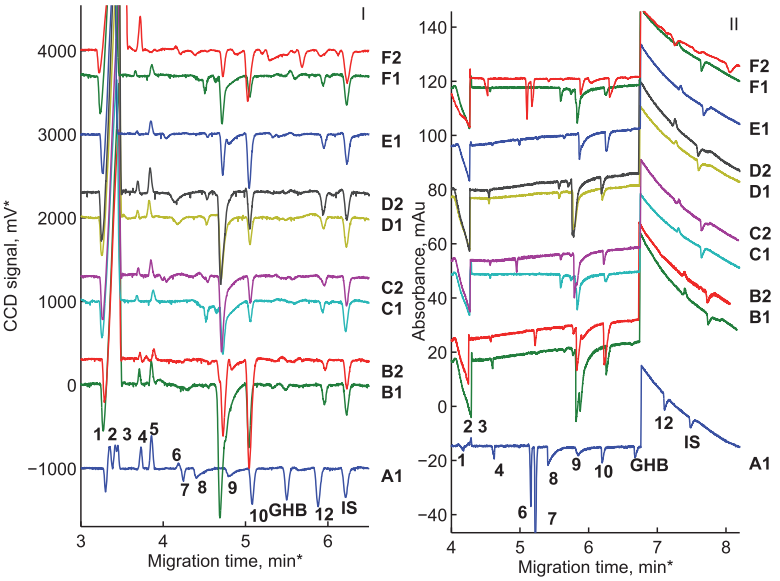

Figure 1. Various profiles of saliva samples: (I) CE-conductivity detection; (II) Indirect absorbance detection (λ_{ABS} = 210 nm). Analytes: 1. Chloride (5 mg/L); 2. Nitrite (10 mg/L); 3. Nitrate (10 mg/L); 4. Sulfate (10 mg/L) and sulfite (5 mg/L); 5. Thiocyanate (40 mg/L); 6. Tartrate (120 mg/L); 7. Succinate (80 mg/L); 8. Citrate (–120 mg/L); 9. Hydrogen phosphate (20 mg/L); 10. Lactate (20 mg/L); 11. GHB (20 mg/L); 12. Glutamate (20 mg/L); 13. PMPA (IS).
Applying the working conditions. BGE: 8.5 mM maleic acid, 17 mM Arg, and 255 μM CTAB, 15% ACN, pH = 7.65; capillary length, 65.0 cm; temperature, 21°C; voltage, –19 kV; hydrodynamic injection, 35 mbar for 10 s; contactless conductivity detection, frequency 150 Hz; absorbance detection, λ_{ABS} = 210 nm; sample acetonitrile ratio, 1:3.

2.6 Software

Data were processed with in-lab-made software for peak acquisition (TUT, Chemistry Department, Estonia), Chemstation (Agilent Technologies, Waldbronn, Germany), and PLS toolbox 6.2 (Eigenvector Research) in Matlab R2011b.

3 Results and discussion

3.1 CE methodology optimization

The preliminary study was conducted using the proposed methodology by Hauser et al. [13]. Unfortunately, the time of analysis was more than 10 min and, therefore, the methodology was not suitable for quick onsite analysis of saliva samples. Therefore, it was decided that the methodology should be optimized for efficient and rapid GHB separation in saliva samples within 10 min. At the BGE pH of 7.65, GHB (p*K*a is 4.71) was negatively charged and migrated as an anion in BGE. Moreover, the high pH value assured that GHB did not convert to the lactone form GBL.

It was found out that the concentration of CTAB, as a cationic modifier, and ACN, as an organic modifier, drastically affected the migration time, resolution (*Rs*), and efficiency in terms of theoretical plates (N) of anions naturally occurring in saliva. First, different concentrations of CTAB, such as 30, 100, and 300 μM, were tested. Upon addition of CTAB to the BGE (ACN0%), there was a rapid transition from normal (cathodic) to reversed (anodic) EOF. The increase of CTAB concentration led to the decrease of EOF migration time from 18 min to 6.2 min, indicating the complete EOF reversal in the BGE. A stable baseline and a good resolution (*Rs* >1.2) between GHB and neighbor peaks, as well as a short separation time, were obtained with 300 μM CTAB in the BGE. Second, the voltage was optimized (–5, –10, –15, and –19 kV). The separation at –19 kV was assumed to be the most suitable, as the separation of GHB in spiked saliva was achieved in 3.6 min (sample: ACN 1:1, BGE + ACN0%) (S – Fig. 1I–A3). The sample preparation was optimized in addition to the optimization of BGE and CE conditions. Whole saliva proteins were precipitated using ACN in ratios of 1:1, 1:2, and 1:3 (sample/acetonitrile). The best resolution, a stable baseline and the most effective precipitation of proteins in saliva were achieved with the ratio of 1:3. Moreover, adding ACN to the saliva sample that contained a high concentration of inorganic ions (chloride presents at about 5 mM in resting and up to 70 mM in stimulated saliva [24]) increased the efficiency of CE separation via a "ACN-salt stacking" effect [25]. The stacking mechanism was similar to the transient isotacophoresis. The salts, due to the limited solubility in ACN, moved rapidly with some water in the front, creating the region with a low field strength and leaving behind a more concentrated zone of ACN (the region with a high field strength), where organic anions were moving faster, slowing down at the region of a low field strength. Furthermore, ACN, as an organic modifier, could affect the capillary surface in the BGE, changing its viscosity by inducing solvent–solute and solute–solute interactions, as well as by modifying the p*K*a of ionization of the silanol group, solute molecules, and analytes—thus resulting in the alteration of electroosmotic and electrophoretic mobility. The addition of ACN to the BGE increased the sensitivity and improved the resolution (Supporting Information

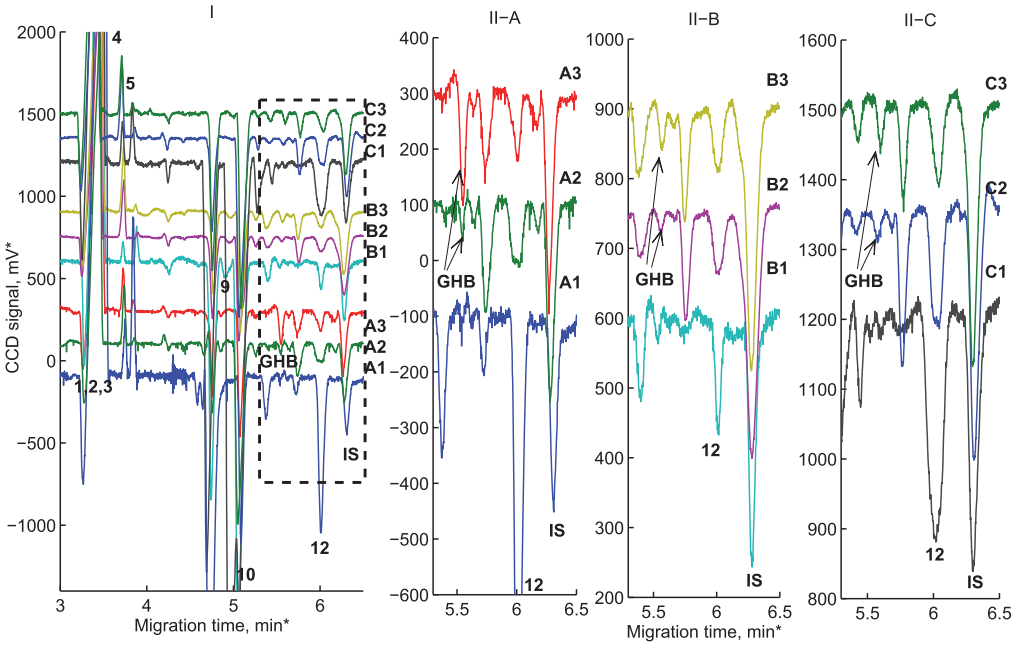

Figure 2. (I) CE-C^{4}D electropherograms of saliva samples after red wine drinking; (II) Zoomed region of electropherogram from 5.3 to 6.5 min: (A1) Blank saliva of volunteer #5; (A2) Volunteer's #5 saliva after drinking of 40 cl of "Le Grand Noir"; (A3) Volunteer's #5 saliva spiked with 20 mg/L GHB (#11) after drinking of 40 cl of "Le Grand Noir"; (B1) Blank saliva of volunteer #6; (B2) Volunteer's #6 saliva after drinking of 30 cl of "Vina Maipo Vitral"; (B3) Volunteer's #6 saliva spiked with 10 mg/L GHB (#11) after drinking of 30 cl "Vina Maipo Vitral"; (C1) Blank saliva of volunteer #5; (C2) Volunteer's #5 saliva after drinking of 30 cl of "Vina Maipo Vitral"; (C3) Volunteer's #5 saliva spiked with 10 mg/L GHB (#11) after drinking of 30 cl of "Vina Maipo Vitral."

Fig. 1, Fig. 1II). The best results were achieved with the BGE containing 15% of ACN. N_{GHB} was increased from 60 000 (ACN0%) to 120 000 (ACN10%) and, finally, to 255 000 (ACN15%) plates per meter. However, because of the added ACN, the GHB migration time increased to 5.5 min (ACN15%) (CV% uncorrected 7.3–9.4%) due to a lower EOF velocity that was caused by a lower dielectric constant and a higher viscosity. Inspite of that, the time required for analysis was within a 10-min timeframe, and the sensitivity improvement (fourfold) was of more importance.

3.2 Validation of CE methodology

3.2.1 Identification

Saliva consists of 99% water, containing inorganic ions (sodium, chloride, nitrate, nitrite, phosphate, and others) and proteins (enzymes, immunoglobulins, and others). The presence of different substances reflects the metabolism processes in the body and, subsequently, shows the body's health and wellbeing. For example, it was found that physical activity changes the sodium and lactate ion contents [24]. Moreover, saliva can indicate medicament or illegal-drug consumption.

Prior to knowledge of possible interferences, the identity of GHB was studied in different samples. The identity study was based on selectivity/specificity, showing the ability of the methodology to distinguish the analyte of interest, GHB, and IS, PMPA (t_{mig} = 6.3 min), from the endogenous matrix components under the optimized methodology. As the composition of saliva varies from person to person, six samples were analysed for possible interferences. Moreover, the saliva variations under different effects were monitored, including intensive physical training, upper respiratory tract infection (URT) with administration of Ospamox (1000 mg of Amoxicillin, Sandoz GmbH, Austria), long-term tobacco smoking, drinking of orange juice, and the consumption of two red wines. Moreover, the possible anions, such as chloride, nitrate, nitrite, sulfate, sulfite, thiocyanate, succinate, tartrate, citrate, hydrogen phosphate, lactate, and glutamate, that can be found in saliva naturally or occur after the administration of different products (juice, coffee, wine, etc.) were identified. Possible comigration with GHB was studied.All samples were suggested as true-negative samples, except for the red wine, in which GHB can be naturally found.

Table 1. CE-CCD and indirect UV absorbance performance characteristics

	Range, mg/L	*n*, *Tn**	R^2	Equation ($Area_{GHB}/Area_{IS}$)100%	IDL (IQL)	ILLOQ (IULOQ)	LoD (LoQ)	Precision (interday/intraday)	Accuracy (recovery), %*	ME %
Conductivity detection										
GHB in water	2.5–400	$n = 6$; $Tn = 36$	0.9906	$y = (3.63 \pm 0.07)x + (-34.5 \pm 10.0)$	0.62	2.5	2.5	2.5: 1.1%/1.6% 7.5: 0.9%/4.3%	2.5: 117.1 ± 1.7 7.5: 100.2 ± 4.8	
GHB in water (small)	2.5–25	$n = 6$; $Tn = 36$	0.9959	$y = (1.97 \pm 0.03)x + (1.13 \pm 0.38)$	(2.1)	(400)	(8.3)	100: 2.2%/6.6% 300: 2.0%/1.5%	100: 106.5 ± 6.4 300: 101.1 ± 1.5	
Saliva	2.5–400	$n = 6$; $Tn = 36$	0.9945	$y = (3.62 \pm 0.03)x + (-12.77 \pm 4.1)$	0.49	2.50	2.0	2.5: 2.6%/5.9% 7.5: 2.4%/3.4%	2.5: 100.1 ± 3.4 7.5: 95.7 ± 4.2	–0.3
Saliva (small)	2.5–25	$n = 6$; $Tn = 36$	0.9923	$y = (3.02 \pm 0.35)x + (6.90 \pm 0.35)$	(1.6)	(400)	(6.5)	00: 5.7%/9.0% 1300: 2.3%/1.6%	100: 87.9 ± 5.6 300: 102.1 ± 2.4	+53.2
UV absorbance-indirect detection (λ_{ABS} = 210 nm)										
GHB in water	12.5–400	$n = 6$; $Tn = 36$	0.9907	$y = (3.78 \pm 0.08)x + (-44.6 \pm 11.5)$	3.7	12.5	14.8	12.5: 1.7%/2.2% 35: 3.0%/5.9%	12.5: 111.2 ± 1.8 35: 100.3 ± 2.1	
GHB in water (small)	12.5–75	$n = 6$; $Tn = 36$	0.9884	$y = (1.95 \pm 0.06)x + (-6.7 \pm 2.6)$	(12.4)	(400)	(49.7)	100: 1.1%/14.6% 400: 4.8%/3.5%	100: 97.6 ± 12.5 400: 100.3 ± 3.5	
Saliva	12.5–400	$n = 6$; $Tn = 36$	0.9956	$y = (3.86 \pm 0.07)x + (-47.2 \pm 11.6)$	5.1	12.5	20.4	12.5: 4.4%/6.1%. 35: 2.1%/ 5.6%	12.5: 104.4 ± 5.5 35: 95.3 ± 1.9	+2.0
Saliva (small)	12.5–75	$n = 6$; $Tn = 36$	0.9926	$y = (2.41 \pm 0.07)x + (-7.9 \pm 2.2)$	(17.0)	(400)	(68.0)	100: 9.3%/10.1% 400: 2.5%/6.3%	100: 87.2 ± 8.0 400: 101.1 ± 2.5	+23.6

**n*, number of concentration levels and *Tn*, total number of measurements used for linear regression construction.

Figure 1 presents the electropherograms of Milli-Q water spiked with different anions found in saliva (A1) and whole saliva blanks (B1, D1, F1) in comparison to saliva after training (D2), long-term tobacco smoker saliva (E1), saliva of a volunteer with a URT infection (C1) and that after treatment (the fourth day of Ospamox administration) (C2) as well as saliva samples after juice (B2) and red wine consumption (F2). There was no comigration observed for the analyte of interest, GHB, with the tested substances under the optimized conditions. Moreover, consumption of 10 cl of red wine has not significantly increased the endogenous GHB content in saliva and remained below instrument detection limit (IDL). Therefore, it was concluded that the optimized system has successfully passed the identity study. Moreover, the system was successful for the codetection of thiocyanate, lactate, and glutamate by C^4D. Furthermore, BGE has proven to be suitable for sequential detection by conductivity and then indirect UV absorbance. It was observed that indirect UV absorbance detection was perfect for tartrate, succinate, citrate, hydrogen phosphate, lactate, glutamate, and GHB (Fig. 1II: A1). C^4D detection was not so sensitive to tartrate, succinate, citrate, or hydrogen phosphate. As saliva contains a very high concentration of chloride ions, it was impossible to separate chloride from nitrate and nitrite in real samples. Nevertheless, the effective separation of the latter ions was not the aim of the current study, but only an additional observed fact.

3.2.2 Limits of detection and quantification

The LOD and LOQ were evaluated in saliva by spiking GHB at different concentration levels in blank saliva: 0, 2.5, 5, 7.5, 10, 12.5, and 25 mg/L for C^4D and higher concentrations were needed for indirect UV absorbance: from 12.5 to 75 mg/L. At least six independent replicates for each concentration were conducted. The linearity was evaluated with higher concentrations up to 400 mg/L.

Linearity was found up to 400 mg/L for C^4D and indirect UV absorbance. The IDL was defined using a 95% prediction interval of the regression line [25]. The IDL was 0.49 mg/L and the instrument quantification limit (IQL) was 1.6 mg/L in saliva for C^4D. The IDL and IQL for indirect detection were lower, as expected, at 5.1 and 17 mg/L in saliva, respectively. Taking into account the sample preparation, the LOD equalled 2.0 mg/L and the LOQ 6.5 mg/L for C^4D and 20.4 mg/L and 68 mg/L for indirect UV absorbance in saliva, respectively.

Despite the fact that the IQL was sometimes lower than the lowest calibration level (for conductivity), the instrument lower LOQ (ILLOQ) was set to 2.5 mg/L and instrument upper quantification limit was set to 400 mg/L. It was found that the LOQ of 6.5 mg/L for the C^4D was suitable for accurate differentiation between exogenous and endogenous GHB levels in the case of a 10 mg/L cut-off limit. Unfortunately, the sensitivity of indirect UV absorbance was not enough, but could be used for the quantification of higher concentrations up to

Table 2. Box–Behnken design of experiments and response values for robustness study (*number of replicates* = 6)

Run order	Design order	[Maleic acid], mM	[Arginine], mM	[CTAB],μM	$R_{U1\text{-}GHB}$	$R_{GHB\text{-}U2}$	N_{GHB} (10^5)	$t_{R\,GHB}$, min
1	1	–1	–1	0	2.2	1.2	1.43	4.3
2	5	0	–1	–1	2.2	1.4	1.46	4.4
3	3	–1	0	1	2.1	1.8	1.19	4.5
4	11	1	0	–1	2.4	1.2	1.24	4.7
5	13	1	1	0	2.4	1.1	0.98	4.4
6	2	–1	0	–1	2.2	1.4	0.77	4.3
7	8	0	1	–1	2.5	1.2	1.15	4.5
8	10	1	–1	0	2.7	1.2	0.90	4.6
9	6	0	–1	1	2.1	1.3	0.69	4.6
10	9	0	1	1	1.6	1.3	0.48	4.5
11	12	1	0	1	1.6	1.7	0.41	4.5
12	4	–1	1	0	1.3	2.3	0.28	4.5
13	7	0	0	0	2.1	1.2	1.07	4.7

257 mg/L of GHB, which can be found in saliva samples after GHB consumption [4].

3.2.3 Accuracy and precision

A precision study was carried out to assure the reproducibility of the proposed methodology. The results were expressed as the coefficient of variation (CV%) and evaluated at four levels: LLOQ, 3 × LLOQ, MLOQ (medium LOQ, 30–50% from calibration range) and at 75% of the upper LOQ (ULOQ) or at the ULOQ for indirect UV absorbance. The interday precision was evaluated using six replicates of spiked blank saliva and Milli-Q for each concentration level and intraday precision was evaluated over 3 days, preparing the same concentration levels. The inter- and intraday precisions (CV%) were below the acceptance level of 15% for the tested concentrations and below 20% for the LLOQ. The interday precision was within 2.3–5.7% and intraday within 1.6–9.0% for C^4D and 2.1–9.3% or 5.6–10.1% for indirect UV in spiked saliva, respectively. The precision results showed good reproducibility and repeatability.

The accuracy was assessed for samples spiked with GHB before the sample preparation at four concentration levels: LLOQ, 3 x LLOQ, MLOQ (30–50% from calibration range) and at 75% of ULOQ or at ULOQ for indirect UV absorbance. The accuracy was expressed as the relative recovery (Rec%). Six replicates for each level were conducted over three different days. The accuracy was within ±20% of the nominal value at the LLOQ and within ±15% of the nominal value for the other concentration levels. The results for each level are presented in Table 1.

3.2.4 Matrix effect

The ME indicates the saliva matrix components effect on GHB signal intensity. The positive ME value emphasizes the enhancement of the signal and the negative one shows the suppression of the signal. The ME was evaluated along a range of concentrations using the slopes of calibration curve of GHB dissolved in water and in the saliva sample using the following equation [26]:

$$ME\% = \left(\frac{slope_{matrix}}{slope_{solvent}} - 1\right) \times 100\%. \quad (3)$$

The ME% in the range of ±20 (±15)% (within accepted intraday precision) was designated as showing no matrix effects, while MEs within ±20 (±15)–50% and higher than ±50% were assigned to a medium and strong ME, respectively [26].

A medium and strong MEs for the low concentration levels were observed for indirect UV absorbance (ME% of +23.6%) and for conductivity (ME% of +53.2%), respectively. Therefore, the concentration levels between 2.5–25 mg/L of GHB for C^4D must be evaluated using either the calibration curve constructed by the standard addition method or applying the correction factor to the traditional calibration curve, thus eliminating ME. No MEs (<15%) were observed for the higher concentration levels (Table 1).

3.2.5 Robustness study

Since only CE-C^4D was capable to differentiate the exogenous and endogenous GHB concentrations in saliva, the robustness study for CE-C^4D was conducted using a saliva sample matrix spiked at the cut-off level of 10 mg/L GHB. The design order was randomized and the run order of the experiments was conducted as shown in Table 2. The responses, such as migration time ($t_{R\,GHB}$) of GHB, the number of theoretical plates (N_{GHB}) and the two resolution values between undefined peaks migrated before and after GHB, U1-GHB ($R_{U1\text{-}GHB}$), and GHB-U2 ($R_{U2\text{-}GHB}$), were recorded and calculated (Table 2). It was found that N_{GHB} is significantly affected by concentration variations of [CTAB] (+1) and [arginine] (+1) and their interactions [maleic acid] x [CTAB] and [maleic acid] x [arginine] (p-value < 0.050). Therefore, CE-C^4D method is sensitive to small variations of BGE at GHB cut-off level and, therefore, extreme attention must be paid to correct BGE preparation.

3.3 Real sample analysis

GHB can be found in food and beverages, that is, in sherry as well as white and red wines. Red wine is a natural source of GHB, and the GHB concentration can be up to 21.4 mg/L (mean 12.6 mg/L) [6]. Therefore, two different red wines (red wine 1 and red wine 2) were chosen for the detection of GHB in saliva samples. The analysis of blank saliva was conducted prior to wine drinking. The GHB endogenous occurrence in saliva was investigated using 10 cl doses of red wine. Each dose was completed during 3–4 min and followed by the new one after sample collection. The saliva sample was collected after 2-min interval of every completed dose. Slight increase of GHB peak height was observed after drinking 30 and 40 cl of red wine. The experiment was finished within 25 min due to short half-life of GHB (10–53 min).

The identity of the GHB peak was further confirmed by spiking GHB into the same sample. It follows that minor quantities in human saliva can be unambiguously detected (Fig. 2). The quantification of GHB was not conducted, as it was below the IQL. But, the detection of the presence of GHB in red wine has proven the possibility to detect GHB in saliva based on CE with C^4D and indirect UV absorbance detection. The analysis of clinical samples after GHB consumption is to be conducted in further research.

4 Concluding remarks

In this paper, CE-C^4D–indirect absorbance was successfully applied for the determination of GHB in saliva samples. The validation results show that CE-C^4D has suitable sensitivity for the differentiation of endogenous and exogenous concentrations of GHB in saliva. The biggest advantages of the proposed methodology are the short time of analysis, sensitivity, and simple sample preparation that can be further automated and miniaturised for on-site confirmation analysis.

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The authors have declared no conflict of interest.

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Publication II

P. Saar-Reismaa, M.Vaher, M. Kaljurand, M. Kulp, J. Mazina-Šinkar, Simultaneous determination of γ-hydroxybutyric acid, ibotenic acid and psilocybin in saliva samples by capillary electrophoresis coupled with a contactless conductivity detector, Analytical Methods. 9 (2017) 3128–3133.

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Simultaneous determination of γ-hydroxybutyric acid, ibotenic acid and psilocybin in saliva samples by capillary electrophoresis coupled with a contactless conductivity detector†

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The aim of the study was to develop a methodology for the determination of γ-hydroxybutyric acid (GHB), ibotenic acid (IBO) and psilocybin (PY) abuse in human saliva. Capillary electrophoresis with a capacitively coupled contactless conductivity detector (CE-C^4D) was used with an optimized background electrolyte (BGE) consisting of 17.9 mM L-arginine (Arg), 9.6 mM succinic acid (Suc) and 0.0019% (w/v) hexadimethrine bromide (HDMB) with an adjusted pH of 7.3. Saliva samples were spiked with all three analytes of interest as well as the internal standard, before protein precipitation with acetonitrile (ACN) (1 : 2, v/v, saliva : ACN). The limits of detection (LOD) and quantification (LOQ) in the saliva were 1.5 and 5.0 mg L^{-1} for GHB, 2.1 and 7.9 mg L^{-1} for IBO and 3.6 and 12.0 mg L^{-1} for PY. The intraday precision varied from 0.7% to 8.6% and interday precision from 3.9% to 11.4% for all substances. The overall accuracy and combined recovery were from 95% to 123% and 98% to 103%, respectively. The present study demonstrates that the use of oral fluid as an alternative sample matrix which is easy and fast to collect and prepare, and in combination with a portable CE instrument built in-house, provides rapid and efficient determination of psychoactive substance intoxication.

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Introduction

Capillary electrophoresis (CE) has become a sustainable method for the determination of a wide range of substances, and is one of the most suitable methods for the detection of small polar molecules. CE has been shown to be useful for analysis of the bioactive constituents of natural plants[1,2] and environmental drug residues[3] and determination of intoxication or drug intake.[4] Recreational use of different psychoactive substances is a rising trend all over the world, where the altering of consciousness can be achieved by intentional consumption of a wide variety of plants or drugs, many of which are already illegal, though a vast majority are still legal.

Some of the compounds have a shorter half-life, making them harder to detect after administration, while still having major impacts on perception of reality, potentially leading to harm to oneself as well as others. One such example is γ-hydroxybutyric acid (GHB), which is a common illegal drug taken to achieve a recreational high, known more widely as a 'date rape' drug; it has a half-life of only 20–50 min.[5] In addition, hallucinogenic substances can be found in mushrooms, such as those containing psilocybin (PY), which has a half-life of 50 min,[6] or those containing neurotoxins such as ibotenic acid (IBO), which is also used as a psychoactive drug.[7] However, these substances can be lethal, especially if consumed by accident or in an unknown dosage.

The dosage for GHB varies from 1 g up to 10 g, and its range of effects extends from mild excitement to coma or even death.[8] GHB is an endogenous substance reaching concentrations up to 3.3 mg L^{-1} in saliva.[9] Therefore, the predefined cut-off limit for the determination of its illegal usage is set at 4–10 mg L^{-1} in different samples (blood, urine and oral fluid).[10] Dosages of mushrooms are harder to estimate, but the amount of PY needed for mind-altering effects starts from a dose over 15 mg,[11] whereas 3–10% of PY is excreted in urine unmodified and is detectable within 20–40 min after intake.[6] IBO is not considered an illegal drug, but has been shown to have hallucinogenic properties and is becoming more popular as it is found in many mushrooms of the *Amanita* genus. The dose for the IBO intoxication begins from 30 to 60 mg, most of it being excreted unchanged,[12] resulting in concentrations from 32 to 55 mg L^{-1} in urine.[13]

Determination of the amount of a psychotropic substance being used recreationally by an individual is of the utmost importance, even more so when the detection window is shortened by the substance's half-life. Our previous study concerning determination of GHB abuse in saliva has proven the

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applicability of a CE-coupled conductivity detector for easy and reliable differentiation between the exogenous and endogenous concentration levels of GHB in saliva samples.[14] IBO and PY have also been studied in mushrooms by CE[13,15] and in biological samples with other methods such as NMR[13] and HPLC.[16]

All above-mentioned substances have an acidic group with low pK_a values (Fig. 1). GHB has only one acidic group with a pK_a value of 4.71.[16] Both IBO and PY have acidic and basic groups, where IBO has an acidic group with a pK_a of 3.0 and two basic pK_a values of 5.0 and 8.2,[17] and PY has two acidic groups with pK_a values of 1.3 and 6.5 and basic one at 10.4.[18] Therefore, at the pH of saliva, which ranges from 6.48 to 7.26 in passive secretion,[20] all analytes of interest are partially or totally negatively charged. This provides the possibility to use CE for saliva sample analysis, as it is a suitable method for the detection of small polar molecules and complex sample matrices in the neutral pH range. As the metabolism of the substances in humans is relatively fast, sample collection and the preparation procedure must be as quick and easy as possible. Moreover, a person, who is substantially impaired by psychoactive drugs or a police officer conducting the drug test on a suspected impaired driver, may not be aware what he or she has consumed before. Therefore, the simultaneous analysis of different psychoactive drugs is of the utmost importance. Utilization of oral fluid for such an objective is favoured as the sample collection is non-invasive and easily achievable on the spot. The presence of psychoactive substances in saliva has been shown to correlate with blood concentrations, therefore providing the opportunity for discovery of drug intake and determination of abuse.[21,22]

To date the methods for determination of PY have not focused on the substance itself, but on the main metabolite psilocin in biological samples, and for IBO the determination of IBO concentration levels following poisoning and hospitalization. To the best of our knowledge, there are no publications dealing with simultaneous determination of GHB, IBO and PY in a complex sample matrix such as saliva by CE coupled to conductivity detection.

Our previous study has shown good applicability of capillary electrophoresis with a capacitively coupled contactless conductivity detector (CE-C^{4}D) for the determination of GHB in saliva. However, it was found that the methodology optimized for the determination of GHB abuse in saliva[14] had several setbacks in its use of CTAB, organic modifiers and emerged eigenpeaks as well as insufficient robustness (due to the instability of the complex multicomponent background electrolyte) for on-site use. Therefore, the aim of the current study was to develop and validate a methodology for simultaneous determination of GHB, IBO and PY in oral fluid by CE, which could be employed for on-the-spot analysis, having sufficient resolution for the simultaneous determination of the analytes of interest in a fast and reliable manner.

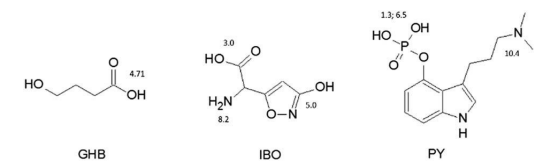

Fig. 1 Chemical structures and pK_a values for GHB,[17] IBO[18] and PY[19] functional groups.

Materials and methods

Chemicals

All chemicals used were of analytical grade. Sodium γ-hydroxybutyrate (Na-GHB) solution (1 mg mL^{-1} GHB salt in methanol) and PY were purchased from Lipomed AG (Germany). IBO, L-arginine (Arg), succinic acid (Suc), hexadimethrine bromide (HDMB) (≥94%), pinacolyl methylphosphate (PMPA), acetonitrile (ACN) (HPLC grade), methanol (MeOH) (HPLC grade) and sodium hydroxide were bought from Sigma-Aldrich (Germany). Ultrapure water (Milli-Q) (resistivity ≥ 18 MΩ cm) was obtained using a Milli-Q integral water purification system (Merck KGaA, Germany).

Capillary electrophoresis

All analyses were performed using a portable CE device built in-house and equipped with a C^{4}D (Department of Chemistry, TUT, Estonia).[23] An uncoated fused silica capillary (Agilent Technologies, USA) with a total length of 55 cm and an effective length of 40 cm for the C^{4}D (i.d. 50 μm and o.d. 360 μm) was used to separate the analytes of interest. The applied voltage was −19 kV. The C^{4}D frequency was set to 200 kHz.

The capillary was activated by rinsing with 1 M NaOH (10 min), Milli-Q water (10 min) and the background electrolyte (BGE) for 15 min. The BGE was composed of 17.9 mM Arg, 9.6 mM Suc and 0.0019% (w/v) HDMB at pH 7.3. The sample was injected by applying −19 kV for 3 s. Between each experiment, the capillary was rinsed with MeOH (2 min), 1 M NaOH (2 min), Milli-Q water (2 min) and the BGE (2 min) to achieve better repeatability and to avoid adsorption of saliva compounds onto the inner wall of the capillary.

Sample preparation

The saliva samples were collected from ten different volunteers (aged 19–70 years). Saliva was gathered in an Eppendorf tube and, if not used right away, was stored in a freezer (−20 °C). The saliva samples were spiked with the analytes of interest at a certain concentration level, and 1.8 g L^{-1} PMPA with a final concentration of 18 mg L^{-1} as the internal standard (IS). Precipitation of saliva proteins was achieved by adding ACN to the sample at a ratio of 2 : 1 (ACN : saliva) and the sample being centrifuged for 5 min at 3000 rpm using a mini-centrifuge (Sarstedt, Germany). The supernatant was used for further CE analysis. The simulated samples for GHB were prepared as for usual samples.

Preparation of mushroom extracts

Mushroom extracts were prepared according to the literature:[24] 0.3 g of air-dried mushrooms *Amanita muscaria* and *Psilocybe*

semilanceata were ground in a mortar and dissolved in 3 mL of MeOH. The solution was sonicated for 15 min and the first extract was transferred to a test tube. An additional 2 mL of MeOH was then added onto the mushroom and again sonicated for 10 min. The first and second extracts were combined and centrifuged for 5 min at 3000 rpm using a mini-centrifuge (Sarstedt, Germany). The *Amanita muscaria* methanolic extract was concentrated 25 times using a rotary evaporator (Laborota 4000, Heidolph, Germany) under reduced pressure.

Software

Data were processed with software written in-house for peak acquisition (Department of Chemistry, TUT, Estonia) and Matlab R2011b (The MathWorks, Inc., United States). Optimization was conducted using JMP® 12 software (SAS, United States).

Results and discussion

Optimization of separation

An optimization study was carried out to determine the optimal composition of the BGE and its pH, the sample preparation and sample injection time. Two designs for optimization were made as shown in Table 1. The first set used a wider range of parameters. As the instrument built in-house had a fixed voltage of −19 kV, no optimization was done for applied voltage. Baseline separation was achieved by using a capillary with an effective length of at least 40 cm for all analytes of interest (resolution, Rs ≥ 1.5 for GHB, IBO and PY).

The results showed that the pH extrema were unsuitable for simultaneous separation of GHB, IBO and PY. Insufficient resolution was observed between GHB and IBO (Rs < 0.4) at pH 7.8. Likewise, PY was not separated at a pH of 7.2. At a pH of 7.5, PY was separated, but insufficient separation was still observed for GHB–IBO (Rs = 1.07). The injection time of 2 s did not provide a consistent sample volume and 4 s injection provided too large an excess of smaller anions into the capillary. Therefore, the best injection time was 3 s.

Based on the results obtained from the first set, the capillary length was set to L_{tot} = 55 cm (L_{eff} = 40 cm) and the pH value was varied from 7.3 to 7.5 for the second set. All BGE concentrations were varied in the same range as before, in an effort to see if they had any additional influence on separation in the second set.

The second optimization set was constructed using the Box–Behnken design of experiments (DOE) to find the optimal BGE composition in JMP® software,[25] applying a minimum amount of experiments and, therefore, reducing time and material resources.

The evaluation of optimal electrophoretic separation conditions was performed by comparing Rs between unidentified peak 1 (U1) and GHB, GHB–IBO, IBO and unidentified peak 2 (U2), as well as unidentified peak 3 (U3) and PY. Moreover, the theoretical plate counts (N) for GHB, IBO and PY were calculated. The DOE scheme with calculated results is shown in the ESI† (Table A.1). All experiments were conducted using a sample spiked with 30 mg L^{-1} of each analyte: GHB, IBO or PY. The results were obtained from the response surface plots (contour profiler) and interaction profiles (ESI Fig A.1†), where the optimal BGE composition was found to be 17.9 mM Arg, 9.6 mM Suc, and 0.0019% (w/v) HDMB and a pH of 7.3.

On the basis of the theoretical results achieved by DOE, the optimal BGE composition was tested using a sample spiked with each analyte of interest and the IS. A BGE of 17.9 mM Arg, 9.6 mM Suc and 0.0019% (w/v) HDMB with the pH set at 7.3 and a capillary with an effective length of 40 cm showed a stable baseline as seen in Fig. 2.

The following Rs values were found between the peaks under optimized conditions: 1.55 for U1–GHB, 1.53 for GHB–IBO, 1.50 for IBO–U2 and 2.33 for U3–PY. The N values were 2.91 × 10^5, 1.89 × 10^5 and 2.48 × 10^5 for GHB, IBO and PY, respectively. Injection of the sample was electrokinetic for 3 s from the cathode and the applied voltage was −19 kV.

Calibration and validation

Determination of detection limits included measurement of the instrumental detection limit (IDL) and the limit of detection (LOD) of the methodology, which took into account the sample preparation procedure. The same was applied for the instrument quantification limit (IQL) and the limit of quantification (LOQ) of the methodology. The IDL was determined as a signal-to-noise (S/N) ratio of 3, and the IQL as ten times the S/N. As the

Table 1 Optimization sets

Parameter	Minimum	Maximum
First optimization set		
Capillary length	L_{eff} = 20 cm	L_{eff} = 40 cm
Injection time	2 s	4 s
pH	7.2	7.8
Arg	16 mM	20 mM
Suc	7.6 mM	9.6 mM
HDMB	0.005% (w/v)	0.0035% (w/v)
Second optimization set		
Arg	16 mM	20 mM
Suc	7.6 mM	9.6 mM
HDMB	0.005% (w/v)	0.0035% (w/v)
pH	7.3	7.5

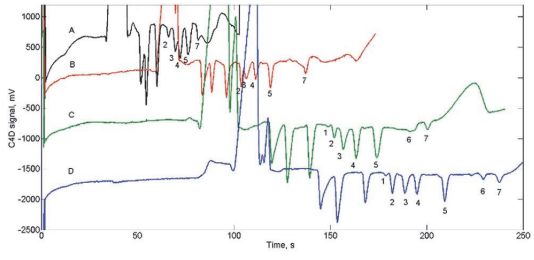

Fig. 2 Effect of the capillary effective length for CE separation and baseline stability: A – 20 cm, B – 25 cm, C – 35 cm, D – 40 cm. Analytes of interest: 1 – U1, 2 – GHB 5 mg L^{-1}, 3 – IBO 5 mg L^{-1}, 4 – U2, 5 – IS 18.0 mg L^{-1}, 6 – U3, 7 – PY 5 mg L^{-1}.

saliva proteins were precipitated by the addition of ACN in the ratio of 2 : 1 (ACN : saliva), the concentrations in real samples were diluted three times. The saliva used for calibration was a pooled sample to ensure a versatile blank.

As the human body naturally produces GHB, it is important to take these low endogenous concentration levels into account. Therefore, a blank saliva sample and the samples spiked with GHB were analysed. The endogenous concentration level was subtracted from the saliva. The LOQs achieved, shown in Table 2, are consistent with limits that can be found for illegal use of GHB, where the recommended cut-off limit is 10 mg L^{-1} in saliva, shown in a previous study.[14] In the case of PY and IBO, all concentrations above zero (LOD) lead to evidence of consumption of such psychoactive compounds.

Representative electropherograms for blank saliva (showing endogenous GHB), saliva spiked with low concentrations (6 mg L^{-1}) and saliva spiked with high concentrations (90 mg L^{-1}) of substances are shown in Fig. 3.

The calibration was accomplished simultaneously for all psychoactive substances, up to 90 mg L^{-1} for each analyte of interest. The IBO peak with a concentration level higher than 90 mg L^{-1} was not separated completely from GHB and peak tailing increased drastically in addition to lack of peak sharpness. The equations were calculated using the peak areas, corrected using the IS peak area. All lower concentration ranges showed good linearity, having R^2 above 0.9, and full ranges had an excellent linearity with R^2 over 0.99. Each calibration curve had at least six concentration points (n) with six independent replicates for each, making the overall number of experiments over 36 (N). All results are presented in Table 2. The calibration curves with low concentration levels were used for evaluation of the LOD and LOQ.

Precision, accuracy and recovery

The validation also included the determination of intraday and interday precision, accuracy and recovery. All parameters were determined at multiple concentrations: low concentrations (6 mg L^{-1} for GHB, 9 mg L^{-1} for IBO and 12 mg L^{-1} for PY); in the middle part of the calibration, two points for GHB and PY (30 and 90 mg L^{-1}) and one point for IBO (30 mg L^{-1}); and another concentration at the end of the calibration curve for all analytes, 600 mg L^{-1} for GHB, 90 mg L^{-1} for IBO and 600 mg L^{-1} for PY.

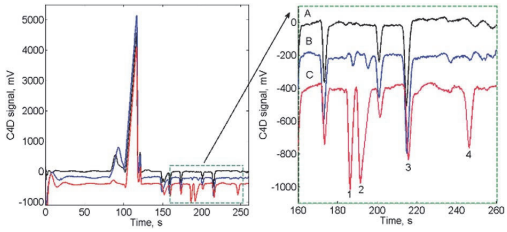

Fig. 3 Electropherograms of saliva and spiked saliva samples: A – saliva + IS; B – saliva spiked with 6 mg L^{-1} GHB, IBO and PY; C – saliva spiked with 90 mg L^{-1} GHB, IBO, PY. The analytes of interest: 1 – GHB, 2 – IBO, 3 – IS and 4 – PY.

The intraday precision was determined using six parallels on the same day for each concentration and for interday precision, experiments were carried out over the course of 7 days. The intraday precision ranged from 0.7% to 8.6%, interday from 3.9% up to 11.4%, demonstrating good overall intraday and interday precision. All results are shown in Table 3.

Accuracy was measured by spiking samples with a desired amount of psychoactive substance and then calculated from the calibration curves. Low concentrations – 6 mg L^{-1} for GHB, 9 mg L^{-1} for IBO and 12 mg L^{-1} for PY – were calculated using the calibration curves with small concentration ranges, with all others using the full-sized ones. The achieved values matched those desired for each substance with derivations for low concentrations from 100% to 123%, thereby showing good

Table 3 Intraday and interday precision, accuracy and recovery

	Added conc, mg L^{-1}	Precision, % intraday/interday	Accuracy, %	Recovery, %
GHB	6	3.3/11.4	123 ± 7	95 ± 4
	30	2.2/4.3	99 ± 5	99 ± 2
	90	0.7/3.9	101 ± 6	101 ± 2
	600	1.0/6.2	100 ± 3	99 ± 5
IBO	9	3.2/8.5	102 ± 7	98 ± 2
	30	1.9/5.6	101 ± 7	102 ± 3
	90	2.2/4.3	95 ± 1	101 ± 4
PY	12	8.6/10.3	100 ± 11	103 ± 6
	30	3.9/8.2	101 ± 9	101 ± 5
	90	4.0/7.7	95 ± 1	99 ± 2
	600	5.1/6.8	100 ± 1	98 ± 4

Table 2 Calibration range, equations and detection limits

Analyte	Range, mg L^{-1}	n, N	R^2	Equations, $area_{analyte}/area_{IS}$	IDL, IQL[a]	LOD, LOQ[b]
GHB, low	0.5–4	6, 36	0.9908	$y = (0.0470 \pm 0.0007)x - 0.005 \pm 0.001$	0.5, 1.7	1.5, 5.0
GHB	0.5–200	8, 48	0.9985	$y = (0.0546 \pm 0.0003)x - 0.067 \pm 0.026$		
IBO, low	0.7–5	6, 36	0.9903	$y = (0.0556 \pm 0.0008)x + 0.004 \pm 0.002$	0.7, 2.3	2.1, 7.0
IBO	0.7–30	8, 48	0.9956	$y = (0.0786 \pm 0.0009)x - 0.067 \pm 0.019$		
PY, low	1.0–6.0	6, 36	0.9569	$y = (0.0369 \pm 0.0016)x - 0.011 \pm 0.006$	1.2, 4.0	3.6, 12.0
PY	1.0–200	8, 48	0.9996	$y = (0.0336 \pm 0.0001)x - 0.003 \pm 0.004$		

[a] 3 times signal-to-noise for the IDL and 10 times for the IQL. [b] LOD and LOQ are three times those for real samples compared to the IDL; all concentrations are in mg L^{-1}.

accuracy. The higher concentrations for GHB, IBO and PY had accuracy ranging from 95% to 101% (shown in Table 3), proving the method to be reliable.

In addition to precision and accuracy, recovery was also studied. Recovery is needed as the samples used are saliva samples, which in turn are precipitated, making it possible for some of the analyte to be lost during the preparation. To calculate the recovery of the proposed method, analysis results of the saliva spiked with analytes before sample preparation were compared with the results obtained when the sample was spiked after precipitation of proteins. Recovery was calculated using three samples for each concentration level; the results obtained are included in Table 3. The recovery for GHB at a low concentration level showed the biggest difference, which could be explained by the subtraction of the natural GHB level, which can be under the LOQ, making the calculation an underestimate; for higher concentrations, the recovery ranged from 99% to 101%. Regarding the recovery for IBO and PY, all values were between 98% and 103%, meaning that the precipitation of saliva did not influence the concentration of substance measured.

The sample stability was not evaluated on the basis of previous studies describing the degradation of PY by light[26] and the volatile nature of ACN, making it important for samples to be protected from sunlight as well as limiting the amount of vaporization. This was achieved by storing the prepared samples at +5 °C (refrigerated) and wrapping the samples in tinfoil to improve stability over the 7 days.

Analysis of the simulated samples

The developed methodology was further employed to identify GHB in a saliva sample obtained after drinking of 400 mL of red wine over a period of 30 min. Since red wine contains GHB,[27] its concentration in saliva is supposed to increase after drinking. Unfortunately, it was impossible to obtain real samples containing IBO and PY. However, to demonstrate the potential of the proposed methodology, saliva samples were spiked with hallucinogenic mushroom extracts (60 μL extract to 200 μL saliva). *Amanita muscaria* extract was used for IBO determination and *Psilocybe semilanceata* extract for PY determination. All samples were also spiked with the IS to determine the relative migration times for peak identification. Although saliva spiked with mushroom extracts does not exactly reflect the composition of saliva after substance intake, it was used for evaluation of IBO and PY in saliva that did not originate from spiking with pure standards, but from a real complex sample matrix.

The electropherograms in Fig. 4 show that the developed method allowed detection of the substances of interest in all samples. Despite some baseline diversity, the peaks of GHB, IBO and PY in samples are clearly identified, confirming that the proposed methodology can be applied for analysis of real saliva samples.

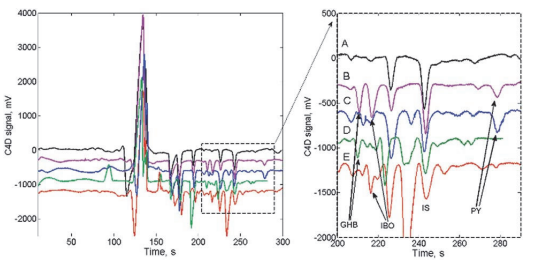

Fig. 4 Electropherograms of simulated samples. A – blank saliva + IS; B – saliva + GHB, IBO and PY standards; C – saliva + *Psilocybe semilanceata* extract; D – saliva after drinking wine; E – saliva + *Amanita muscaria* concentrated extract.

Conclusions

CE coupled with conductivity detection is a rapid method for the determination of small anions in biological samples, including psychotropic substances such as GHB, IBO and PY. Due to the nature of the use of these substances as recreational drugs or hallucinogens, their detection is of interest and importance.

This work presented an optimized and validated methodology for simultaneous determination of GHB, IBO and PY in saliva samples within 10 min. The CE-C^4D application for simultaneous determination of psychoactive substance abuse in saliva is novel and appropriate for cases where the substance of abuse is unknown and rapid detection is needed. The developed methodology involves very simple sample preparation without any derivatization steps or complex purification. Moreover, the miniaturization of the CE instrument makes it portable, allowing it to be employed on site.

Conflict of interest

The authors declare that there is no conflict of interest.

Compliance with ethical standards

This study has been approved by the appropriate ethics committee and has been performed in accordance with the ethical standards. Informed consent was obtained from all volunteers prior to sample collection.

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Publication III

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Rapid and sensitive capillary electrophoresis method for the analysis of *Ecstasy* in an oral fluid

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ABSTRACT

In the present study, a capillary electrophoresis method, with a native fluorescence detection for the quantification of three amphetamine derivatives, methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA) in an oral fluid is described. The reported CE method has made it possible to assess *Ecstasy* abuse in approximately 15 min, including a saliva sample collection, pretreatment procedures and capillary electrophoresis (CE) analysis. The proof of the principle that was demonstrated on a home-made lab scale instrument has had the potential to be easily translated onto a truly portable instrument for on-site measurements. The baseline CE separation of the three illegal drugs was achieved in 10 min, by applying an aqueous background electrolyte (BGE) that was composed of 40 mM phosphoric acid and 10 mM triethanolamine. The amphetamine derivatives were detected at their $\lambda_{ex}/\lambda_{em}$ maximum (280/326 nm) with LOD values of about 3 ng/mL for each amphetamine. The recovery of the compounds from the collection pad was about 40% of the LOQ concentrations and the inter-day precision was less than 6% for all of the analytes. The procedure was applied to a quantitation of oral fluid (OF) samples that were collected during the Baltic Weekend Music Festival that was held in Estonia.

1. Introduction

During the last decade, the diffusion of synthetic amphetamine derivatives is dramatically increasing in the European illegal markets. Amphetamine and 3,4-methylenedioxy-methamphetamine—the latter is also known as “ecstasy” or “MDMA”—remain the most widely used synthetic stimulants in Europe and they compete to some extent with cocaine in this respect [1]. This trend has been confirmed by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), indicating that there has been a steadily increased use of these drugs since the year 2010. Thus, 9.8 million ecstasy tablets were seized in Europe in 2015 and the availability of high-MDMA content products has prompted joint alerts from Europol and the EMCDDA [2].

MDMA (Fig. 1) is the prototype for a large series of phenethylamine designer drugs, together with some homologous compounds that have similar effects, such as methylenedioxyamphetamine (MDA) and methylenedioxyethylamphetamine (MDEA). These are commonly taken orally, whether in a tablet form or swallowed as a powder - and also, less often, they are snorted or even injected. An estimated 2.1 million young adults in Europe used ecstasy in 2015 [1].

The need of detecting illicit drugs at trace levels in biological matrixes has challenged the scientific community into developing new sampling and detection methods, with an improved sensitivity and selectivity [3–6].

The quest for the development of an analytical method has been targeted at the ability of identifying the narcotics that are used by drivers/criminals as quickly as possible, so that appropriate preventive measures can be taken. For such a method to be useful, several factors have to be considered, including non-invasive sampling, sample preparation times, the portability of instruments, instrument start-up times and the actual analysis time.

The use of oral fluids (OF) as an alternative biological matrix for drug abuse testing has received increased attention in forensic and clinical chemistry [7–10]. In fact, oral fluids can be collected in a simple, inexpensive and non-invasive manner by nonmedical personnel. The sampling can be closely supervised, without an invasion of privacy, in order to prevent a substitution, an adulteration, or a dilution of the sample.

To date, the assessment of *ecstasy* use that is based on an oral fluid analysis employs on-site screening tests. In the case of positive results, there is a follow-up confirmatory analysis in the lab. Screening

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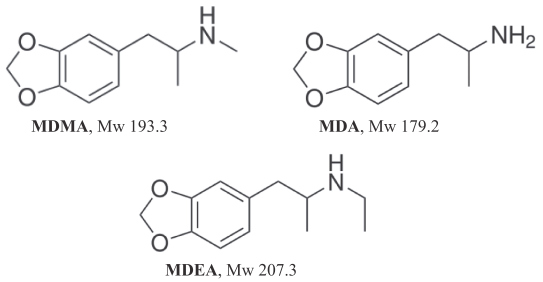

Fig. 1. The chemical formulae and the molecular masses of the studied synthetic stimulants.

methodologies are typically based on molecular recognition and ligand binding, with immunoassays as the most popular choices. Several immunoassay devices for on-site testing are available commercially [10,11]. The main drawbacks of the immunoassay tests are a cross-reactivity and a poor analyte recovery from the devices, which leads to a low diagnostic sensitivity (about 60%) and quite often, to inadequate performances [12,13]. In the confirmation analysis of drug abuse, chromatographic separation techniques play a dominant role. Liquid (HPLC) and Gas Chromatography (GC), with a mass spectrometric detection [3,14–16], provide for very reliable identifications of the separated compounds. However, their applicability for an on-site analysis is rather limited. GC can be made portable and the separation times are relatively short [17]. Nevertheless, the analysis typically requires a sample derivatisation. HPLC, on the other hand, cannot readily be made portable, thus, it requires the sample to be delivered to an analytical laboratory.

Capillary Electrophoresis (CE) and related techniques are increasingly being employed in forensic analysis, as documented in several recent studies [18–20]. The unique features of CE, such as a high separation efficiency, a rapid analysis, as well as a low solvent and sample consumption, have made this technique a valid alternative to LC for the determinations of the drugs of abuse [21,22]. In contrast to these advantages, the primary disadvantage limiting its use is its poor concentration sensitivity, particularly when it is applied with an on-column ultraviolet (UV) absorbance detection, due to the low sample-injection volume and the short optical path length. More adequate detection limits for the abused drugs, in the context of miniaturised analytical techniques, can be obtained by light-emitting diode (LED) induced fluorescence detection [23,24]. The combination of extremely highly stable, long lifetime, small size, low cost and the commercial availability at wavelengths ranging from deep-UV to near-IR regions, make LEDs an attractive light source for these abuse detections [25–28].

The primary purpose of this study was to evaluate the hypothesis that the capillary electrophoresis method, with an LED-induced fluorescence detector (LED-IF), is an alternative analytical technology, to the laboratory GC/LC-MS and roadside drug tests, for the quantitative detection of ecstasy abuse. The proof of the principle was demonstrated on a home-made lab scale instrument, with the goal of transforming it further, to a portable format for on-site measurements. The LED-fluorescence detector that was used in this work operated at a deep UV wavelength, providing for a sensitive detection of the three amphetamine derivatives, MDA, MDMA and MDEA, at their excitation/emission maximums, without the need for any derivatisation. A saliva sample collection/preparation/pre-concentration procedure, combined into one step, by using a Salivette® sampling device and a CE separation of analytes, were carried out in under several minutes. The developed method was thoroughly validated, according to the EMEA Guidelines [29]. This procedure was used for the analysis of real oral fluid specimens that were collected by law enforcement officers during the Baltic Weekend Festival that was held in Estonia in August 2016.

2. Materials and methods

2.1. Chemicals, reagents and standards

The amphetamines, namely, 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxyethylamphetamine (MDEA), in standard solutions of 1 mg/mL of methanol and powders, were purchased from Lipomed AG (Switzerland). Internal standard (IS) allocryptopine (ALC), as well as the background electrolyte compounds, including phosphoric acid and triethanolamine (TEA), sodium hydroxide and acetonitrile (ACN), were all obtained from Sigma-Aldrich (USA). All of the chemicals were ACS grade and the organic solvents were HPLC grade. Milli-Q water was used for the preparations of the solutions.

2.2. Calibrator and quality control solutions

The MDMA, MDA and MDEA in 1 mg/mL of methanol standard solutions were diluted with acetonitrile, in order to prepare the 100 μg/mL stock solutions, which were stored at −20 °C. A stock solution of 100 μg/mL allocryptopine was used as an internal standard (IS) and this was also prepared in acetonitrile. Pooled OF samples for the preparation of the working standards and the quality controls (QC) were prepared from the salivas that were donated by 10 people from the staff personnel. The calibration solutions of 10, 20, 40, 80 and 150 ng/mL were prepared in pure ACN and in the saliva by the addition of the appropriate amounts of the analytes stock solutions and IS (35 ng/mL) to the blank oral fluid samples, all when using the following sample preparations (as described below). The QC solutions were prepared in a similar way from the powdered MDMA, MDA and MDEA standards, but differently to those that were used for the calibrations.

2.3. Oral fluid collection and the sample preparation

The oral fluid collection and the sample preparation procedure was carried out according to the researchers' previous study [22]. The salivas were collected by Salivette® devices (Sarstedt, Nümbrecht, Germany), by sucking on the cotton pads for two minutes. The pads were placed back into the tubes and they were centrifuged at 8000 rpm for 2 min. The centrifugates were discarded and 1000 μL of acetonitrile were added to the pads. The Salivette tubes were centrifuged again under the same conditions and the obtained centrifugates were injected for the CE analyses. The collected samples were stored at − 20 °C.

2.4. CE apparatus and analysis

The CE apparatus was constructed in-house. Uncoated fused silica capillaries, I.D. 75 μm and O.D. 360 μm (Polymicro Technologies, Phoenix, AZ, USA), were used for the analyses. The total capillary length was 62 cm, with the detection zone placed at 15 cm from the end of the capillary. Prior to the injections, the capillary was rinsed sequentially with 1 M NaOH, Milli-Q water and the background electrolyte (BGE), for 2 min of each one. The separation electrolyte consisted of 20 mM phosphoric acid and 6 mM TEA (pH 2.5). The samples were injected into the capillary by a hydrodynamic flow at a height differential of 15 cm for 30 s. The separations were performed at + 17 kV. Before the measurements, the new capillaries were conditioned by rinsing them sequentially with 1 M sodium hydroxide, Milli-Q water and the background electrolyte for 10 min of each one. Between analyses, the capillaries were rinsed again with the electrolyte solution for 2 min.

The LED-fluorescence detector that was used for the determinations of the synthetic stimulants was designed and constructed by Laser Diagnostic Instruments AS (LDI), Estonia. This instrument has been described in detail in the researchers' previous studies [22,28]. Briefly, a UV-LED (Roithner Lasertechnik, Austria) was used as the fluorescence

excitation source (λ = 280 nm). An interference filter of 326 nm (Andover Corporation, USA) was used to block the reflected UV radiation and to select the required spectral region for the fluorescent signal registration.

2.5. Method validation

The developed procedure was validated according to the EMEA Guidelines on bioanalytical method validation [29]. The CE method was evaluated for its selectivity, limits of detection and quantification (LOD and LOQ), linearity, inter-day precision, accuracy, carry-over and extraction recoveries. The robustness of the method was also determined.

Selectivity was defined as the ability of the analytical method to differentiate and quantify an analyte of interest and an internal standard from the endogenous components in the matrix, or other components in the sample. The potential endogenous interferences were assessed by the analyses of six OF samples from the volunteers that were fortified with the IS. In order to assess some of the exogenous interferences that were caused by the smoking of tobacco or eating, the saliva samples were taken at different times during the day (before and after eating) from the smokers and the non-smokers.

For the extraction recovery (R%) evaluations, two different calibration curves were constructed. The first calibration was performed with pure standards that were dissolved in the ACN; the second one was carried out by the method of standard additions. For this, pooled saliva samples that were obtained from the ten volunteers were divided into aliquots, which were spiked with five different concentration levels, before the extractions with the Salivette® device. IS was added to the extracted saliva samples before each analysis. Each concentration level was injected five times. The analyte responses were normalised to an internal standard and they were quantified by linear least-squares regression. The linearities were checked from 10 ng/mL to 150 ng/mL for all of the amphetamines. The determination coefficients (r^2) were required to be at least 0.99 and the regression errors to be < 20% at the LOD and < 15% at the other concentrations. The extraction recoveries (R%) were calculated from the slope ratios of the calibration curves that were obtained in solvents (ACN) and in the OFs.

The LOD and LOQ values were determined by measuring a series of the decreasing concentrations of the fortified saliva samples. The LOD values were determined at the lowest analyte concentrations, with an S/N ratio of at least 3 for all of the analytes, all with acceptable peak shapes. The LOQ values were the lowest concentrations that could be quantified with an acceptable precision (%CV$^{<}$20%) of the target concentrations.

The precisions and accuracies were assessed at low (10 ng/mL), medium (40 ng/mL) and high (150 ng/mL) QC concentrations in the oral fluid matrices. The precisions and the accuracies were studied by analysing three replicates on ten different days (n = 30). The experimental precisions were expressed as the relative standard deviation of replicates. The accuracies were calculated as the differences between the mean and targeted concentrations (Ct) (A% = mean/Ct × 100).

The fortified OF samples that exceeded the linear ranges for MDMA, MDA and MDEA (150 ng/mL) were extracted and analysed, in order to evaluate the carry-overs. The blank samples containing IS were injected after each carry-over challenge, so as to quantify the potential carry-overs from the previous injections.

The robustness of the analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters [30].

The robustness of the developed CE method was determined by using the Plackett and Burman two-level fractional design, being centred on the optimum. In this case, modified experimental conditions were introduced, coded as − 1 (lower value) and + 1 (upper value). Code 0 was reserved for the nominal values in the procedure (central value). The Plackett and Burman design was used for the evaluations of 11 factors (10 real factors and one dummy), with 12 experiments. The factors analysed were: the hydrodynamic injection time and the injection height, which determine the injection volumes and may affect the resolution and peak area of the analytes; the applied voltage, which may affect the plate counts and the resolutions; the capillary rinse time with the Milli-Q water, the NaOH and the BGE, which may affect the capillary equilibrations; the capillary length and the temperature, which may affect the mobility of the species; the concentration of TEA and phosphoric acid in the background electrolyte, which may have an impact on the electroosmotic flows; and on the plate numbers by the stacking or the destacking effects. The responses that were evaluated were the ratios of the analyte and the IS peak areas (A/A_{IS}). After the calculations of the effects for each parameter (by the sums of the responses of the positive and negative levels), the statistical interpretation (*t*-test) allowed for the determination of the similarity or the difference of the results. The intermediate precision estimates were used in order to estimate the standard error and to identify the significant effects.

3. Results and discussion

3.1. Method development

3.1.1. Fluorescence emission spectra of the Ecstasy Amphetamines

The fluorescence emission spectra of the MDMA, the MDA and the MDEA were first investigated. It was found that all of the amphetamines emitted a fluorescence at wavelengths of around 320 nm, when excited at 230 nm and 280 nm with a similar quantum yield [31]. Therefore, it was possible to use a 280 nm light-emitting diode, with an interference filter of 326 nm, in order to detect them in the CE, without the need for any fluorescent derivatisation.

3.1.2. CE conditions

All of the compounds under examination in this work were primary or secondary amines, with pKa values from 9.7 to 10.2. Thus, in the acidic electrolytes, all of the amphetamines were protonated at the nitrogen atom and migrated as cations towards the cathode. The acidic medium also provided an enhanced selectivity. At low values of the pH electrolytes, with little or no electroosmotic flow, possible interferents with acidic or neutral characteristics would bear negative, or even no charge at all, therefore, they would not pass the detector at measurable values. Another parameter that was considered in the method development was the nature of the counter ion. On account of its good buffering capacity at low pH values, 40 mM phosphate with a pH value of 2.5 was chosen as a promising counter-ion.

In capillary zone electrophoresis, it is routinary for the use of additives, in order to reduce the interactions of certain analytes with the capillary walls. The analytes that were composed of a lipophilic backbone, with a cationic aminic moiety, tended to adsorb on the negatively charged wall of the silica capillary, which led to the formation of asymmetrical peaks and tailing, affecting both the qualitative and quantitative analyses. The adsorption effects were able to be minimised by the addition of polyamine or alkylammonium salts to the BGE [32,33]. Amines are well-known for blocking the silanol groups, making the undesirable interactions with the analytes less pronounced. In the present study, 10 mM TEA was used as a BGE additive for the separations of the three amphetamine derivatives and the internal standard (Fig. 2*a*). A *Celandine* alkaloid allocryptopine was used as an internal standard, due to its structural similarity and its sufficient quantum yield at working on the excitation/emission wavelengths. The baseline separation of all of the substances was achieved in 14 min. All of the amphetamine derivatives under study had the same charge at a pH of 2.5 and the migration time depended proportionally upon the molecular weight of the drugs.

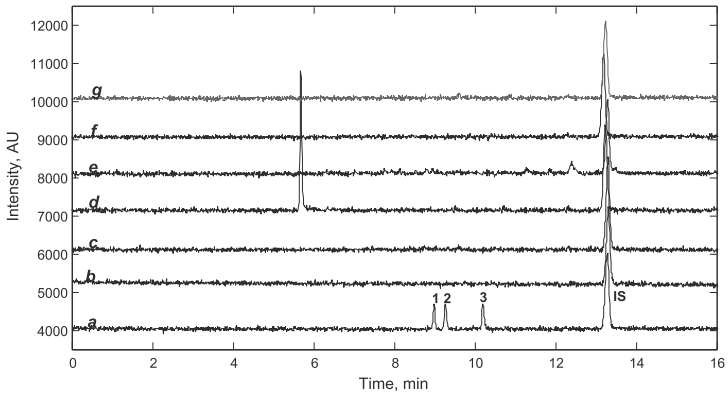

Fig. 2. CE separations of the MDA, MDMA and MDEA standards (a) and analyses of the independent saliva samples from the six drug-free volunteers (b-g). Separation conditions: BGE – 40 mM phosphate containing 10 mM TEA, pH 2.5, capillary I.D. 75 μm, field intensity 315 V/cm, λex/λem = 280/326 nm. Analytes: 1 –MDA (10 ng/mL), 2 – MDMA (10 ng/mL), 3 – MDEA (10 ng/mL), IS – allocryptopine (35 ng/mL).

3.2. Method validation

The selectivity of the method was controlled by the evaluation of the endogenous matrix interferences when analysing the six OF samples that were collected from the drug-free volunteers and fortified with IS. No endogenous signal contributions for any analyte of interest were observed (Fig. 2, *b-g*).

Table 1 depicts the results of the method validation regarding the linearities, the limits of detection and quantification and the extraction recoveries. The plots of the peak area ratios (analyte to IS) versus the concentrations of the standards in pure solvent (1) and the standards in the OF after the extraction procedures (2) exhibited adequate linearities for the studied analytes, all with acceptable statistical parameters: the coefficients of the determinations were > 0.99 and the regression errors were ˂10%.

The limits of detection and quantification were estimated by analyses of the fortified saliva samples of 1 mL volume. LOD and LOQ values of around 3 ng/mL and 6 ng/mL were achieved for all of the amphetamine derivatives. It was important to note that 'dry mouth' effects of the abused individuals can cause a lack of saliva at the sampling moment, thus influencing the dilution factor of the sample. Despite this, even a sample dilution factor of 10 would provide LOQ values of around 60 ng/mL, which meant that the proposed method was capable of detecting and quantifying the MDA, MDE and MDMA drugs in concentrations much lower than their cut-off values [34].

The extraction recoveries (R) from the Salivette® cotton swabs were assessed by the slope ratios of the calibration curves (1) and (2). The recovery values for all of the analytes were below 50%, which were due to the comparably weak adsorptions of the amphetamine derivatives by the Salivette® cotton swabs. Despite this, these Salivette® devices for sample collections and extractions can still be successfully used for *ecstasy* impairment detections, providing that the LOQs were below the recommended cut-offs.

The inter-day reproducibility (precision) and the accuracy were assessed by using a fortified drug-free matrix at three concentration levels (Table 2). The inter-day reproducibility results that were expressed as a residual standard deviation (RSD%) were constantly ≤ 5%. The accuracies were calculated as the percent differences between the mean and the targeted concentrations of each analyte – and while taking into account the recovery correction factors, they ranged from 98% to 109% for all of the concentration levels.

Table 2
Analytical reliability parameters of the method.

	MDA	MDMA	MDEA
Precision (RSD%)			
20 ng/mL	3.2	4.8	2.4
80 ng/mL	2.3	3.4	5.0
150 ng/mL	2.9	3.5	4.3
Accuracy (%)			
20 ng/mL	98	102	104
80 ng/mL	109	103	107
150 ng/mL	106	105	105

The negative samples that were injected immediately after the samples containing 100 μg/mL of each analyte showed no evidence of carry-over (the signals were below the LOD for both of the analytes).

3.2.1. Robustness

For the testing of the robustness of the analytical method, a multivariate approach, in which the variations in the factor levels of the method were simultaneously introduced into the matrix of the experiments, was a better choice, since the influences of each factor were calculated from several experiments. This was because in the capillary electrophoresis method, there were a large number of factors which were potentially critical for the assay. As a result, a Plackett and Burman (PB) fractional design, which requires a minimum number of experiments, was used, in order to estimate the robustness of the developed method. Ten real factors and one dummy were analysed (Table 3) and the effects for each factor were calculated. The limit values to identify the statistically significant effects were derived from *t*-test statistics [35].

Table 1
Linearities, limits of detection (LOD) and quantification (LOQ) of CE method.

Analyte	Regression (1)		Regression (2)		R ± U[a] (%)	LOD (ng/mL)	LOQ (ng/mL)	DRUID recommended cut-offs (ng/mL) [34]
	r^2	Regression error (AU/%)	r^2	Regression error (AU/%)				
MDA	0.995	0.039/4.8	0.993	0.017/4.7	40 ± 4	3.3	5.5	220
MDMA	0.993	0.047/5.9	0.991	0.022/6.0	43 ± 3	3.8	6.4	270
MDEA	0.994	0.065/6.5	0.992	0.024/5.3	35 ± 3	3.6	6.1	270

[a] Expanded uncertainty, k = 2, norm.

Table 3
Analysed factor levels and calculated *t*-values for the MDA, the MDM and the MDE with the statistical results.

Factor	Level (low; high)		$t_{calculated}$			Non-significance interval		
			MDA	MDMA	MDEA	MDA	MDMA	MDEA
A	Injection time, s	27; 33	−4.77*	−3.67*	−5.01*	± 2.0	± 2.7	± 1.9
B	High voltage, kV	15; 19	0.55	2.02	2.06			
C	Rinse time (BG), s	108; 132	−0.97	1.64	0.23			
D	Rinse time (Milli Q), s	108; 132	0.27	−0.41	2.34			
E	Rinse time (NaOH), s	108; 132	−0.40	−0.58	−0.01			
F	[H_3PO4], mM	18; 22	3.85*	5.67*	4.20*	± 1.7	± 1.1	± 1.5
G	[TEA], mM	5.4; 6.6	2.39	1.34	2.71			
H	Capillary length, cm	60; 64	−4.71*	−5.29*	−3.89*	± 1.4	± 1.2	± 1.7
I	Injection height, cm	13.5; 16.5	2.81	2.16	0.68			
J	Temperature, °C	21; 25	5.91*	8.77*	7.45*	± 1.1	± 0.8	± 0.9
K	Dummy		−2.00	−2.24	−2.16			
			$t_{critical\ (0.01;9)}$ **3.25**					

* Significant at $P = 0.01$.

$$t = \frac{|E_x|}{(SE)_e} \leftrightarrow t_{critical}, \tag{1}$$

where $(SE)_e$ was the standard error of an effect E_x, which represented the experimental variability within the design. In this case, the $(SE)_e$ was estimated from the intermediate precision results as:

$$(SE)_e = \sqrt{\frac{4s^2}{N}}, \tag{2}$$

with s^2, being the variance that was determined from the replicated experiments at nominal levels, performed on different days and *N*, being the number of experiments in the design. A negative sign meant that changing the factor from a low to a high level had a negative effect on the assay and vice versa.

The corresponding standardised plot (Fig. 3) represents the absolute values of *t*-calculated for an assay of the investigated factors (A-K) and *t*-critical, and gives a rapid visual information on the magnitudes of the effect.

The factors that most influenced the assays (A/A_{IS}) were the separation temperature, the injection time, the concentration of phosphoric acid in the background electrolytes and the capillary length. For all of the significant factors, the non-significance intervals were estimated and they provided the intervals of the factor levels, by which they should be controlled, in order to eliminate the effects. These levels were estimated as:

$$\left[X_{(0)} - \frac{|X_1 - X_{(-1)}|E_{critical}}{2|E_x|}, \quad X_{(0)} + \frac{|X_1 - X_{(-1)}|E_{critical}}{2|E_x|}\right], \tag{3}$$

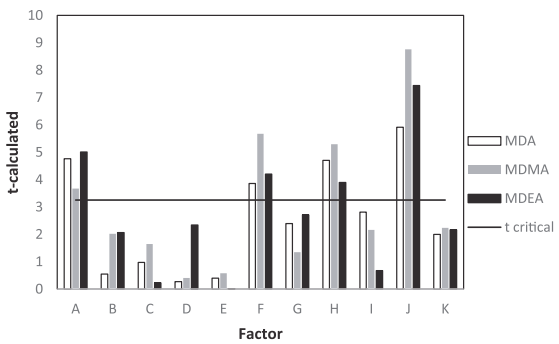

Fig. 3. Bar charts representing the t-calculated values for an assay of the investigated factors (A-K) in the Plackett-Burman experimental design and the t-critical, represented by the horizontal line.

where $X_{(0)}$, $X_{(1)}$, $X_{(-1)}$ were the real values of factor *X* for the nominal (0), high (1) and low (-1) levels [35]. When the factors with significant effects were controlled, within the non-significance intervals, as presented in Table 3, no significant effects on the assays were found.

3.3. Application for analysis of the real OF samples

The developed CE method was employed in order to quantify the *ecstasy* in the OF specimens that were collected with the Salivette® devices during the annual Baltic Weekend Festival that was held in Pärnu, Estonia. The saliva samples were collected in a cooperation with the police officers from the Police and Border Guard Board. The typical electropherograms of the suspects' OF samples that were submitted to the proposed analytical method with positive results for the MDMA are presented in Fig. 4(A-D). Here, the electropherograms (*a*) depict real OF samples and (*b*) the same samples that were spiked with an analyte standard mixture containing MDA (50 ng/mL), MDMA (50 ng/mL), MDEA (100 ng/mL), cocaine (200 ng/mL) and cocaethylene (200 ng/mL). The OF samples that were collected during the Weekend Festival from the suspects mostly contained amphetamine, MDA, MDMA, cocaine and cocaethylene. It was easily seen from the electropherograms that cocaine and cocaethylene, which is formed if cocaine is being consumed with alcohol, were determined by the developed CE method as well. The amphetamines ($\lambda_{ex}/\lambda_{em}$ 255/315 nm) could not be detected with the 280 nm excitation source, therefore their presence was confirmed by another fluorescence detector with a xenon lamp working at 230 nm. The research results of the OF samples that were collected at the Weekend Music Festival in the years 2016–2018 are to be published separately. The measured concentrations of MDA and MDMA (n = 3) were correspondingly in sample A 75 ± 6 and 2668 ± 85 ng/mL, in sample B 448 ± 56 and 7126 ± 142 ng/mL, in sample C <LOQ and 137 ± 8 ng/mL, in sample D 42 ± 5 and 634 ± 44 ng/mL. The contents of MDEA were below the LOQ values.

4. Conclusions

The proposed separation protocol, in a combination with the highly selective native fluorescence detection, made it possible to quantify the *Ecstasy* in the biological matrices, with minimum sample pretreatments and with excellent detection limits. No separate precipitation of proteins or derivatisation were needed. The procedure of saliva sample collection and clean-up took 10 min.

The validation results have shown that the detection limits of the CE-LED method were almost 100 times lower than the recommended cut-offs and because the method was based on a distinct separation mechanism, it can be considered complementary to the well-established liquid chromatography methods in forensic studies. Moreover, the

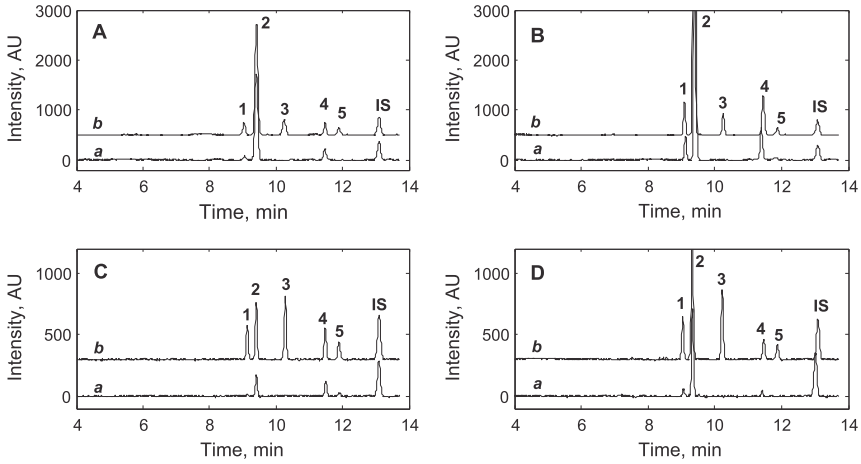

Fig. 4. CE analyses of the street OF samples (A-D). The experimental conditions were as in Fig. 1. Electropherograms: a – real OF, collected from the suspects, b – spiked OF samples. Peaks: 1- MDA, 2 – MDMA, 3 – MDEA, 4 – cocaine, 5 – cocaethylene, IS – internal standard.

method provided acceptable performance characteristics, as well as identity confirmation for the toxicologically relevant substances. In respect to the simplified operating conditions, excellent selectivity and miniaturisation benefits of the proposed CE technology, it would seem to be a very promising and reliable method for roadside or clinically-based drug testing. The construction of a novel portable CE instrument with two more powerful LEDs operating at 280 nm and 250 nm would be an interesting direction for further development.

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Publication IV

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In Situ Determination of Illegal Drugs in Oral Fluid by Portable Capillary Electrophoresis with Deep UV Excited Fluorescence Detection

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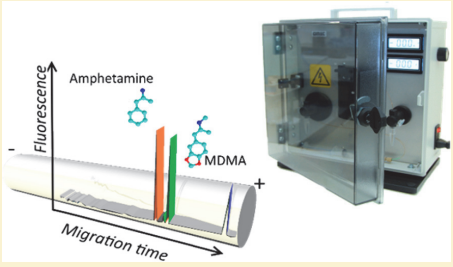
ABSTRACT: The present study demonstrates the potential of a portable capillary electrophoresis (CE) instrument, coupled to deep UV fluorescence detector (FD) with a 230−255 nm excitation wavelength range, for the determination of the abuse of illegal drugs in oral fluids *in situ*. CE was introduced in this study due its exceptional power of separation and resolution, short analysis time, and ability for miniaturization for on-site assessment of different substances. The deep UV fluorescence detector was equipped with five interchangeable emission filters, in the emission wavelength range from 278 to 600 nm, and was successfully employed for determination of natively fluorescing illegal drugs, such as cocaine, cocaethylene, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxeamphetamine (MDA), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA), para-methoxyamphetamine (PMA), para-methoxy-*N*-methylamphetamine (PMMA), amphetamine (AMP), methamphetamine (METH), tetrahydrocannabinol (THC) and cannabidiol (CBD). The developed FD showed impressive sensitivity. The instrumental detection limit was 0.5 μg/L for MDMA. It also showed broad linearity, up to 50 mg/L for MDMA. The noise CV% was 1.1% for an empty capillary and 0.6% for a capillary filled with acetonitrile. The portable CE-FD with developed electrophoretic methodologies was successfully utilized for the determination of illegal abuse of drugs during "Weekend" 2016 and 2017 Music Festivals (Estonia). Moreover, CE-FD can be employed for detection of other natively fluorescing compounds in the proposed range (e.g., for different phenolic compounds, BTEX, naphthalene derivatives, and others), significantly widening the applicability of developed CE-FD instrument.



The analysis of oral fluid (OF) for illegal drug abuse determination offers different advantages compared to blood and urine. Nonmedical personnel can collect it in a simple, inexpensive, and noninvasive manner. Oral fluid sampling can be closely supervised without an invasion of privacy and to prevent substitution, adulteration, or dilution of the sample, which could happen with urine analysis. Oral fluid sampling also avoids the risk of infection, which is possible during a blood draw.[1] Several approaches for detecting drugs in oral fluid have been developed. The majority of these are based on immunological procedures[2,3] or chromatographic techniques, coupled with mass spectrometry or tandem mass spectrometry.[4−6] The problem with immunoassays is that there is a great probability of obtaining a false negative or false positive result due to the ambiguity of detection (in the form of faint stripes), degradation of antibodies used, and cross-reactivity with other analytes.[7,8] Some studies performed with various commercially available assays revealed a 70% false positive and sometimes 50% false negative detection accuracy.[3,9] Thus, immunoassays are used as preliminary screening approaches, in situ, which are then followed by a chromatographic technique to confirm the results. Besides the well-known GC/MS methods, some LC−MS methods have been described.[8,10] In comparison to the immunoassays, chromatographic techniques require sample pretreatment, which makes the analysis time-consuming.

Until now, capillary electrophoresis has received less attention as a tool for determination of illegal drugs.[11,12] Advantages of CE are well-known and are mainly associated with the small sample size. Still, one must consider the detection limits of the CE, which are generally several orders higher than in the case of other chromatographic and spectroscopic techniques. However, one attractive feature of CE is the compactness and robustness of the equipment, which would open the opportunity for the construction of portable instruments. These could be used as a confirmation tool by law enforcement agencies at the point of care, if the detection limits of the CE could be reduced to the required cutoff levels.[13] Such

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an instrument could become an attractive alternative to the immunoassays.

One candidate to such detection could be fluorescence. Recently, we proposed a CE method with a light emitting diode (LED) (λex = 280 nm) that induced native fluorescence detection for the analysis of cannabinoids in oral fluid.[14] The validation results showed that the detection limits of the CE-LED induced fluorescence method were less favorable than GC/MS in terms of LODs, although it could be considered acceptable for the determination of cannabinoids in saliva during a short time after smoking (a couple of hours). The lack of detection sensitivity can be overcome by using an excitation source at the first λex/λem maximum of cannabinoids (230/307 nm).[14] Unfortunately, there are no still commercially available fluorescence detectors with lasers or LEDs with longer wavelengths that can be coupled to the portable CE instruments. For instance, Picometrics Technologies SAS (France) offers two models of detectors, such as Zetalif LED with possible excitation wavelengths 365, 450, 480, 530, or 640 nm and a Zetalif Laser from 266 to 785 nm. Both detectors are not compact enough (43 cm × 23 cm × 34 cm, 12 kg) for incorporation inside the portable CE instrument and do not provide the desired excitation wavelength in the deep UV range around 230 nm for detection of the first spectral maximum of analytes, that has predominantly higher quantum yield. Recently, a 235 nm prototype LED was studied and used for construction of an UV-LED-based on-capillary photometric detector.[15] Although LEDs have several major advantages over conventional light sources (mercury, mercury−xenon, xenon, xenon flash, deuterium lamps) such as extended lifetime, a narrow emission band, low parasitic emission light, lower costs, and increased portability, the optical power of the 235 nm, LED prototype was assumed to be too low (0.052 mW) for the construction of a sensitive drug of abuse (DOA) analyzer.

In this paper, we report on the implementation of a miniature flash Xe-lamp with excitation broadband from 230 to 255 nm, instead of a LED that still lacks enough optical efficiency for the current application. The radiation bandwidth and intensity of such a source can be widened or cut to the specific wavelength range, and thus greater flexibility can be achieved. We demonstrate that a portable CE instrument can be built on such a lamp. Sample preparation and protocols for the separation of common drugs of abuse found in saliva will be reported. Finally, we demonstrate testing of this instrument at the "Weekend" electronic music festival (Pärnu, Estonia, August 2016 and 2017), where among 10 000 attendants, 31 of 36 in 2016 and 32 of 37 in 2017 tested positive for the use of various drugs of abuse. To the best of our knowledge, this work reports the first field use of deep UV, with a peak emission wavelength 240 nm, for fluorescence detection incorporated into a portable CE instrument.

EXPERIMENTAL SECTION

Chemicals. Standard solutions of tetrahydrocannabinol (THC) (10 mg/mL in ethanol), cannabidiol (CBD) (1 mg/mL in methanol), 3,4-methylenedioxymethamphetamine (MDMA) (1 mg/mL in methanol), 3,4-methylenedioxeamphetamine (MDA) (1 mg/mL in methanol), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA) (1 mg/mL in methanol), para-methoxyamphetamine (PMA) (1 mg/mL in methanol), para-methoxy-*N*-methylamphetamine (PMMA) (1 mg/mL in methanol), cocaine (1 mg/mL in methanol), cocaethylene (1 mg/mL in acetonitrile), D,L-amphetamine (AMP) (1 mg/mL in methanol), D,L-methamphetamine (METH) (1 mg/mL in methanol), and powders were purchased from Lipo ed AG (Switzerland). The internal standards of allocryptopine (IS1) and benzylamine (IS2) were from Sigma-Aldrich (USA). Background electrolyte constituents, including sodium hydroxide, methanol, and acetonitrile (ACN), phosphoric aci , (85%) and Tris(hydroxymethyl)aminomethane were obtained from Sigma-Aldrich (USA). All chemicals were ACS grade and the organic solvents were of HPLC grade. Deionized water was purified with Millipore (USA) Milli-Q equipment.

Fresh OF samples were collected, preconcentrated, and extracted with the Salivette tube (Sarstedt, Numbrecht, Germany) according to the procedure described in our previous study[14] with minor modification. The OF samples were collected during a 2 min period and centrifuged at 8000 rpm for 2 min. The first aqueous phase centrifugate was discarded and 1 mL of acetonitrile was added to the pad, providing the denaturation of the mucosa proteins and release of analytes absorbed through the oral mucosa remained inside the pad after the first centrifugation. The Salivette tube was centrifuged again under the same conditions, and the obtained centrifugate was injected for CE analysis. The collected samples and centrifugates were stored at −20 °C. This research was performed in accordance with the ethical standards and approved by the appropriate ethics committee. Informed consent was obtained from all volunteers prior to sample collection.

Fluorescence Detector. The xenon flash lamp module L9455-02 containing a 5 W flash lamp along its power supply and trigger socket was obtained from Hamamatsu, as well the photosensor module H10720-210 containing a metal package photomultiplier tube (PMT) and a high-voltage power supply circuit. Aspherical and spherical UV fused silica optical lenses, with high numerical apertures (NA) (0.3−0.63), and the spherical concave mirror were sourced from Edmund Optics. Three optical filters were used in the excitation channel for blocking the parasitic long wavelength radiation from xenon lamp: (a) interference bandpass filter 228FS25 from Edmund Optics (no. 68-337), (b) hard-coated bandpass filter ET240/40pb from Chroma Technology Corporation, and (c) hard-coated short-pass filter FF01-276/SP from Semrock. Hard-coated emission filters FF01-285/14, FF01-302/10, FF01-315/15, and FF01-267/LP were sourced from Semrock and nos. 84-704, 65-128, and 84-710 from Edmund Optics. The mechanical parts were made from aluminum (black anodization) and black ertacetal-C (POM-C).

The PMT signal was scaled with a simple transimpedance amplifier, converted to digital with a CY8CKIT-059 PSoC 5LP board (Cypress Semiconductor) and sent over USB to a PC with a virtual COM port and interface program. The experimenter can use this program for controlling measurement parameters and visualizing and storing results.

Design of Detector. The optical layout of the detector is shown in Figure 1A. The xenon flash lamp (1) delivered 0.5 μs light pulses at a repetition frequency of 450 Hz; the pulse input energy was 11 mJ. The xenon flash lamp had strong emission bands in the region of 230−260 nm, but it irradiated also in the broad spectrum range until NIR.

Therefore, excitation filters with very high blocking were required to cutoff the parasitic radiation higher than 260 nm. A set (3) of two bandpass filters and a short-pass filter were used to provide optical density better than OD11 above 275 nm. Data transmitted through the set of excitation filters spectral

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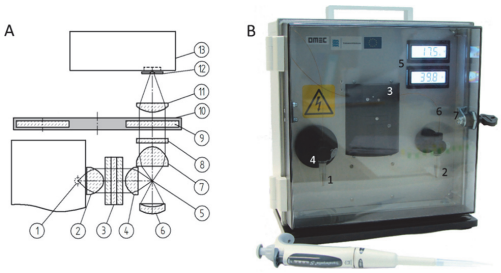

Figure 1. (A) Optical layout of the detector: (1) flash xenon lamp, (2, 4, 7, 11) lenses, (3) set of two bandpass filters and a short-pass filter, (5) capillary, (6) spherical mirror, (8) long-pass filter, (9) changeable emission filters, (10) filter wheel, (12) diaphragm, (13) PMT window. (B) CE-FD instrument: (1, 2) sample/background electrolyte inlet and outlet ports with Pt electrodes, (3) fluorescence detector, (4) electrode with high voltage power source, (5) digital voltammeter and ammeter, (6) capillary, and (7) door lock.

band is shown in Figure 2A. Aspherical lenses (2) and (4) were used to collect and focus the excitation light to the capillary (5) with high efficiency. Radiation emitted by the solution inside the capillary was collected and focused on the cathode of the PMT by lenses (7) and (11). A spherical mirror (6) was used to increase the light collection ability of the emission optical channel. Five emission filters (9) were mounted on a filter wheel (10): (a) CWL 285 nm, fwhm 18 nm (F_{EM1}); (b) CWL 302 nm, fwhm 10 nm (F_{EM2}); (c) CWL 315 nm, fwhm 20 nm (F_{EM3}); (d) CWL 337 nm, fwhm 10 nm (F_{EM4}); and (e) broadband 300−600 nm (F_{EM5}). A common long-pass filter (8) was used to enhance blocking. A diaphragm (12) was mounted in front of the PMT window (13) to suppress stray light.

An optical reference channel was introduced to eliminate the xenon lamp aging effect on measurement accuracy. The detector head contained a moveable light guide, which could be manually shifted into the reference signal measurement position for detecting the excitation intensity by the PMT. The reference signal was measured each time after turning on the detector, and its value was recorded in the memory and used for correction of measurement results.

Portable CE Instrument. The CE instrument was built around the fluorescence detector. It consisted of a high-voltage power supply for the detector (EMCO 250 DXn, USA) and a chemical compartment, consisting of a sample/buffer inlet and outlet ports. The overall size of the instrument was 20 cm × 10 cm × 30 cm and it weighed about 3 kg. The CE-FD instrument is shown in Figure 1B. Uncoated, fused-silica capillaries, i.d. 75 μm and o.d. 360 μm (Polymicro Technologies, Phoenix, AZ, USA), were used for the analyses.

The total capillary length was 58 cm, with the detection zone placed at 18 cm from the capillary end. Prior to injection, the capillary was rinsed sequentially with 0.1 M NaOH and the background electrolyte for 2 min each. The samples were injected into the capillary by hydrodynamic flow at a height differential of 15 cm for 10 s. Separations were performed at +20 kV. Before the measurements, new capillaries were conditioned by rinsing them sequentially with 1 M sodium hydroxide and deionized water. Between analyses, the capillaries were rinsed with the background electrolyte (BGE) solution for 2 min.

Two BGEs were used for analysis of illegal drugs. The first BGE (BGE1) was utilized for determination of amphetamine type stimulants (ATS), such as AMP, METH, MDMA, MDA, MDEA, PMA, PMMA and also cocaine and its metabolite cocaethylene. The second BGE (BGE2) was applied for analysis of cannabinoids.[14] BGE1 consisted of 42.5 mM phosphoric acid, 30 mM TRIS (pH 2.5), and BGE2 2.5 mM NaOH dissolved in MeOH/ACN (1:1) (pH* 12).

RESULTS AND DISCUSSION

Performance Characteristics of CE-FD. The construction of the fluorescence detector in the deep UV range was a challenging task for many reasons, such as the absence of optically powerful LEDs and filters with high optical density for blocking undesired parasitic light. It was even more challenging to combine such a fluorescence detector with a capillary electrophoresis system, due the complexity of focusing the light source inside the round capillary with a short light path (10−150 μm internal diameter). Besides this, it was also difficult to register classical angle (90°) fluorescence, along with reducing the scattered light from round capillary external and internal surfaces, elastic scattering from the background electrolyte, light

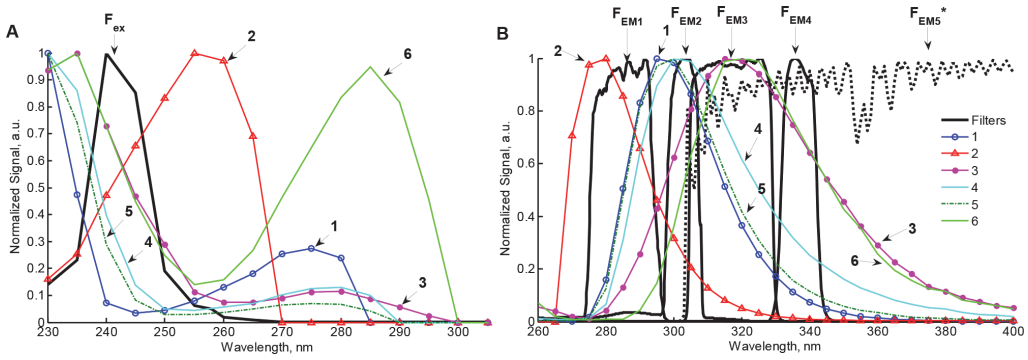

Figure 2. (A) Excitation spectra of illegal drugs and excitation filters sets (228FS25, FF01-276/SP and ET240-40bp), (B) emission spectra of illegal drugs and emission filters (F_{EM1}, F_{EM2}, F_{EM3}, F_{EM4}, and $*F_{EM5}$ is up to 600 nm): 1, PMA and PMMA in water; 2, AMP, METH, 3, cocaine in water; 4, THC in ethanol; 5, CBD in ethanol; 6, MDMA, MDEA, MDA in water.

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refraction in the capillary, or light scattering due to the irregularities of the capillary surface.

The optimal set of excited filters was found for blocking dominant parasitic light from the xenon lamp in the wavelength range of 270−600 nm. The choice of filters was based on illegal drug excitation and emission characteristics, manufacturer declared specifications, and the performance of the optical layout confirmed by experiments. The illegal drug excitation and emission characteristics are shown in Figure 2, along with filters sets utilized in the CE-FD instrument. The excitation and emission spectra of illegal drugs were obtained using NarTest NTX2000 Drug Analyzer (NarTest AS, Estonia) with operation parameters as previously described.[16] The spectra of illegal drugs are presented in Figure 2 for the 230−300 nm excitation wavelength range and 260−400 nm emission wavelength range with 5 nm step on excitation (EX) and emission (EM) modes, respectively. All fluorescence data were presented in normalized arbitrary units. The following performance characteristics for the designed CE-FD analyzer were evaluated: specificity, detector linearity, noise, and sensitivity.

The specificity of the CE-FD analyzer was assured by the properly utilized excitation/emission filters in FD and which properties were suited to the native fluorescence characteristics of illegal drugs in the specific region under excitation within the wavelength range of 230−255 nm. Moreover, the specificity was achieved by utilized CE mode with the specific electrophoretic separation conditions and a special sampling/extraction/preconcentration procedure. Therefore, the probability of comigrating of the fluorescing interference from another substance and their registering at the certain region of emission wavelength controlled by filters and CE conditions was minimized.

The linearity was evaluated according to the ASTM E578-07 (2013)[17] using MDMA solution in ACN as a standard test solution instead of quinine sulfate dihydrate solution in sulfuric acid, as ASTM proposed. MDMA was an appropriate substitute standard due to large quantum yield at very high dilution, high stability to the exciting radiation during spectral measurements, small overlap of fluorescence, and its absorption spectra, weak concentration dependence on the quantum yield.

Finally, MDMA was suited for the designed optical layout. The linearity was evaluated from 0.25 μg/L to 100 mg/L of MDMA in ACN in triplicate using static linearity measurements, i.e., without electrophoretic separation. The fluorescence of MDMA was corrected by subtraction of background fluorescence (acetonitrile). The lower limit of linearity was found to be 0.5 μg/L, and the upper limit of linearity was 50 mg/L. Figure 3 presents the linearity range using filter F_{EM3}.

The noise level was evaluated both in static (i.e., without electrophoretic separation) and dynamic (i.e., within electrophoretic separation) modes using filter F_{EM3}. The root-mean-square (RMS) of the noise was estimated as a peak-to-peak value, divided by 5.16 ($\pm 2.58\sigma$) (99% confidence level).[18] The noise CV% of the dynamic mode was evaluated for different PMT voltages from 500 to 800 V, with a 100 V step using the illegal drug's standard solution at the level of quantification. The digital noise was found at a low level of PMT gain 500 V.

Therefore, it was assumed that using a Savitzky-Golay filter that has increased the signal-to-noise ratio (1.2−1.7 times) would not greatly distort the signal. The noise of dynamic mode was evaluated between 2 and 5 min. The noise CV% for 500 V, 600 V, 700 V, and 800 V were 0.95%, 0.70%, 0.90%, and 0.80%, respectively. It was assumed that using 500 V would work, as it is more suitable for a high concentration range of illegal drugs that is unpredictable in the real oral fluid samples. The static mode was evaluated during 10 min using an empty capillary with a burnt detection window and filled with ACN. The noise CV% was 1.1% for an empty capillary and 0.6% filled with for ACN for static mode.

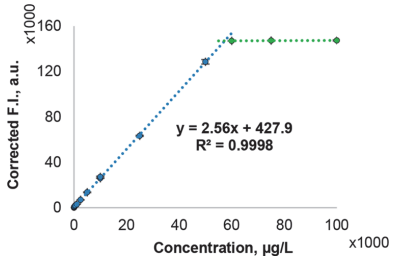

Figure 3. Detection fluorescence linearity depicted as a background corrected fluorescence signal vs concentration graph, estimated in the static mode using MDMA in ACN at filter F_{EM3} and 500 V.

The instrumental detection (IDL) and quantification (IQL) limits of the illegal drugs were evaluated in acetonitrile using developed and optimized CE methodologies, excluding the matrix effect of OF and sampling/extraction/preconcentration procedure recoveries. The exception was made for THC and CBD evaluation. The separation of these two cannabinoids using nonaqueous capillary electrophoresis (NACE) was described in the previous study[14] and drastically depends on the sample matrix.

The instrumental detection and quantitation limits were found using the signal-to-noise (S/N) approach.[20] The S/N ratio for IDL level equaled 3:1, proving the presence of the analyte in the test sample with a probability larger than 99%. The S/N ratio for IQL level was set to 10:1, respectively. The analysis of samples containing the analytes at the level of IDL and IQL was repeated 18 times, confirming the calculated results. The results are presented in Table 1. The electrophoretic separation of illegal drugs at IQL level using capillary zone electrophoresis is shown in Figure 4A.

Table 1. Detection Capability of CE-FD Analyzer for Illegal Drug Determination in ACN (N = 18)

substance	F_{EM}, nm	IDL, μg/L	IQL, μg/L	recommended cutoff limit, μg/L[19]
THC[a]	302	25	83	27
CBD[a]	302	25	83	N.D.
AMP	285	3000	10000	360
AMP	315	9500	31670	360
METH	285	3000	10000	410
METH	315	9500	31670	410
MDA	315	0.5	1.6	220
MDMA	315	0.5	1.6	270
MDEA	315	0.5	1.6	270
PMA	315	10	33	N.D.
PMMA	315	10	33	N.D.
cocaine	315	13	42	170
cocaethylene	315	15	50	N.D.

[a]Using BGE of 1:1 ACN−MeOH with addition of 2.5 mM NaOH, injection 45 s, spiked blank saliva sample. N.D. − not defined.

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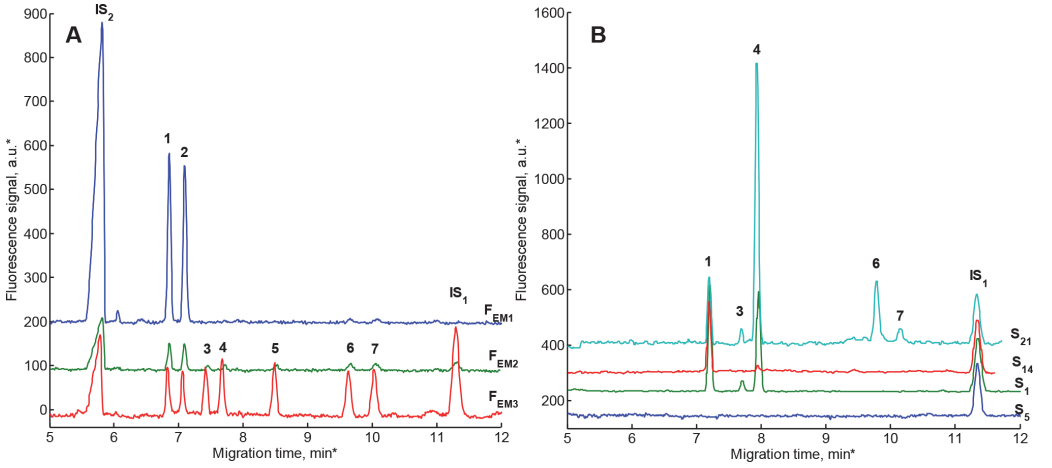

Figure 4. (A). Electropherograms of illegal drugs in acetonitrile obtained with emission filters F_{EM1}, F_{EM2}, and F_{EM3}: 1, AMP (66 mg/L); 2, METH (66 mg/L); 3, MDA (3.3 μg/L); 4, MDMA (3.3 μg/L); 5, MDEA (3.3 μg/L); 6, cocaine (83 μg/L); 7, cocaethylene (100 μg/L); IS1, allocryptomine (33 μg/L); IS2, benzylamine (300 mg/L). (B) Drug abuse suspected user's oral fluid samples eletropherograms at emission filter F_{EM3} no. S5, no. S1, no. S14, and no. S21. *, corrected by IS1 and IS2 peaks' migration time and IS1 peak height.

The results showed that the designed CE-FD instrument was able to detect MDMA, MDA, MDEA, cocaine, and THC at the recommended by DRUID project cutoff limits[19] for illegal drug abuse determination in oral fluids. Unfortunately, there are still no defined cutoff limits for PMA, PMMA, and cocaethylene for drug abuse determination in oral fluids. The last one is a metabolite of cocaine that appears when cocaine is used together with alcohol. CBD is a legal cannabinoid that is found both in illegal and predominantly legal cannabis plants. However, it is worth mentioning that CBD, and also other cannabinoids, may produce a "false positive" reading for illegal cannabis abuse using the immunoassay tests.[21] The developed CE-FD methodology eliminates this possible "false positive". Despite the impressive sensitivities for the above-mentioned substances, the sensitivity for amphetamine and methamphetamine still requires improvement to achieve the recommended cutoff limit for the DRUID project.

However, the real field testing showed that the drug concentration levels of amphetamine abuse users were much higher than recommended limits (100−2500 mg/L Weekend 2017 samples) than the cutoff limit. Therefore, such concentration levels of amphetamines were possible to detect even using filter F_{EM3}. However, the sensitivity of F_{EM3} was three times worse than at the filter F_{EM1}. It should also be highlighted that filter F_{EM1} was more suitable for AMP and METH fluorescence properties, but the sensitivity of F_{EM1} was affected by unideal blocking of parasitic light from the xenon lamp emission range. Obviously, customized filters with better blocking properties can improve the sensitivities, but it is still a challenging task in the CE system.

Analysis of Oral Fluid Samples. The oral fluid samples were provided to us by police officers from the Police and Border Guard Board (PBG) of Estonia (Pärnu Road 139, 15060 Tallinn, Estonia). In cooperation with PBG, their samples from suspects taken from the "Weekend 2017" electronic music festival were analyzed. There were around 40 from the 10 000 participants suspected and screened for illegal drug use. Typical electropherograms of suspects' oral fluid samples are presented in Figure 4B. Sample 5 was one of the negatives; samples 1, 14, and 21 were positives. The suspected users' oral fluids contained AMP, MDA, MDMA, cocaine, and cocaethylene. Also, the analysis was performed by local police officers using lateral flow chromatographic immunoassays DOA Urine Drugtest used with a Hand-held Reader Touch or multi drug urine cups (Ultimed, Belgium). In the cases when the official screening method was able to detect illegal drug presence in the suspect samples, the median coincidence rate was higher than 80%.

The distinction occurred due the differences in the illegal drug determination principle. The oral fluids contain the psychoactive illegal drug, while the urine samples contain predominantly illegal drug metabolites. The latter gives an overview of the illegal drug use timeline, even up to weeks for some drugs, but not the present drug intoxication. Therefore, the suspected user cannot be charged at the time of testing using urine tests, while the presence of the illegal drug in oral fluid indicates the current intoxication state and its level. Therefore, the differences in the results between the oral fluid and urine samples were expected. In addition to the oral fluid samples collected during the music festivals, the CE-FD instrument was successfully utilized for analysis of oral fluid samples collected by police during the roadside testing. The research results on the "Weekend" music festival samples of 2016 and 2017 and samples collected during the roadside testing are to be published separately.

■ CONCLUSION

This study demonstrates the first application of a deep UV excited fluorescence (excitation maximum around 240 nm) as a valuable tool for portable capillary electrophoresis instruments for the detection of drug abuse by analyzing oral fluid of suspects in situ. An approximate 6−25-fold decrease in LOD compared to our early result was achieved.

With respect to the simplified operating conditions, excellent selectivity, and miniaturization benefits of the proposed CE

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technology, it seems to be a very promising and reliable alternative to conventional laboratory GC/LC−MS for on-site analysis of drug abuse. The developed CE-FD instrument was capable of detecting several illegal drugs: marijuana (THC, CBD), AMP, METH, PMA, PMMA, MDMA, MDA, MDEA, cocaine, and cocaethylene. The results were illustrated also by field studies. Electropherograms of illegal drugs appeared to be free of interferences in the form of overlapping peaks. The tested drugs can be quantified using peak areas when required. The overall analysis time (15 min) is acceptable for use by law enforcement officers acting in the field. Altogether, the portable CE with deep UV exited fluorescence provides a platform for instrument as a quick, selective, and sensitive screening tool and even can be applied for confirmatory studies with a sufficient limit of detection, thus removing the time between suspicion and confirmation.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Use of a newly-developed portable capillary electrophoresis analyser to detect drugs of abuse in oral fluid: A case study

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ABSTRACT

The aim of the current study was to develop and validate an analytical method to determine whether drugs of abuse (DOA) were present in oral fluid (OF) using a newly-developed, portable capillary electrophoresis (CE) instrument coupled to a deep ultra-violet fluorescence detector (FD). The performance of this portable CE-FD DOA analyser was tested at the Weekend Festival Baltic (Pärnu, Estonia) between 2016 and 2018 as well as on the roadside OF samples collected by the police. The study reported 128 analysed cases in which persons were allegedly found to have been under the influence of DOA. The samples were analysed for amphetamine (AMP), methamphetamine (METH), 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), cocaine (COC) and cocaethylene (COET). Subsequent toxicological reports revealed that 26% cases were negative, and 74% were positive. The most frequently detected and quantified DOA was MDMA (68 cases, 62%). A comparative study was conducted to validate the accuracy of using the CE-FD DOA analyser versus classic high-performance liquid chromatography coupled to mass spectrometry (HPLC-ESI-MS). Diagnostic statistics for CE-FD DOA were also evaluated and were higher than 99.5%. In addition, all zeta-scores were lower than 2 when both methods were compared, showing that the CE-FD analyser can be implemented as a reliable, sensitive and convenient tool for roadside and workplace testing for DOA.

1. Introduction

Drug use and its misuse, facilitates the spread of infectious diseases such as hepatitis C and B, HIV and tuberculosis as well as being a public safety hazard (drugged driving, violence and accidents in safety-critical industries) [1,2]. Random testing of drivers on the roadside and in the workplace is used for the prevention of drug abuse. Such testing must be rapid and produce accurate results, which means sampling and detection should not take days or weeks, but minutes.

First, researchers must determine the best way to assess whether an individual is currently under the influence of drugs and poses a possible risk. Drugs of abuse (DOA) testing can be conducted using various biological samples like urine [3], blood [4–6], exhaled breath [7,8] and oral fluid (OF) [6,9]. The pharmacokinetics must be considered depending on sample matrix characteristics as some samples, (urine and hair) reveal retrospective information about past drug use but cannot be used to determine whether alleged individuals are currently under the influence of drugs. Although blood is most frequently used and reflects the presence of DOA, the drawbacks include invasive sample collection and long processing time. Therefore, urine and blood are not preferred sample matrices for the determination of the present state of intoxication of an individual, which requires rapid, on-site testing. Consequently, matrices like exhaled breath and OF have increasingly been used for DOA testing. OF is a mixture of the excretions from the major and minor salivary glands, gingival crevicular fluid, oro-nasopharyngeal secretions and cellular debris [10]. OF has gained popularity with respect to on-site testing because sample collection is easy, can be performed by non-trained personnel, and has minimal privacy issues. In addition, it provides information about the current state of intoxication, not retrospective information. Despite these advantages, some drugs such as THC, cocaine and methamphetamine decrease the flow of, leading to a dry mouth [11]. Thereupon, sample collection can be challenging when individuals are intoxicated. In addition to OF collection issues, reports analysing how levels determined via OF are correlated to other biological fluids have produced variable results [6,12]. It is not expected that there will ever be 100% correlation between drug test results from different body fluids.

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Generally, OF testing tools have been based on immunoassay methods, since they are highly sensitive, simple to administer, and disposable testing kits can be constructed in a relatively cheap manner. The interpretation of the results of these tests is usually visual and qualitative (indicators will show whether a sample is present or not). The main drawback associated with immunoassay testing is the high rate of false-positives that can occur due to the cross-reactivity of prescribed medication, tobacco, food etc. [13–15]. Despite its low specificity and inherent qualitative nature, one device, Dräger DrugTest® 5000 (Draeger Safety AG & Co. KGaA, Luebeck, Germany), is now approved for illegal drug abuse determination in OF and can be officially employed by police for roadside testing in Canada [16].

In addition to the immunoassay method, mass-spectrometry (MS) [5,17], infrared spectroscopy [18,19] and ion mobility spectroscopy [20,21] can be utilised to detect DOA. Separation techniques such as capillary electrophoresis (CE) and chromatography, are widely used in clinical, forensic and chemical laboratories [4,22]. Indeed, the high resolution, short time requirement, minimal volume of sample and the possibility to miniaturise the apparatus makes CE ideal for on-site testing. CE is also gaining prominence in, for example, the European Guidelines for Workplace Drug Testing, for its reliability [23]. The native fluorescence of DOA including amphetamine and derivatives (*Ecstasy* and its analogues), coca alkaloids, cannabinoids, and opioids [24] in the deep ultraviolet (DUV) range enables the fluorescence detection and quantification of the drugs at the low concentrations found in OF post drug use [25,26]. In addition, fluorescence provides high selectivity as excitation and emission wavelengths are specific to particular drugs.

This study aimed to evaluate the performance characteristics of a newly-developed, illegal DOA analyser that was based on CE and fluorescence detection in DUV excitation wavelength range (λ_{EX} = 230–255 nm). The thorough validation of the developed analytical procedure was conducted according to European Medicinal Agency (EMA) guidelines regarding bioanalytical method validation [27], quality management was assured according to ISO 17 025 requirements. The reliability of the quantitative results and the diagnostic performance of the analyser were estimated by the measurement of 128 real oral fluid samples by an independent reference HPLC-MS method. An overview about the DOA usage trends at the Weekend Festival Baltic over the three-year period (2016–2018) has been also provided.

2. Materials and methods

2.1. Chemicals and reagents

Standard solutions and/or powdered drugs were purchased from Lipomed AG (Switzerland) and included the following chemicals and reagents in 1 mg mL^{-1} in methanol and/or 10 mg free base powder: 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxeamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), *para*-methoxyamphetamine (PMA), *para*-methoxy-*N*-methylamphetamine (PMMA), cocaine (CO), cocaethylene (COET), D,L-amphetamine (AMP), D,L-methamphetamine (METH), d,l-fentanyl, 3-methyl-fentanyl, 4-OH-amphetamine, 4-hydroxy-3-methoxyamphetamine (HMA), 4-hydroxy-3-methoxymethamphetamine (HMMA), benzoylecgonine (BZE), heroin, morphine, codeine, psilocin, and lysergic acid diethylamide (LSD). D,l-amphetamine-d3 (AMP-d3), D,L-3,4-methylenedioxymethamphetamine-d3 (MDMA-d3), and cocaine-d3 (COC-d3), 3-monoacetylmorphine (3-MAM), hydromorphone, methadone, ecgonine, ephedrine, norephedrine. 4-OH-methamphetamine (1 mg mL^{-1} in acetonitrile) named Pholedrine®, was purchased from Canada (Toronto Research Chemicals, Canada). All were purchased according to the Estonian Agency of Medicine license IN-3-8.1/5520–2.

The internal standards allocryptopine (IS1) and benzylamine hydrochloride (IS2), were purchased from Sigma-Aldrich (USA). The background electrolyte constituents, including sodium hydroxide (NaOH), acetonitrile (ACN), tris(hydroxymethyl)aminomethane (Tris) and formic acid (FA) were obtained from Sigma-Aldrich (USA). All chemicals and organic solvents were ACS and HPLC grade, respectively. Ortho-phosphoric acid (H_3PO_4) (85%) was purchased from Fluka (Germany). Milli-Q water was used for preparation of the solutions.

All pharmaceuticals, used to assess the selectivity of the method, were purchased from the local drugstore, shown in supplementary data Table S1. Tyramine, dopamine, phenylacetic acid were also bought from Sigma-Aldrich (USA).

2.1.1. Oral fluid sample preparation

The OF samples were collected using Salivette® oral fluid collectors using a cotton pad (Sarstedt, Numbrecht, Germany) and centrifuged (EBA 200S, Hettich, Germany) at 6153 g for 10 min. The first centrifugate was discarded and 1 mL of ACN was added to the pad. The Salivette tube with cotton pad was centrifuged again at 6153 g for 2 min 900 μL of centrifugate was transferred to a 1.5 mL Eppendorf tube, already containing an air-dried mixture of IS1-IS2 stock. The sample was then thoroughly mixed and used for CE-FD or HPLC-MS analysis. The spiked samples were stored at 5 °C prior to analysis and at −20 °C for overnight storage.

2.2. Field testing oral fluid samples

Overall, 112 oral fluid samples were collected from donors at the Weekend Festival Baltic from 2016 to 2018 in Pärnu, Estonia. In addition, 18 OF samples were collected in the same period of time by traffic police throughout daily roadside DOA testing of allegedly intoxicated drivers. To assess the selectivity of the method the testing included the collection of samples from 15 non-drug users, 18 smokers, 2 snuff users and 2 people after physical training, 3 taking different prescription drugs, 20 eating and drinking (n = 60). The samples were collected using a Salivette® pad for 2 min. The samples were stored in the refrigerator (5 °C) prior to the extraction procedure or immediately processed according to the sample preparation procedure described above. This research has been performed in accordance with ethical standards and was approved by the appropriate ethics committee. Informed consent was obtained from all volunteers prior to sample collection.

2.3. Calibrator and quality-control solutions

The calibrator solutions in ACN (regression I) were prepared at nine different concentrations. For the preparation of calibrator solutions in pre-processed oral fluid (PPOF) (regression II) the pooled sample matrix was prepared after collecting the saliva from six volunteers (two samples from each) and processed as described above. Thereafter, samples were thoroughly mixed together providing the PPOF matrix. This pooled PPOF was divided into aliquots, which were spiked with same concentrations as were used for ACN solutions. The third calibration (regression III) method was carried out by adding standard into the unprocessed, pooled OF sample matrix. OF samples were collected using the passive drool technique from six volunteers (3 mL OF was collected from each volunteer) and combined into pooled sample, which was thoroughly mixed by shaking. Then pooled OF was divided into 1 mL aliquots, which were spiked with nine different concentrations of standard. These spiked aliquots were pipetted onto Salivette® cotton pads prior to processing as described above. Each concentration was injected six times.

The stock solution of internal standards IS1 and IS2 (IS1-IS2 stock) contained 735 ng mL^{-1} of allocryptopine (IS1) and 6000 μg mL^{-1} of benzylamine (IS2) diluted in ACN. Then, 45 μL of IS1-IS2 stock solution was pipetted to a 1.5 mL Eppendorf vial and air-dried using an Eppendorf concentrator (Concentrator plus, Eppendorf AG, Germany) at 20 °C for 30 min. The ready-to-use Eppendorf vials were stored at −20 °C prior use.

2.4. Electrophoretic and fluorescence protocol specifications

All CE experiments were carried out using the portable CE-FD DOA analyser [28]. A fused silica uncoated capillary with an inner diameter of 75 μm and outer diameter of 360 μm (Polymicro Technologies, Phoenix, AZ, USA) with an effective length (l_{ef}) of 35 cm and a total length (L_{tot}) of 53 cm was used for the analyses. Prior to use, the new capillary was activated with a 20-min rinse with 1.0 M NaOH solution, followed by a 20-min rinse with Milli-Q water and, finally, a 30-min rinse with background electrolyte (BGE). Prior to analysis, an already activated capillary was rinsed for 10 min with 1.0 M NaOH and Milli-Q water, followed by a 10-min rinse with BGE. Between every run, the capillary was rinsed for 2 min with the BGE. The BGE consisted of 36 mM Tris, 38 mM H_3PO_4 in water, pH 3.3. The samples were hydrodynamically injected at a height differential of 20 cm for 10 s. The applied voltage for the separation was −20 kV and the current was from 55 to 65 μA during the CE run.

The CE-FD DOA analyser had excitation spectra from 230 to 255 nm, with the peak at 240 nm, and five emission filters in the wavelength range from 285 to 600 nm. Detailed information regarding the CE-FD DOA construction was previously published in Ref. [25]. One of the emission filters with a wavelength range from 305 to 325 nm was chosen as a default emission filter for all analytes of interest. The voltage of the PMT (photomultiplier tube) detector was set to 600 V.

2.5. Liquid chromatography mass spectrometry conditions

Liquid chromatography mass spectrometry (HPLC-MS) was performed using a 1200 Series HPLC instrument (Agilent Technologies, Inc., Palo Alto, CA, USA) using Eclipse Plus C18 2.1 × 150 mm 3.5 μm column (Agilent Technologies, USA). A binary gradient was used, consisting of mobile phase A (Milli-Q with addition of 0.1% FA) and B (ACN with addition of 0.1% FA) at a flow rate of 0.4 mL min^{-1}. The gradient of the mobile phase was as follows: 0 min 0% B; 3 min, 15% B; isocratic for 0.5 min; 4.5 min, 27% B; isocratic for 6 min; 12 min, 100% B; hold 3 min; 20 min, 0% B. Equilibration time was 5 min prior to the next injection. Total run time was 25 min and 5 μL was injected into the chromatographic system.

The HPLC system was coupled to a 6300 Series Ion-Trap mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionisation (ESI) source working in positive mode. Compounds were detected by multiple reaction monitoring (MRM), with the protonated molecular ion $[M+H]^+$ employed as the precursor. The quantification the precursor ion was extracted in the form of an extracted ion chromatogram (EIC), all the chosen precursors have been shown in Table 1. Fragmentation of each protonated compound was used to confirm the identity of analytes. Nitrogen was used as nebulisation and desolvation gas, helium (purity 5.0) as collision gas. The optimal MS parameters were as follows: drying gas temperature was 350 °C, dry gas flow was 10 L min^{-1}, nebuliser was at 50 psi, capillary voltage was 3780 V and the skimmer was set to 25.0 V. Deuterated internal standards (AMP-d3, MDMA-d3, and COC-d3 at 0.1 mg L^{-1}) were added to each sample, to compensate for possible interferences in the ESI source.

2.6. Method validation

The CE analytical procedure was validated according to EMA guidelines for bioanalytical method validation [27]. The following parameters were assessed: selectivity, limits of detection (LOD), lower limits of quantification (LLOQ), linearity, matrix effect (ME), extraction recovery (R), stability of analytes, precision, accuracy and carry-over effect. In addition, uncertainty was also evaluated.

Selectivity of the method was studied using blank OF samples collected from 35 volunteers. OF samples from volunteers were collected from both males and females aged between 21 and 70 years. Among the volunteers, 18 were smokers and two were snuff users, additional OF samples were collected after 2 h of physical workout, after drinking tea, coffee, coke, alcohol and energy drinks as well as from people who have taken prescription medication. The selectivity of the procedure was also studied by deliberate spiking of PPOF samples with potential interfering agents. Altogether, 58 different substances were studied, namely 8 metabolites of drugs under investigation, 14 other DOA, 33 pharmaceutical drugs and three substances that are presented in Table S2 (supplementary data). If a potentially interfering compound was detected, a LOD value of the interferent was assessed and compared to possible concentration levels in OF according to published data.

Three calibration curves, demonstrating the relationship between the nominal concentration of analytes and the response of the analytical method to the analyte, were constructed using a linear least-squares regression in ACN (I), PPOF (II) and OF (III). The responses of analytes were normalised to an internal standard (area of analyte/area of internal standard IS2).

The limit of detection (LOD), and lower limit of quantification (LLOQ), were determined by measuring a series of decreasing concentrations of fortified OF samples. The LODs and LLOQs were determined by signal-to-noise (S/N) ratios of 3*S/N and 5*S/N for LODs and LLOQs, respectively, for all analytes with acceptable peak shapes. The calibration range was defined by the lowest limit of quantification (LLOQ) and upper limit of quantification (ULOQ).

Linearity, or ability of a method to produce results directly proportional to analyte concentration within a given range, was estimated using lack-of-fit ANOVA test for all regressions [29]. If the estimated probabilities (P) were greater than 0.05, the hypothesis that a linear relationship exists was accepted.

The extraction recoveries (R%) were determined as ratios of normalised response in fortified pooled OF samples to the normalised response in fortified PPOF. Matrix effect (ME%), that shows suppression/enhancement of analyte response by co-migrating compounds, occurred in the OF matrix and was calculated using the ratio of the normalised response in PPOF and the normalised response in ACN.

Accuracy was estimated by root mean square (RMS) of single

Table 1
Time segment limits of dynamic MRM.

Analyte	Time segment (number of ions selected)	Time segment (min)	Selected precursor *m/z* ratios $[M+H]^+$	Retention time (min)	Corresponding IS
AMP	7 *m/z*	0.0–7.1	136.4	5.7	AMP-d_3
MDA			180.4	6.0	MDMA-d_3
METH			150.4	6.1	AMP-d_3
MDMA			194.4	6.3	MDMA-d_3
MDEA			208.4	6.7	MDMA-d_3
AMP-d_3			139.1	5.7	
MDMA-d_3			197.1	6.3	
COC	4 *m/z*	7.1–10.0	304.4	7.6	COC-d_3
COET			318.4	8.3	COC-d_3
FEN			337.5	9.1	COC-d_3
COC-d_3			307.1	7.6	

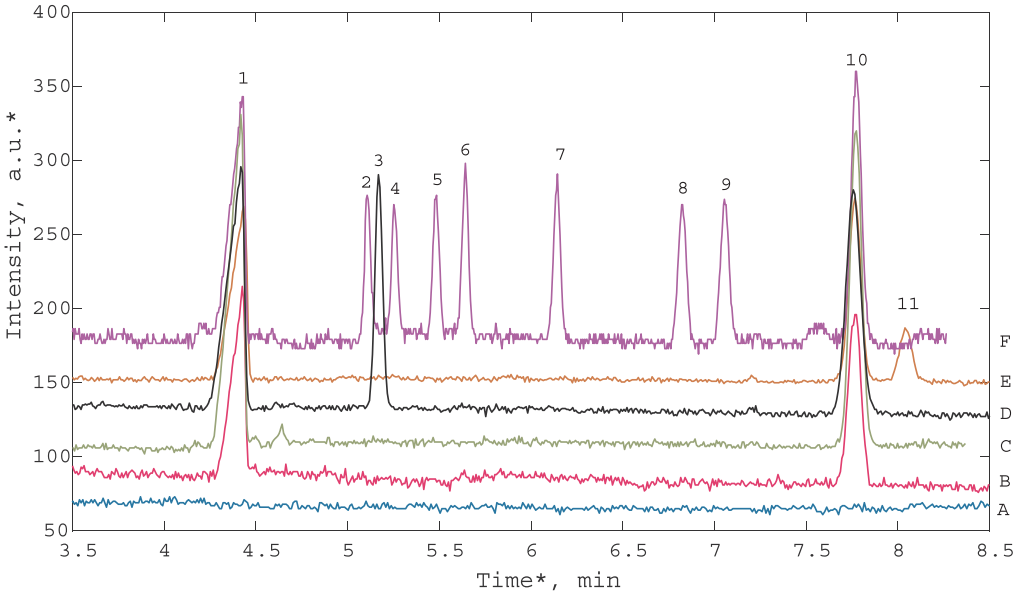

Fig. 1. Electropherograms of different OF. A – Blank OF, B – Blank OF with IS, C – OF from a snuff user with IS, D – OF from a smoker with IS, E – OF from volunteer, F – OF with all analytes of interest. Peaks: 1 – benzylamine (IS1) 300 μg mL^{-1}, 2 – AMP 133 μg mL^{-1}, 3 – tyramine, 4 – METH 133 μg mL^{-1}, 5 – MDA 20 ng mL^{-1}, 6 – MDMA 20 ng mL^{-1}, 7 – MDEA 20 ng mL^{-1}, 8 – COC 95 ng mL^{-1}, 9 – COET 90 ng mL^{-1}, 10 – allocryptopine (IS2) 35 ng mL^{-1}, 11 – Metoprolol.

measurement bias (n = 10). To estimate bias, pooled OF was spiked throughout eight-week period at two different concentrations. Quantitative results were obtained by using regression (III). Bias considered the stability/instability of the analyte during the analysis, recovery, matrix effect and other forms of bias (standard purity, volumetric ware, pipetting, etc.).

Between-run precision was determined by the analysis of seven samples during a one-month period. Each sample was analysed five times (n = 35). Within-run precision was evaluated by analysing 6 samples at each concentration (n = 6). Precision was measured at two concentrations for all analytes of interest. Using between-run precision (random error) and accuracy (systematic error) data, measurement uncertainty (*U%)* (k = 2, norm) was calculated. According to the EMA guidelines, uncertainty should not exceed 30%.

A thorough investigation of the stability (ST) of analytes ensured that they remained stable during sample preparation, processing and sample storage within the prescribed time intervals. Short- and long-term stabilities (ST) were assessed at two concentrations according to EMA specifications. The stability of analytes was evaluated in pre-processed OF stored at 5 °C and −20 °C for 12 weeks and at room temperature for 12 days. In addition, the number of freeze-thaw cycles that could be handled by samples were evaluated.

The correlation between measurements was estimated using z-scores calculated using the average of three replicates and the standard deviations for each sample measured using two different methods.

2.7. Diagnostic performance parameters

Diagnostic performance characteristics such as sensitivity (SE), diagnostic specificity (SP) and diagnostic accuracy (DA) show how reliably a method can accurately identify the presence or absence of an analyte in the sample [30]. The number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) were used to calculate sensitivity (SE = TP/[TP + FN] × 100%), diagnostic specificity (SP = TN/[TN + FP] × 100%) and diagnostic accuracy (DA = [TP + TN]/[TP + TN + FP + FN] × 100%). TP and TN were evaluated using a reference method. If the concentration of DOA exceeded the LOD of the reference method, it suggested the sample was a true positive. The sample, containing at least one analyte of interest, was assumed as positive for certain analyte of interest and negative for the rest of the analytes of interest.

3. Results and discussion

3.1. CE method development and optimisation

Following a previously published procedure for determination of ecstasy levels in oral fluid [31] revealed a certain instability for triethylamine (TEA), which was one of the main components of the background electrolyte (BGE). The proposed BGE in the previous study was sensitive to even small temperature fluctuations and rather unstable when stored for extended periods. Also, short analysis time is crucial for on-site analysis, these finding suggested that more stable BGE, better suited for the rapid analysis of illegal DOA in OF, must be found.

During the optimisation of the BGE composition, the TEA additive was exchanged to Tris, which was the most stable as well as enhancing sensitivity among the tested chemicals. Also, effects of altering the concentration of H_3PO_4 (from 36 to 45 mM) and of Tris (from 36 mM to 42 mM) and pH (from 2.3 to 4.5) on separation efficiency/time were studied. The effective length of the capillary was assessed from 30 cm to 40 cm. The optimal results were obtained with a BGE composition of 38 mM Tris, 36 mM H_3PO_4 at pH = 3.3 and capillary length of l_{ef} = 35 cm and L_{tot} = 53 cm. Under these conditions an efficient peak separation (Rs > 1.2) for all analytes of interest, even at high concentrations, was achieved and the electrophoretic run took less than 8 min. The stability of the BGE was tested over a period of six months of storage at room temperature and showed sufficient stability to provide reproducible separation.

3.2. Validation study

3.2.1. Selectivity

The selectivity of the method was evaluated through assessing endogenous matrix interferents by analysing the 15 samples collected from the DOA-free volunteers and fortified with IS. Most of the collected samples had stable baselines and no endogenous signal contributions were observed (Fig. 1A–C). Additional peaks were observed in only two cases. One of them was identified as Metoprolol; a medicine, prescribed for patients with hypertension (Fig. 1E). Another sample, obtained from a smoker, produced an extra peak which was identified as tyramine (Fig. 1D). Tyramine is an endogenous amine, a metabolite of tyrosine, and is also found in many fermented foods and drinks [32]. Tyramine migrates between amphetamine and methamphetamine peaks and could produce false positive results based on visual estimation. However, the in-house developed expert system for peak recognition has efficiently differentiated tyramine from other analytes under study. Utilising chemometric algorithms for peak recognition are not discussed in this publication, as a publication on the matter is forthcoming.

Selectivity was also assessed by fortifying PPOF samples with 58 potentially interfering substances (shown in Supplementary Table S2). The peak recognition system was used to determine if the compounds would overlap with the DOA. Only two possible interferences were found from tested compounds, codeine and citalopram. Several other analytes (alprazolam, sulfamethoxazole, pseudoephedrine and dopamine) migrated similarly to analytes of interest. Despite this, their response was lower than 20% of the response of studied drugs at the LLOQ level.

3.2.2. Calibration curves, linearity, limits of detection and quantification

The calibration range was determined for all calibration curves. The calibrations (I) and (II) ranged from 1 to 10 000 ng mL^{-1} for MDA, MDMA and MDEA, and from 20 to 10 000 ng mL-1 for CO, COET, respectively. The calibration curve (III) ranged from 10 to 10 000 ng mL^{-1} for MDA, MDMA and MDEA, and 50 to 10 000 ng mL^{-1} for CO and COET. Regarding the calibration of AMP and METH, the calibration (I) and (II) ranges were from 20 to 500 μg mL^{-1} and for calibration (III) from 70 to 500 μg mL^{-1}.

Table 2 depicts method validation results regarding linearities, limits of detection and quantification. The plots of the peak area ratios (analyte to IS2) versus the concentrations of the standards in three different matrices exhibited adequate linearities for studied analytes, all with acceptable statistical parameters. The coefficients of the determinations (r^2) were > 0.99 and the regression errors were below < 10%. The results of the statistical lack-of-fit ANOVA test also confirmed the linearity of all described regressions, since estimated probabilities (*P*) were all greater than 0.05.

Several international projects aimed, inter alia, to elaborate on recommended cut-off values for DOA in OF have been carried out during the last decade. Cut-off values are used to define the lowest concentration levels from which a sample is considered positive. The best-known projects DRUID [33], for roadside testing, and ROSITA-2 [34] provided cut-off values for a list of drugs for both screening and confirmation analysis. Two other regulatory documents, the European guidelines for workplace drug testing in oral fluid (EWDTS) [40] and American SAMSHA Mandatory Guidelines [35] propose cut-off levels for screening tests which are similar to the ROSITA-2 project results (Table 3).

Table 3
Comparison of detection limits of CE-FD to proposed cut-off values in OF.

Substance	CE-FD, ng mL^{-1}	DRUID, ng mL^{-1} [33]	EWDTS, ng mL^{-1} [23]	ROSITA-2, ng mL^{-1} [34]	SAMSHA, ng mL^{-1} [35]
AMP	40[a]	360	40	25	25
METH	40[a]	410	40	25	25
MDA	6	220	40	25	25
MDMA	6	270	40	25	25
MDEA	6	270	40	25	25
COC	28	170	30	8	15
COET	27	–	30	–	–

[a] μg mL^{-1}.

CE-FD analyser LOD and LLOQ values for MDA, MDMA, MDEA and COC met proposed cut-off levels by both DRUID and EWDTS studies, and without any additional sample pre-treatment would also fit the SAMSHA and ROSITA-2 limits for MDA, MDMA and MDEA. The oral fluid LODs were 6 ng mL^{-1} for MDA, MDMA, MDEA, around 30 ng mL^{-1} for COC, COET and 40 μg mL^{-1} for AMP and METH. However, for amphetamines the proposed method LODs obtained using a default emission filter still need to be significantly improved for amphetamine and methamphetamine determination in OF. The AMP and METH LODs were improved when the amphetamine emission filter was applied when taking measurements. However, this step required additional analysis, thus increasing the overall analysis time per OF sample, and failed to meet proposed cut-off values. Therefore, it was suggested that the development of the next generation of CE-FD DOA analyser should be initiated.

3.2.3. Recovery and matrix effect

Extraction recoveries depended on concentration level. To ensure the reproducibility of the sample processing steps all swabs were weighed before use, after the OF collection, and after the first and after second centrifugations. The retained amount of in the Salivette® pad after the first centrifugation was 0.28 ± 0.04 g (CV% 14.1%, n = 39) and did not depend on whether the OF was pipetted on the pad or collected from the oral cavity from a suspected user or a drug-free volunteer. OF sample weighs from Weekend Festival Baltic 2018's and six lab comparisons are presented in Table S3 (supplementary data).

The extraction recoveries for amphetamines ranged from 11 to 13%, for amphetamine derivatives (MDMA, MDA, MDEA) up to 35% and for cocaines from 19% to 25%. The loss of analytes of interest was caused

Table 2
The calibration curves and detection limits of substances in OF.

Analyte	Regression (I)			Regression (II)			Regression (III)			LOD, ng mL^{-1}	LLOQ, ng mL^{-1}
	RE (AU, %)	r^2	P	RE (AU, %)	r^2	P	RE (AU, %)	r^2	P		
AMP	0.03/0.5	0.9957	0.754	0.07/1.2	0.9989	0.690	0.050/0.1	0.9966	0.901	40[a]	66[a]
METH	0.02/0.5	0.9967	0.677	0.05/1.0	0.9992	0.711	0.040/0.2	0.9969	0.863	40[a]	66[a]
MDA	1.05/1.6	0.9993	0.764	0.13/2.0	0.9997	0.817	0.119/1.1	0.9995	0.928	6	10
MDMA	1.38/1.8	0.9990	0.487	0.32/1.4	0.9998	0.996	0.307/2.2	0.9991	0.928	6	10
MDEA	0.91/1.1	0.9997	0.586	0.42/1.5	0.9996	0.828	0.197/1.8	0.9992	0.905	6	10
COC	0.42/3.6	0.9967	0.993	0.10/0.9	0.9997	0.646	0.026/1.2	0.9998	0.840	28	47
COET	0.23/1.9	0.9991	0.936	0.22/1.5	0.9991	0.625	0.068/2.2	0.9992	0.882	27	45

[a] μg mL^{-1}.

Table 4
Performance parameters (precision, recovery, ME, accuracy) in OF.

Analyte	Conc., ng mL^{-1}	Within-run precision, (CV %)	Between-run precision, (CV %)	R% (CV%)	ME% (CV%)	Accuracy, (RMS bias, %, n = 10)	Uncertainty, k = 2
AMP[a]	200[a]	1.0	8.5	11 (7.0)	113 (2.6)	5.2	19.9
	400[a]	0.7	8.6	11 (3.7)	119 (2.4)	5.0	19.8
METH[a]	200[a]	1.9	7.3	13 (4.2)	111 (1.0)	9.1	23.4
	400[a]	1.0	5.2	13 (3.7)	113 (2.7)	7.8	18.7
MDA	100	0.7	5.5	35 (12.0)	106 (2.2)	5.1	15.1
	1000	2.8	9.1	17 (5.3)	99 (2.5)	7.0	22.9
MDMA	100	1.2	7.2	31 (12.5)	98 (1.9)	7.4	20.6
	1000	2.9	9.1	19 (3.8)	98 (1.3)	7.9	24.1
MDEA	100	2.4	8.0	28 (15.2)	94 (2.1)	6.6	20.8
	1000	1.6	5.6	16 (3.3)	103 (1.6)	7.6	18.9
COC	300	3.1	9.2	25 (3.8)	104 (3.3)	8.6	25.2
	2000	3.5	9.0	19 (3.2)	103 (1.1)	8.2	24.3
COET	300	2.5	5.8	25 (2.8)	110 (2.7)	7.6	19.1
	2000	3.1	11.1	22 (2.5)	117 (1.3)	8.5	27.9

[a] μg mL^{-1}.

mainly by the weak adsorption of the analytes by the Salivette cotton pad. Nevertheless, the discharge of the first centrifugate was not influential. First, the weight of remaining inside the pad upon the extraction with ACN during the second centrifugation remained stable. Second, the OF samples were filtered by the cotton swab from large mucinous material that could interfere with CE analysis. The extraction recoveries (R%) are shown in Table 4.

3.2.4. Analytical reliability parameters

According to EMA requirements, variation in the precision and accuracy of the procedure should not exceed 15%, and expanded uncertainty should not exceed 30%. The within-run and between-run precision, accuracy, and uncertainty results for all analytes of interest, at two different concentrations, are presented in Table 4. The within-run precision varied from 0.7% to 3.5%, while between-run precision varied from 5.2 to 11.1%, accuracy varied from 5.1% to 9.1%, and uncertainty values were consistently below 30%, therefore EMA requirements were satisfied.

3.2.5. Stability and carryover effects

Analysis of the stability of analytes revealed that the analytes of interest were stable during the testing period, while storing at 5 °C up to 12 weeks and even at room temperature up to 12 days. The results are presented in Table S4 (Supplementary data).

Higher variability of quantitative results were observed for all analytes of interest stored at −20 °C than other temperatures. Visual inspection of the samples showed that OF became heterogeneous after the thawing. It was determined that a minor amount of the peptides remained unprecipitated and were denatured inside the cold acetonitrile while stored at −20 °C. The residues of peptides affected separation of compounds, producing inconsistent CE results. Nevertheless, this issue can be overcome using an additional step in which samples are filtered through a 0.45 μm cellulose syringe filter before injection into the analyser.

A freeze-thaw stability study demonstrated that the samples could be frozen and thawed up to three times before the concentrations of analytes measured are more than ± 15% of the initial analyte concentrations. The DOA-free samples were injected immediately after samples with ULOQ concentrations of all DOA and showed no evidence of carry-over (the fluorescent signals were below the LOD for all analytes).

3.3. Weekend Festival Baltic results

In cooperation with the Estonian Police, 110 real samples were collected at the Weekend Festival Baltic, Pärnu, Estonia throughout 2016–2018 from people suspected of being under the influence of drugs. All samples were voluntarily provided using a Salivette device and analysed for AMP, METH, MDA, MDMA, MDEA, COC and COET. Since AMP and METH concentrations reported in OF were in the ranges of 230 ng mL^{-1} to 10 000 ng mL^{-1} [36,37], which was lower than the LOD of the described CE-FD method, their detection was not possible. Therefore, AMP and METH were left out of the comparative analysis. The rest of the analyte concentrations found in OF using the CE-FD DOA analyser were compared to HPLC-MS results. The summarised results of samples collected at Weekend Festival Baltic from 2016 to 2018 have been presented in Table 5. All results of CE-FD analysis of samples collected throughout the Weekend Festival Baltic from 2016 to 2018 have been presented in Tables S5–S7 (Supplementary data).

Of the 37 samples collected from the Weekend Festival Baltic in 2018, 25 were positive (67.6%) for at least one narcotic substance and 12 (32.4%) with no detected DOA. The most frequently detected drug was MDMA, which was found in 21 samples (56.8%) at concentrations ranging from 34 ng mL^{-1} to 767 ng mL^{-1} The next most frequently detected substance was MDA. MDA is the first metabolite of MDMA and can also be taken on its own. Cocaine was detected in the OF samples of three individuals, and in one case COET was also present, indicating that alcohol was consumed along with COC. In 2017 and 2016 MDMA was also the most commonly identified DOA, identified in 25 and 23 OF samples, respectively. Overall, trends of COC use from 2016 to 2018 showed a sharp decline. In 2016, 48.3% of samples tested positive for COC, while only 11% were positive in 2018. Fig. 2 shows a representative electropherogram of an MDMA sample (Fig. 2D), and a COC with COET sample (Fig. 2E).

Tyramine was found in OF along with analytes of interest. Higher tyramine levels are associated with smoking (up to 400-fold higher than for non-smokers) [38] and alcohol use, which are both common in summer festivals. Tyramine migrates between AMP and METH and is easily distinguished by migration time ratio to IS as well as by using a standard addition method (Fig. 2B and C). Tyramine was detected in 60 of the Weekend Festival Baltic samples, a finding that was confirmed by HPLC-MS.

Comparative analysis with HPLC-MS showed a good correlation between both qualitative and quantitative results. The quantitative results for MDA were tightly correlated (r^2 = 0.9937; concentrations were from 15 ng mL^{-1} to 400 ng mL^{-1}). For MDMA, r^2 = 0.9983 (from 15 ng mL^{-1} to 7000 ng mL^{-1}) and for COC, r^2 = 0.9935 (from 20 ng mL^{-1} to 250 ng mL^{-1}). In addition, z-scores were calculated to compare the two methods and resulting z-scores were lower than 2, confirming that the reported results and reference method results were in the agreement. The following min-max z-scores of 0.1–1.4 (n = 13), 0.1–1.9 (n = 17) and 0.2–0.8 (n = 3) for MDA, MDMA and COC, respectively. This comparative study between CE-FD and HPLC-MS showed that proposed DOA analyser is capable reliably and accurately

Table 5
Weekend music festival results during 2016–2018.

Substance	2016	2017	2018	2016–2018
MDA:				
Positive (n)	21	18	16	55
Median ± MAD[a], ng mL^{-1}	302 ± 312	213 ± 141	266 ± 264	259 ± 231
Min – Max, ng mL^{-1}	10–1469	39–778	10–1807	10–1807
MDMA:				
Positive (n)	23	25	20	68
Median ± MAD[a], ng mL^{-1}	2894 ± 2246	4451 ± 4385	4602 ± 5042	3967 ± 3855
Min – Max, ng mL^{-1}	13–9773	43–31 400	43–34 767	13–34 766
COC:				
Positive (n)	15	4	3	22
Median ± MAD[a], ng mL^{-1}	766 ± 4253	223 ± 85	669 ± 1600	654 ± 689
Min – Max, ng mL^{-1}	95–4253	128–327	81–1469	81–4253
COET:				
Positive (n)	10	2	1	13
Median ± MAD[a], ng mL^{-1}	117 ± 38	162 ± 26	27 ≤ x ≤ 45	124 ± 41
Min – Max, ng mL^{-1}	69–268	136–188	27 ≤ x ≤ 45	69–268
Total number of samples	36	37	37	110
Total number of positives (at least one analyte of interest)	30 (83.3%)	27 (73%)	25 (67.6%)	81 (73.6%)
Total number of true negatives	6 (16.7%)	10 (27%)	12 (32.4%)	29 (26.3%)

[a] MAD or median absolute deviation, p = 99.7%.

identifying and quantifying DOA in OF individuals.

3.4. Diagnostic performance in the field study

In addition to the Weekend Festival Baltic samples, police provided 18 OF samples that were collected from drivers suspected to be under the influence of DOA in 2017. These results have been presented in Table S8 (Supplementary data). In addition, 60 samples were collected from volunteers to test the selectivity of the CE-FD. A total of 188 (positive and negative) samples were analysed using the CE-FD DOA analyser during present study. There were 91 positive samples (Weekend Festival Baltic and roadside samples), containing at least one analyte of interest, and 97 samples not containing any analytes of interest (Weekend Festival Baltic, roadside and the study of selectivity). The diagnostic performance characteristics of CE-FD DOA were assessed based on the results obtained from the reference HPLC-MS

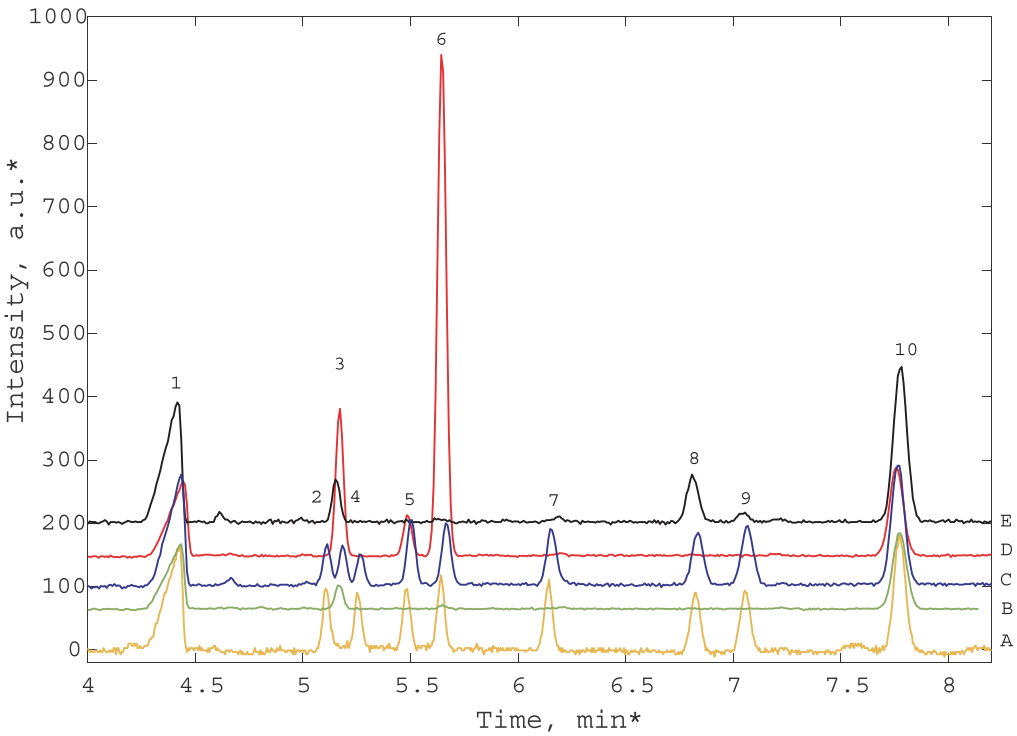

Fig. 2. Examples of Weekend Festival Baltic 2018 samples. A – Standards in OF (2x LLOQ levels), B – w18–012, c – W18-012 with spiked standards, D - W18-009, E – W18-024. Analytes of interest: 1 – benzylamine (IS1), 2 – AMP, 4 – METH, 5 – MDA, 6 – MDMA, 7 – MDEA, 8 – COC, 9 – COET, 10 – allocryptopine (IS2).

Table 6
Diagnostic statistics for CE-FD DOA analyser.

Substance Parameter	AMP	METH	MDA	MDMA	MDEA	COC	COET	At least one drug
TP[a], (n)	6	6	55	77	0	25	13	92
TN[a], (n)	182	182	133	111	188	163	175	96
Positives[b], (n)	0	0	56	77	0	25	13	91
Negatives[b], (n)	188	188	132	111	188	163	175	97
FP, (n)	0	0	1	0	0	0	0	1
FN, (n)	6	6	0	0	0	0	0	0
FP rate, %	0	0	0.8	0	0	0	0	1.0
FN rate, %	–	–	0	0	–	0	–	0.0
SE, %	–	–	100	100	–	100	–	100.0
SP, %	100	100	99.2	100	100	100	100	99.0
DA, %	–	–	99.6	100	–	100	–	99.5

[a] ≥LOD, detected by reference method.
[b] Suggested by CE-FD DOA.

method and are presented in Table 6. The LLOQ values of the HPLC-MS method were around 10 ppb for all studied psychoactive drugs.

One sample was assumed to be a false positive for MDA, since the presence of the compound was only detected using CE-FD DOA, and could not be confirmed using the HPLC-MS method. The estimated concentration of MDA was higher than LOD, but lower than the LOQ of CE-FD DOA. Unfortunately, there was not a great enough sample volume available to conduct further research and identify possible causes of interference for MDA. Diagnostic statistics were evaluated for analytes with at least 20 true positive and 20 true negative samples. There was no AMP, METH and MDEA found in real OF samples and too few COET samples were collected to meet statistical requirements. Therefore, the statistical parameters (FN rate, SE and DA) were not given for AMP, METH, MDEA and COET.

There were nine samples containing AMP and/or METH, AMP in six and METH in seven samples, detected by HPLC-MS at concentrations lower than the LOD of the CE-FD DOA. These samples would be considered to be false negatives for amphetamine and methamphetamine if evaluated separately, but eight of nine samples contained MDMA and/or cocaine and would, therefore, be considered positives using CE-FD to identify DOA. In addition, the sample that would have been counted as a false negative contained under 10 ng mL^{-1} of METH, which indicates that the person had consumed the drug, but was not currently impaired (the concentration was lower than all recommended cut-off limits). Therefore, this sample was not considered an actual false negative when the CE-FD failed to detect a DOA.

The overall diagnostic statistics for CE-FD DOA analysis was higher than 99.5%, showing a good capacity to identify recent DOA usage and resulting impairment. Nevertheless, the CE-FD DOA requires additional improvements regarding its sensitivity with respect to the detection of AMP and METH.

4. Conclusions and perspectives

A portable, sensitive and selective CE-FD DOA analyser was successfully developed and validated for the detection and quantification of AMP, METH, MDA, MDMA, MDEA, COC, and COET in OF. The validation results revealed that the proposed method was precise, accurate, selective and sensitive with LOD values of 6 ng mL-1 and 30 ng mL-1 for ecstasy and its derivatives (MDA and MDEA), and cocaine in OF, respectively. The achieved LODs were below the cut-off values for roadside testing as proposed by the DRUID project and EWDTS. The selectivity was evaluated for more than 118 samples (58 substances in PPOF, 60 OF samples from volunteers), showing only two possible interferents. The portable analyser was successfully applied on-site for analysis of 128 real samples during years 2016–2018 at the Weekend Festival Baltic (Pärnu, Estonia). The results were confirmed by a reference HPLC-MS method and were in good agreement with all analytes of interest (z-score ≤ 2).

The proposed CE-FD DOA analyser has shown great diagnostic performance with respect to diagnostic accuracy, sensitivity and specificity (> 99% for all DOA). Thus, the proposed analyser is very attractive for the on-site quantitative detection of DOA. In regard to future perspectives, the described CE-FD analyser could be further developed to extend the application and analyse DOA in other sample matrices like exhaled breath condensate, sweat, plant materials and powders. Furthermore, the analyser could be used to monitor therapeutic medicines (tramadol, venlafaxine, citalopram, and metoprolol) in OF.

CRediT authorship contribution statement

Piret Saar-Reismaa: Validation, Investigation, Methodology, Conceptualization, Writing - original draft, Writing - review & editing. **Chelsa-Ann Brilla:** Validation. **Kristiina Leiman:** Validation, Investigation. **Mihkel Kaljurand:** Software, Investigation, Conceptualization. **Merike Vaher:** Investigation, Validation, Resources, Project administration. **Maria Kulp:** Methodology, Writing - review & editing. **Jekaterina Mazina-Šinkar:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120662.

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Appendix 2

Table A1. Definitions of the validation parameters from the EMA guidelines [205]

Accuracy	The accuracy of an analytical procedure expresses the closeness of the determined value to the value which is accepted either as a conventional true value or an accepted reference value. Accuracy is defined as (determined value/true value) x100%.
Analytical Procedure	The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analysis.
Carry over	Carry-over is the appearance of an analyte signal in blank sample after the analysis of samples with a high analyte concentration
Cross validation	Comparison of validation parameters of two bioanalytical methods
Instrumental detection limit (IDL)	The instrumental detection limit is the smallest concentration or absolute amount of analyte that provides a signal significantly larger than the signal arising from a standard solution blank
Instrumental quantification limit (IQL)	The instrumental quantification limit is the smallest concentration or absolute amount of analyte that can be quantitatively determined in a standard solution with a pre-defined precision and accuracy
Internal standard (IS)	Test compound(s) (e.g. a structurally similar analogue, or stable isotope labelled compound) added to calibration standards, control samples and study samples at a known and constant concentration to correct for experimental variability during sample preparation and analysis.
Limit of determination (LOD)	The limit of determination in the analytical procedure is the lowest amount of analyte in a sample matrix after processing which has a signal significantly larger than the signal arising from a standard solution blank
limit of quantification (LOQ)	The limit of quantification in the analytical procedure is the lowest amount of analyte in a sample matrix after processing which can be quantitatively determined with a pre-defined precision and accuracy
Matrix effect	The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.
Precision	The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained under the prescribed conditions. Precision is defined as the ratio of standard deviation/mean (%).
Recovery	The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.
Selectivity	Selectivity is the ability of the bioanalytical method to measure and differentiate the analyte(s) of interest and internal standard in the presence of components which may be expected to be present in the sample.
Stability	The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Appendix 3

Table A2. Pharmaceutical drugs and their active ingredients.

Active compound	Content mg/tbl	Producer used	Usage
Alprazolam	0.5	Pfizer, Italy	anxiety, panic disorder
Aspirin	500	Bayer, Germany	fever, pain relief
Azithromycin	500	Teva, Poland	antibiotic
Carbamazepine	200	Nycomed, Estonia	anti-epileptic drug
Cetirizine	10	UCB Pharma, Finland	antihistamine
Citalopram	10	Teva, Poland	antidepressant
Clindamycin	300	Pfizer, Germany	bacterial infections
Diclofenac	100	Ratiopharm Germany	arthritis, pain
Doxazosin	4	Teva, Poland	Hypertension, enlarged prostate
Doxycycline	100	Ratiopharm Germany	anti-bacterial, malaria prophylactic
Dydrogesterone	10	Abbott, Holland	menstrual disorders, miscarriage prevention
Enalapril	10	Merck, Holland	high blood pressure, heart dysfunction
Finasteride	1	Accord Healthcare Limited, UK	enlarged prostate
Fluoxetine	20	Vitabalans, Finland	depression, OCD, bulimia
Ibuprofen	400	Takeda, Estonia	pain
Indapamide	2.5	Teva, Poland	high blood pressure
Loperamide	10	Ratiopharm, Germany	diarrhea
Loratadine	10	Bayer, Lithuania	antihistamine
Metamizole	500	Analgin, Russia	painkiller, fever, spasms
Metoprolol	50	Ratiopharm, Germany	hypertension, chest pain
Metronidazole	500	Nycomed, Estonia	against infections, bacterial and parasitic
Nitrofurantoin	50	Nycomed, Estonia	urinary tract infections
Olanzapine	5	Orion, Finland	schizophrenia, depression
Paracetamol	500	GSK, UK	Pain relief
Phenobarbital	0.1	GMBH, Germany	seizures
Prednisolone	5	Gedeon Richter Plc., Hungary	arthritis, immune disorders, cancer
Pseudoephedrine	60	McNeil, UK	common cold, allergies
Sertraline	25	Teva, Poland	depression, OCD, PTSD
Sildenafil	100	Pfizer, UK	high blood pressure in lungs
Sulfamethoxazole	800	BerlinChemia, Germany	antibiotic
Tramadol	50	Lannacher, Austria	pain
Trimethoprim	100	Vitabalans, Finland	bacterial infections
Venlafaxine	75	Ratiopharm, Germany	depression

Appendix 4

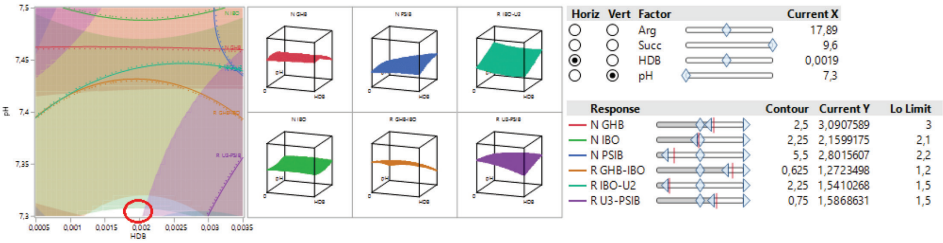

Figure A4.1 – 2D plot of surface plots

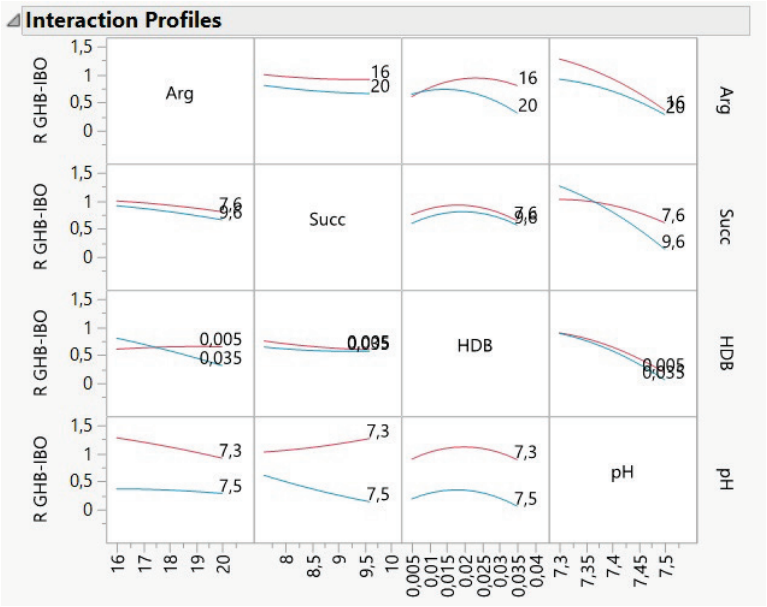

Figure A4.2- Interaction profiles from DOE of CE-C^{4}D method

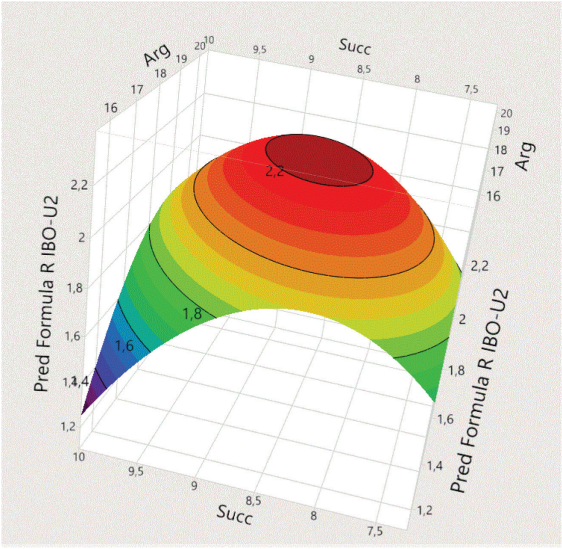

Figure A4.3 – Response surface of the prediction formula from DOE results of CE-C^{4}D

Appendix 5

Table A.3. Selectivity of CE-FD method, F_{EM3}

Substance	PPOF LOD, ng/mL	Max conc. Tested, ng/mL	Substance	PPOF LOD, ng/mL	Max conc. Tested, ng/mL
Metabolites			*Pharmaceutical drugs*		
4-OH-AMF	175		Alprazolam	2 000	
4-OH-METH		1 000	Aspirin		10 000
HMA	75		Azithromycin		10 000
HMMA	75		Carbamazepine		10 000
Benzoylecognine	100 000		Cetirizine	20 000	
3-MAM	1 000		Citalopram	40	
Other DOA			Clindamycin		
PMA	35		Diclofenac		10 000
PMMA	35		Doxazosin		10 000
Heroin	30 000		Doxycycline	2000	
Hydromorphone		10 000	Dydrogesterone		12 500
Methadone		10 000	Enalapril		10 000
Methylone	3 300		Finasteride		10 000
Ecgonine		200 000	Fluoxetine		10 000
MDVP	3 000		Phenobarbital		10 000
LSD	2 000		Ibuprofen		10 000
Psilocin	6 000		Indapamide		10 000
Morphine	500		Loperamide		10 000
Codeine	500		Loratadine		10 000
Fentanyl	160 000		Metamizole	12 500	
3-methyl-fentanyl	300 000		Metoprolol	7	
Ephedrine	75 000		Metronidazole		10 000
Norephedrine	75 000		Nitrofurantoin		10 000
Various substances			Olanzapine		10 000
Tyramine	100		Paracetamol		10 000
Phenylacetic acid		500 000	Prednisolone	2 000	
Dopamine	500		Pseudoephedrine	75 000	
			Sildenafil		10 000
			Sertraline	20	
			Sulfamethoxazole	10 000	
			Tramadol	5	
			Trimethoprim		10 000
			Venlafaxine	4	

Curriculum vitae

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