FACULTY OF
PHARMACEUTICAL SCIENCES

APPLICATION OF ACTIVITY-BASED BIOASSAYS FOR THE DETECTION OF SYNTHETIC CANNABINOID RECEPTOR AGONISTS IN EMERGENCY DEPARTMENT SERUM SAMPLES FROM DRUG-RELATED CASES

Marthe Vandeputte

A Master dissertation for the study programme Master in Drug Development

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SUMMARY

Synthetic cannabinoid receptor agonists (SCRAs) represent one of the largest groups of new psychoactive substances (NPS) in Europe. Their rapid emergence and chemical diversity substantially challenge the detection and monitoring of these compounds. Immunoassays and mass spectrometry-based methods continue to fall short for this purpose, as they are targeted, structure-based methods, hampered by e.g. a lack of cross-reactivity and the need for prior knowledge of molecular identity. These and other limitations to the current detection techniques lay at the root of recent research on the use of "non-targeted", activity-based screening methods for SCRAs (and other NPS). Cannaert *et al.* previously proposed bioassays that are based on the functional complementation of a split Nanoluc® luciferase (NanoBiT® Technology, Promega). Activation of a cannabinoid receptor (CB_1/CB_2), fused to one part of the luciferase, leads to recruitment of β-arrestin 2, fused to the other part. The resulting restoration of luciferase activity leads to measurable bioluminescence. This way, the bioassays allow detection purely based on cannabinoid activity, requiring no prior knowledge about structure.

In the present dissertation, the previously developed CB_1/CB_2 bioassays were evaluated through application on a large set (n = 471) of serum samples acquired from patients presenting with drug-related toxicity to a UK Emergency Department in the second half of 2016. The outcome of the bioassays (SCRA-positive/-negative) was compared to the declared substance use and the results from mass spectrometric analysis. With a final sensitivity and specificity of respectively 100% (52/52) and 97.9% (330/337), the applicability of the CB_1/CB_2 bioassays was convincingly demonstrated. Reducing the sample volume lowered these values to 88.9% (16/18) and 96.6% (56/58), respectively. Interestingly, two cases in which SCRA intake was claimed by the patient, gave rise to a (weakly) positive signal in the bioassays despite a lack of bioanalytical confirmation. Re-evaluation of the bioanalytical data against a future updated library may retrospectively uncover the presence of SCRAs in these patients, further increasing the specificity. It may also be possible that low-level SCRAs exerting additive bioactivity had been picked up. A secondary objective consisted of exploring the potential correlation between the strength of the bioassay's signal and the total SCRA concentration of the sample. This resulted in a plot in which the initial upward trend appeared to saturate at higher concentration levels. However, due to the many encountered uncertainties, the latter remains a preliminary observation. Finally, a tertiary objective was to portray the different clinical presentations following SCRA use. The (limited) clinical profile of the studied SCRA user cohort appeared to be in line with that reported in previous research. However, in contrast to the results of a similar study effected in 2015, 5F-ADB was the most frequently encountered SCRA in the present patient cohort.

In conclusion, the potential of bioassay-based screening for SCRAs was convincingly demonstrated via the successful application on a large set of authentic serum samples. This approach offers the opportunity to serve as an alternative first-line screening tool for the presence of SCRAs in biological matrices.

SAMENVATTING

Synthetische cannabinoïdreceptoragonisten (SCRA's) vormen één van de grootste groepen van nieuwe psychoactieve stoffen (NPS) in Europa. Hun snelle opkomst en structurele diversiteit bemoeilijken detectie en monitoring. De huidige immunologische en massaspectrometrische methoden schieten steeds vaker tekort, onder andere doordat ze gebaseerd zijn op het detecteren van een specifieke structuur. Kennis van deze problematiek heeft geleid tot de ontwikkeling van alternatieve, *"non-targeted"* screeningsmethoden voor de detectie van SCRA's (en andere NPS). Cannaert *et al.* ontwikkelden activiteits-gebaseerde screeningstesten op basis van functionele complementatie van Nanoluc® luciferase *(NanoBiT® Technology*, Promega). Twee inactieve luciferase-subeenheden worden hierbij gekoppeld aan respectievelijk een cannabinoïdreceptor (CB_1/CB_2) en een β-arrestine 2-eiwit. Receptoractivatie resulteert in de recrutering van β-arrestine 2 naar de receptor. De daaropvolgende functionele complementatie van het enzym veroorzaakt een meetbaar lichtsignaal. De detectie van SCRA's gebeurt zo op basis van cannabinoïdactiveit en vereist geen voorkennis over chemische structuur.

De experimenten in deze thesis werden uitgevoerd met als primair doel de toepasbaarheid van de ontwikkelde CB_1/CB_2-screeningstesten te evalueren. Hiervoor werden de testen toegepast op een grote set serumstalen (n = 471) afkomstig van drug-gerelateerde spoedopnames in een ziekenhuis in het Verenigd Koninkrijk in de tweede helft van 2016. Het resultaat van de testen (SCRA-positief/-negatief) werd vergeleken met zelfgerapporteerd gebruik en de resultaten van massaspectrometrische analyse. De bruikbaarheid van de testen werd overtuigend aangetoond met een finale gevoeligheid en specificiteit van respectievelijk 100% (52/52) en 97,9% (330/337). Indien werd uitgegaan van een kleiner staalvolume, veranderden deze waarden naar respectievelijk 88,9% (16/18) en 96,6% (56/58). In twee gevallen waarin de patiënt SCRA-gebruik rapporteerde zonder dat dit bioanalytisch bevestigd werd, gaf de CB_1-test een (zwak) positief signaal aan. Herevaluatie van de bioanalytische data met behulp van een geüpdatete bibliotheek zal mogelijk de aanwezigheid van één of meer SCRAs alsnog kunnen bevestigen. Dit zou de specificiteit van de test verder doen stijgen. Een tweede objectief bestond erin de potentiële correlatie te onderzoeken tussen de sterkte van het signaal uit de CB_1-test en de totale SCRA-concentratie van het staal. Met een aantal beperkingen in het achterhoofd, resulteerde deze evaluatie voorlopig in een plot die, na een stijging bij lagere concentraties, afvlakte naar een maximum. Als laatste werden de klinische symptomen van de SCRA-gebruikers in beeld gebracht: die leken in dezelfde lijn te liggen als eerder gerapporteerde gegevens. In tegenstelling tot een gelijkaardige studie met een patiëntencohorte uit 2015, werd in de huidige studie 5F-ADB als meest voorkomende SCRA aangetroffen.

De resultaten in deze thesis bewijzen de bruikbaarheid van activiteits-gebaseerde SCRA-screening via de succesvolle toepassing op een grote set serumstalen. De beschreven testen kunnen potentieel een belangrijke rol spelen als alternatieve eerstelijnsscreeningsmethoden voor het opsporen van SCRA's in biologische matrices.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

5F-ADB	Methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate
5F-AKB-48	N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
AB-CHMINACA	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide
AB-FUBINACA	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
AMB-CHMICA	Methyl (1-(cyclohexylmethyl)-1H-indole-3-carbonyl)-valinate
AMB-CHMINACA	Methyl (1-(cyclohexylmethyl)-1H-indazole-3-carbonyl)-valinate
AMB-FUBINACA	Methyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate
AUC	Area under the curve
β-arr, β-arr2	β-arrestin, β-arrestin 2
BB-22	Quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate
CB_1/CB_2 receptor	Cannabinoid receptor 1/2
Cumyl-5F-PINACA	1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide
DNA, cDNA	Deoxyribonucleic acid, copy deoxyribonucleic acid
dNGFR	Truncated nerve growth factor receptor
EC_{50}	Half maximal effective concentration
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EGFP	Enhanced green fluorescent protein
EMB-CHMINACA	Ethyl (1-(cyclohexylmethyl)-1H-indazole-3-carbonyl)-valinate
EMB-FUBINACA	Ethyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FBS	Fetal bovine serum
GC	Gas chromatography
GCS	Glasgow Coma Scale/Score
GHB	Gamma-hydroxybutyric acid
GPCR	G-protein coupled receptor
HEK 293T	Human embryonic kidney 293T cells
HRMS, HRMS/MS	High-resolution mass spectrometry, high-resolution tandem mass spectrometry
JWH-018	Naphthalen-1-yl-(1-pentyl-1H-indol-3-yl)-methanone
LC	Liquid chromatography
LgBit	Large BiT
MDMB-CHMICA	Methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate
MS, MS/MS	Mass spectrometry, tandem mass spectrometry

NPS	New psychoactive substances
NR	Nuclear receptor
RGBA	Reporter gene bioassay
RLU	Relative light units
ROC	Receiver operating characteristic
SCRA	Synthetic cannabinoid receptor agonist
SmBiT	Small BiT
SRE	Steroid response element
Δ^9-THC	(-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
UNODC	United Nations Office on Drugs and Crime

1 INTRODUCTION

1.1 NEW PSYCHOACTIVE SUBSTANCES

Over the last decade, new psychoactive substances (NPS) have been flooding the drug market (**Figure 1.1**). This novel class of abused substances is described through numerous synonyms, including "designer drugs", "legal highs", "bath salts" and "research chemicals", often accompanied by the phrase "not for human consumption" to hide their intended use (1). NPS are defined by the United Nations Office on Drugs and Crime (UNODC) as *"substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat"* (1). The term applies to newly misused substances, although not necessarily new compounds, that have recently found their entry into the drug market. The emergence of NPS has undeniably become a global issue: according to the UNODC, up to December 2017, more than 800 NPS have been reported to the UNODC Early Warning Advisory on NPS by more than 110 countries and territories worldwide (2). As the manufacture of these synthetic drugs is not restricted to certain parts of the world with optimal conditions for the cultivation of psychoactive plants, clandestine "NPS laboratories" indeed seem to have appeared everywhere across the globe (1).

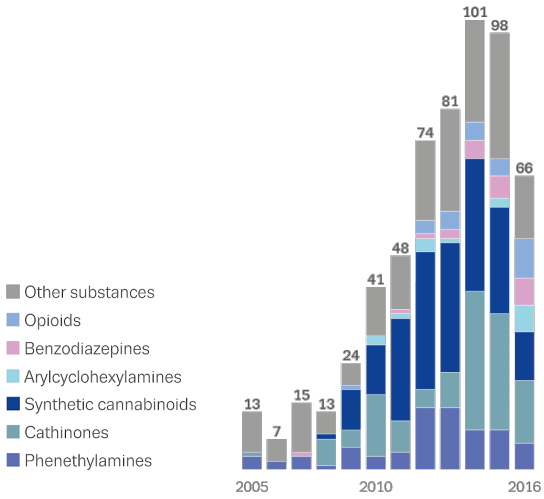

Figure 1.1. Number and categories of new psychoactive substances notified to the EU Early Warning System for the first time (2005-2016). Adapted from EMCDDA, 2017.

In a frantic effort to keep up with this rapidly expanding spectrum of new highs, law- and policymakers have responded in different ways to the challenges presented by NPS (2,3). However, control strategies are hampered by the fact that NPS are purposely designed to evade detection and bypass the existing legislation. Laws based on lists of individual chemical structures are continuously lagging behind as the creativity of clandestine medicinal chemists allows them to quickly replace a recently banned substance by a new (i.e. at that time still unregulated) agent, simply by applying slight modifications to their chemical structure (3,4). As a response, some countries seek refuge in more generic legislations that make use of broader terms such as "cannabimimetic agents" and "psychoactive substances", as well as adding all variants of certain generic structures to the list of controlled substances. Both the UK (2016) and Belgium (2017) are examples of countries that have adopted such generic laws (5,6). However, pitfalls of legislations implying activity include the fact that they are based on the pharmacology of the new compounds, which is usually unknown due to the numerous

minor structural alterations (7,8). Additionally, generic laws place high responsibility with toxicological laboratories, which are burdened with the seemingly impossible task of detecting all these (current and future) NPS variants within a standard screening test.

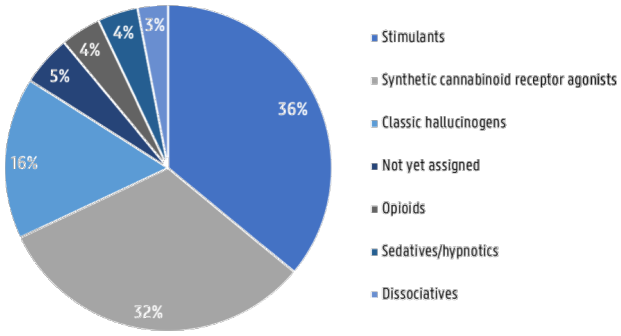

Figure 1.2. Proportion of new psychoactive substances registered by the UNODC Early Warning Advisory on new psychoactive substances (NPS) up to December 2017. The chart represents a classification of NPS in terms of pharmacological effects. Adapted from UNODC, 2018 (9).

Owing to their high structural diversity, it should be clear that a classification of NPS based on their chemical structure is often non-comprehensive. NPS can also be categorised into broadly six groups according to the "parent drug" they are intended to mimic (10). The proportion of the total NPS drug market represented by each class, is illustrated in **Figure 1.2**. By the end of 2017, stimulants, synthetic cannabinoid receptor agonists (cf. infra) and hallucinogens combined, accounted for 84% of the total registered NPS (1).

The "attractiveness" of NPS abuse lies mainly in their easy availability, primarily through the Internet (both the generally accessible as the dark web), market stalls or specialised "head shops", where they are sold at fairly low prices (1,11–13). High-risk groups, such as the homeless and marginalised, are often drawn in by this competitive price. In addition, NPS are often not detected by the current routine drug tests, making them appealing to user groups subjected to such screening tests (e.g. prisoners). In addition, NPS use for recreational purposes is often seen among youth attracted by the applied marketing strategy (e.g. "legal highs"). Other high-risk groups include people who inject drugs, men who have sex with men and so-called "psychonauts", who are actively seeking new mind-altering experiences (1,14).

Most users, however, fail to acknowledge that, contrary to what the marketing of NPS might imply, no new compound can be deemed "safe" (4). Owing to their rapid emergence and high diversity, very little scientific information on the pharmacological effects and both acute and long-term risks of NPS use is available. Users are potentially exposed to very serious health risks as the composition of the products containing NPS is often inconsistent and limitedly, wrongly or simply not mentioned on the package. Additionally, the potency of the individual substances is often very high but unpredictable. Reported side effects range from agitation to aggression and acute psychosis, to seizures and even death (1,2,4,11). It should, however, be stressed that it remains challenging to assess the actual consequences of NPS use. Data from self-reports are often incomplete as, for example, most users do not know exactly which substances they have been consuming. Our knowledge

about adverse effects mostly originates from Emergency Department admissions or fatal case reports, which may paint a picture that is not representative of a typical user's clinical profile (15,16). The increasing problem of polydrug use further complicates the assessment of which effects are attributable to (exactly which) NPS (1,2,4,11).

Despite the numerous NPS that are increasingly synthesised and detected every year, the overall size of the NPS market fortunately remains relatively small compared to the older, more established drugs. However, an important caveat lies with the high levels of harm that have been associated with the use of NPS, highlighting the need to closely monitor and further study every new drug on the block (1,4).

1.2 SYNTHETIC CANNABINOID RECEPTOR AGONISTS

1.2.1 The story of cannabinoids: from plant to laboratory

The medicinal and recreational use of *Cannabis sativa,* commonly known as marijuana, can be traced back to as early as 2600 BC (18). The identification of the most potent psychoactive compound, (-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol (Δ^9-THC) (**Figure 1.3**) in the plant in 1965, paved the way towards the characterisation of the natural endocannabinoid system of the body, encompassing endocannabinoids – whose effects are mimicked by the exogenous components in marijuana - and the cannabinoid receptors, CB_1 and CB_2, all named after the plant that led to their discovery (18–20). An important tool in the elucidation of this endocannabinoid system was the synthesis of high-affinity synthetic CB_1 and CB_2 receptor ligands (e.g. synthetic cannabinoid receptor agonists or SCRAs). Scientists saw potential in the endocannabinoid system as a possible target in the treatment of a myriad of conditions, such as neurodegenerative diseases and pain disorders (13). Examples of novel cannabinoids synthesised in this context, are the CP-series by Carl Pfizer, the HU-series created at the Hebrew University or the AM-compounds from Alexandros Makriyannis' group. It was John W. Huffman's research team that synthesised the broad group of JWH cannabinoid receptor agonists (**Figure 1.3**) (13,17,21).

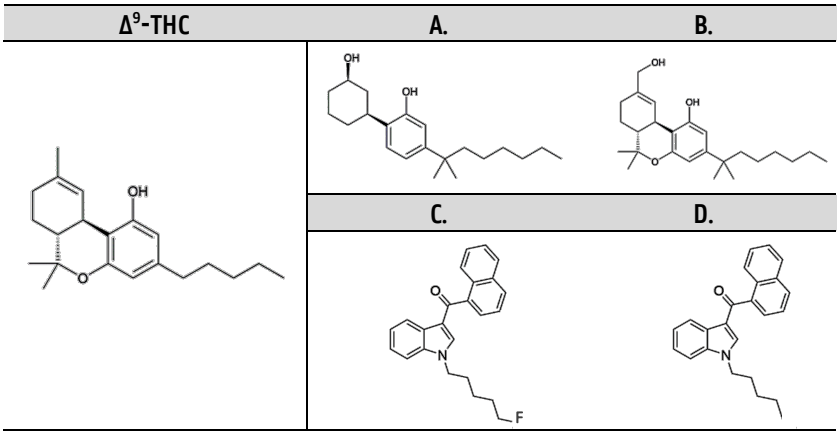

Figure 1.3. Molecular structures of (-)-Δ^9-6a,10a-trans-tetrahydrocannabinol (Δ^9-THC) and early SCRAs originally synthesised for research purposes. (A) CP 47,497; (B) HU-210; (C) AM-2201; (D) JWH-018. Structures from Kevin R. C., 2017 (17).

Clandestine medicinal chemistry laboratories, mainly based in China, gradually took interest in these SCRAs as they mimic the action of Δ^9-THC while evading legislation owing to their structural diversity. As a consequence, the abuse of SCRAs as so-called "legal highs" started expanding and the drugs now constitute the largest NPS segment in terms of number of different substances reported and substances monitored in Europe by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (1,13). Bulk powders are shipped from China to Europe and are then combined with herbs to mimic the appearance of marijuana. This is done by mixing the crystalline powder with plant material or tobacco, although more often the powder is dissolved in solvents such as acetone or methanol. The obtained solution is then used to soak or spray inert plant material. Next, the mixture is dried, packaged and distributed via the Internet and specialist shops, where it is sold as "incense product" or "legal herbal smoking mixture" (13,22,23). Contrary to popular belief, it should thus be stressed that SCRAs are not simply synthetic versions of naturally occurring substances in herbal cannabis, but chemically synthesised products (1). In 2008, German forensic investigators were the first to detect the highly potent synthetic cannabinoids JWH-018 and CP 47,497 in a product marketed as "Spice" in Europe. The same compounds were found in products named "K2", "Yucatan Fire", "Sence" and "Skunk" (22). Illustrating the maddening cat-and-mouse game between drug manufacturers and legislative forces, four weeks after banning JWH-018, newly obtained "Spice" samples were found to contain the unregulated homologue JWH-073 (4). Many other products containing a high diversity of SCRAs later appeared (4,13,14,18,21,24,25). SCRAs are usually smoked, although some reports on oral or snorted SCRAs exist as well (1,26).

1.2.2 Cannabinoid pharmacology

Cannabinoids exert their cannabimimetic effects primarily through interaction with CB_1 and CB_2 cannabinoid receptors present in the body. Both receptors differ in their tissue distribution, signalling mechanisms and sensitivity to certain selective cannabinoid ligands (27). CB_1 and CB_2 receptors are both G-protein coupled receptors (GPCRs), coupled mainly through the $G_{i/o}$ family of G-proteins. Endogenous activation of CB receptors is largely mediated by two endogenous ligands (i.e. endocannabinoids), anandamide (derived from the Sanskrit word "ananda", meaning bliss) and 2-arachidonoyl-glycerol (19,28). Upon interaction with a ligand, a conformational change of the receptor is induced, which allows coupling to a specific intracellular G-protein heterotrimer. After subsequent dissociation of the G-protein into its subunits, the alpha-subunit exchanges guanosine-5'-diphosphate for guanosine-5'-triphosphate and effector molecules (mostly enzymes) can be stimulated or inhibited. Activation of the CB receptors results in inhibition of adenylyl cyclase and thus reduced intracellular cyclic adenosine monophosphate levels. Other transduction mechanisms signalled by the CB receptors are activation of mitogen-activated protein kinase and regulation of calcium and potassium channels, both the latter signalled by CB_1 only (28–30). Upon activation, GPCRs are rapidly phosphorylated by G-protein

coupled receptor kinases and subsequently desensitised by high-affinity binding of β-arrestins (β-arr), obstructing the G-protein binding site of the GPCR and therefore preventing further signalling. The GPCR-arrestin complex then interacts with clathrin, which initiates receptor endocytosis via targeting of the GPCR to clathrin-coated pits (31–33).

CB_1 receptors are predominantly expressed in the central nervous system. They are particularly present in the brain in regions of higher cognitive functions (e.g. cerebral cortex) and areas important in reward, food and drug intake (e.g. nucleus accumbens), where cannabinoids appear to increase dopaminergic signalling (4,19,27,34). CB_1 receptor modulation influences other neuronal systems by inhibiting the release of key neurotransmitters such as gamma-aminobutyric acid and glutamate. Modulation of neurotransmitter release and action has indeed been suggested as one of the main roles of the endocannabinoid system (35). It is generally considered that CB_1 receptor activation is primarily responsible for the psychoactive effects of cannabinoids (4,19,27,34). Although to a lesser extent, CB_1 receptors are also found in the peripheral nervous system and, among others, in the reproductive system, the heart and the lung (19,21,27). Owing to this wide distribution, several therapeutic applications have been proposed for CB_1 ligands, such as neuroprotection or treatment of obesity. However, the search for potential therapeutics targeting the CB_1 receptor has been hampered by the issue of unwanted central effects caused by CB_1 stimulation in the brain (19).

Whereas expression of CB_1 receptors in cells of the immune system has also been reported, their quantity is markedly lower than that of CB_2 receptors (27). Indeed, the latter receptors have been localised in a wide range of cells and tissues associated with the immune system. CB_2 receptors have particularly high expression levels in mature B-cells and macrophages (27). CB_2-selective agonists presumably lack psychoactive effects and their expression increases under certain disease states, making them interesting potential therapeutic targets (19,34,36–38).

1.2.3 Finding order in chemical chaos: a common pharmacophore

Originally, SCRAs were divided into classical cannabinoids, defined by the dibenzopyran scaffold as found in Δ^9-THC; cyclohexyl-substituted phenols including the CP-series; naphtoylindoles exemplified by JWH-018; and benzoylindoles such as AM-694 (12,18,39). These four chemical classes, used to classify the first SCRAs, now tend to fall short in capturing the increasing structural complexity of the newly emerging SCRAs. Looking past this high variability caused by small modifications, the structures of most SCRAs appearing on the market today can be simplified into a common pharmacophore (39–41). The recurring structural features that allow binding and activation of CB_1 and/or CB_2 receptors, are (i) an indole or indazole core; (ii) a linker group containing a carbonyl group or equivalent; (iii) a linked, often “bulky” group such as cyclohexane, a naphthalene ring, substituted

butanamide, or another equivalent moiety; and (iv) a hydrophobic tail attached to the nitrogen atom of the indole or indazole ring (**Figure 1.4**) (17,39–41). The structural diversity is further established by introduction of bioisosteres, e.g. halogenation of alkyl tails (40).

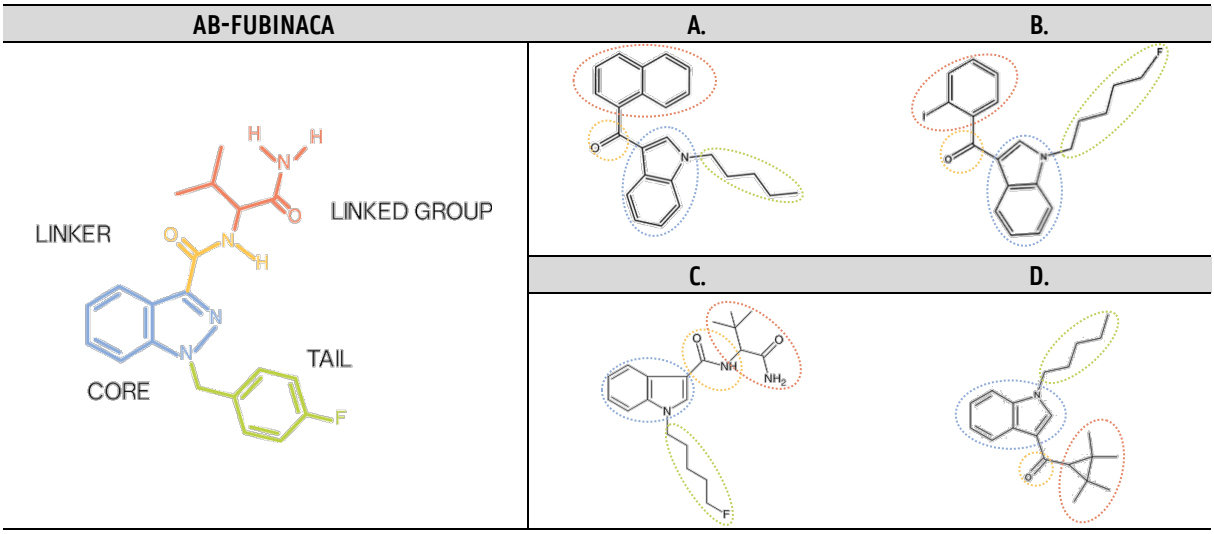

Figure 1.4. Chemical structure of the synthetic cannabinoid AB-FUBINACA (left panel), illustrating the recurring structural features that can be recognised in many SCRAs, as exemplified by (A) JWH-018; (B) AM-694; (C) 5F-ADBICA; (D) UR-144. All structures are composed of a core, a linker, a tail and a linked group. Small structural alterations result in a myriad of possible SCRAs (17). Left panel, figure from EMCDDA, 2017 (13); right panel, molecular structures adapted from Smith *et al.,* 2015 (A + B + D) (12), Worst and Sprague, 2014 (C) (41).

1.2.4 "Not for human consumption": effects and risks

The effects of SCRA intake are qualitatively similar to those experienced after intake of natural cannabis, although often they are of greater magnitude and duration (21). Commonly reported acute toxicity has been characterised by agitation, seizure, nausea/vomiting, hypertension, tachycardia and/or kidney failure. Less common, although serious reported side effects are psychosis in susceptible individuals, cardiotoxicity and even death (13,17,18,21,24,42). Furthermore, it has been reported that repeated use can lead to tolerance and withdrawal symptoms, which some users claim to be more severe than for natural cannabis (17).

Data suggest that the prevalence of SCRA use in the general European population currently remains relatively low. This may be attributed to the apparent preference of most users for natural cannabis, since SCRAs exhibit more adverse effects (13,21). SCRAs have indeed proven to be potentially very dangerous: their use is estimated to be associated with a relative risk of requiring emergency medical treatment that is 14- to 30-fold higher than the same risk after traditional marijuana use (42,43).

Many factors have been suggested to contribute to the greater SCRA toxicity relative to the use of marijuana. Most SCRAs inherently have a higher affinity towards CB receptors, as well as greater potency and full efficacy

compared to the partial agonist Δ^9-THC (13,18,24). The partial agonism of Δ^9-THC may increase the activity of CB receptors in some but not all target tissues, the latter due to competition with endocannabinoids (4). Moreover, most SCRAs are strongly metabolised and, in contrast to most Δ^9-THC metabolites, it has been reported that numerous SCRA metabolites remain highly active (30,44–47). Active metabolites prolong the drug's half-life, adding to the SCRAs increased harmfulness. Some metabolites (e.g. a major metabolite from JWH-018) act as antagonists, which could lead to increased consumption by the user in an attempt to "spice up" the diminished psychoactive effects (8,18,30). Furthermore, it is possible that some SCRAs also act on certain cellular targets other than the CB receptors, thereby causing off-target action that may contribute to their higher toxicity and unpredictability as compared to Δ^9-THC. In addition, Δ^9-THC is only one of at least 113 phytocannabinoids that have so far been identified from *C. sativa*, some of which are non-psychoactive and potentially mitigate the effects following marijuana intake. SCRA products, on the other hand, generally do not contain these potentially "soothing" compounds. Instead, a combination of different potent SCRAs, as is commonly observed in SCRA preparations, may add to the marked toxicity of these products (18,29,30).

Since most compounds originate from scientific research, appropriate clinical trials testing their safety for human consumption have never been established (22). As is the case for most NPS, the SCRA field is dynamic and rapidly evolving. Slight structural alterations to existing (detected and banned) SCRAs, allow manufacturers to cleverly evade routine detection and legislation, while providing individual users with more intense highs (18). However, the lack of appropriate quality control during manufacture results in substantial variability between the different sold units, even within the same brand. The method of preparation, i.e. spraying of chemicals onto herbs, also adds to the variable distribution and concentration within one or between different batches of supposedly "identical" products. This increases the risk of serious adverse effects due to contaminated mixtures or unintended overdosing (13,18,24,42).

To date, no specific treatment for complications following SCRA use has been established. Research on the potential of cannabinoid receptor antagonists in light of acute or chronic SCRA toxicity, has been limited since the withdrawal of the inverse agonist/antagonist rimonabant from obesity clinical trials due to adverse psychiatric consequences (4,48). Currently, treatment is primarily based on supportive care, including symptom management and hydration of the patient. In case of severe intoxication, close monitoring in intensive care units may be necessary. Both benzodiazepines and antipsychotics are sometimes considered to treat agitation and onset of psychotic symptoms (4,22).

1.3 DETECTION OF ABUSED SUBSTANCES

1.3.1 Screening methods for abused substances

Forensic toxicologists typically make use of a two-step strategy for the detection of abused substances in biological matrices (49). The first step comprises a rapid screening of a large number of samples, aimed at discriminating between compliant and suspect samples that need further investigation. Screening is typically performed by means of either chromatographic approaches or immunoassays (49). Following this initial sorting, a confirmatory method is applied to the samples that screened positive, to both identify and, in most cases, quantify the detected substance(s). This confirmation step is based on highly specific methods such as gas chromatography (GC) or liquid chromatography (LC) coupled to (tandem) mass spectrometry (MS or MS/MS) (49). As such methods are expensive and time-consuming, they are less suitable for high-throughput analysis. This stresses the importance of the initial multi-analyte screening method, which should allow rapid and reliable elimination of compliant samples so that only suspect samples proceed to confirmatory analysis (50).

In the context of the rapidly (dis)appearing and structurally diverse NPS, it should be clear that the current screening techniques suffer from some important limitations. For immunoassays, the choice of the immunogen (i.e. the abused NPS) to which the antibody is directed, is complicated when the compound has not been detected and identified before (i.e. is "unknown"). Moreover, the emergence of new NPS may outrun the time-consuming process of producing new antibodies, causing developed immunological tests to be quickly outdated. Even though some antibodies may show cross-reactivity with metabolites or analogues of known immunogens and could therefore theoretically detect structurally similar NPS, this "group specificity" is difficult to predict, resulting in unreliable screening (49). An additional limitation, characteristic to immunological detection, is that the endpoint of analysis reflects the combined immunoreactivity of all compounds structurally related to the immunogen, rather than representing the total level of bioactivity in the sample (51). Targeted chromatographic systems coupled to (tandem) MS suffer from the same flaw in that they are typically set up for the detection of specific ions or MS/MS transitions of known substances only. These methods may fall short in detecting "unknown" compounds that have not yet been included in mass spectral libraries or for which no pure reference standards are available yet. Again, the time it takes for a new drug to be included in the spectral libraries, may take longer than for an NPS to (dis)appear and be replaced by a new compound (49,52). A possible solution when screening for unknown substances, is the use of "non-targeted" screening methods, particularly by means of high-resolution mass spectrometry (HRMS) (52–54). Owing to its high accuracy, HRMS allows detection and identification based on the elemental composition of compounds. Databases of elemental formulae are easily expanded, premising HRMS as the method of choice for multi-analyte screening. However, this technique is not routinely implemented for screening in most laboratories as it is limited by its time-

consuming and expensive character (49,54). In addition, analytes must be present at sufficiently high concentrations to allow acquisition, which may hamper the detection of low concentration SCRAs in biological matrices (52–54).

Offering a promising solution to the emerging problems associated with the detection of newly abused substances, a spotlight is currently on the use of bioactivity-based screening methods (bioassays). By "sensing" biological activity, i.e. the pharmacodynamics of drug action, such assays allow to detect the presence of compounds showing affinity for a certain receptor, independent of prior knowledge about their structure. This way, bioassays may be implemented as first-line screening tools for the detection of abused substances, complementing the existing (non-)targeted analytical methods (8,54,55).

Characteristics of an "ideal" *in vitro* bioassay intended for screening purposes are outlined below (54).

- *Assay duration:* As screening should go fast, the bioassay should yield rapid results and/or allow the analysis of multiple samples in one run. The shorter the duration of the assay, the more samples can be analysed per time frame.
- *Simplicity:* An ideal screening test does not require a lot of technical experience or highly sophisticated equipment. For cell-based assays, the generation of stable cell lines may help to improve the simplicity of the assay.
- *Sensitivity:* As the aim of the screening assay is to detect physiologically relevant concentrations of abused substances in (extracts of) biofluids, its sensitivity is of utmost importance.
- *Selectivity:* Screening requires a method generating a minimum amount of false negative results. False positives may be allowed up to a certain extent, although the assay should be as selective as possible.
- *Reproducibility:* The results acquired from independently performed assays should be consistent.
- *Cost and throughput:* Ideally, the assay should allow high-throughput screening of samples, preferably at low consumable cost and with the possibility of automation.

The remaining sections in this chapter focus on reporter bioassays for the screening of synthetic cannabinoids and steroid hormones[i] in biological matrices.

[i] The use of reporter gene bioassays for the detection of steroid hormones was added to this dissertation in the light of a review to which the author had the opportunity of contributing during her time at the Ghent University Laboratory of Toxicology: *Looks don't matter, it is what you do that counts: Activity-based reporter assays as a new concept for abused substance screening in biological matrices* (manuscript provided in **Appendix I**). Her contribution consisted of compiling and summarising existing literature on the subject (**Tables 1.1 + 1.2**), as well as writing parts of the text (which was revised by Dr. A. Cannaert and Prof. Dr. C. Stove).

1.3.2 Bioassays for the screening of synthetic cannabinoids in biological matrices

Cannaert *et al.* recently reported on the development of cell-based cannabinoid reporter assays for the activity-based detection of SCRAs and their metabolites in biofluids (8,30,53). A similar bioassay has been developed for the detection of opiates and synthetic opioids and has been evaluated on blood samples (56). The use of such activity-based bioassays for the detection of NPS has the potential of being implemented as a screening tool in the context of recent generic NPS legislations based on (psychoactive) activity rather than on the chemical structure of compounds (30).

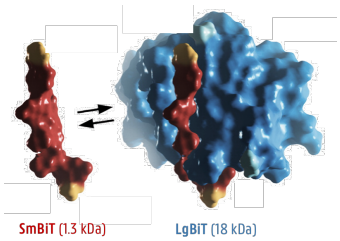

Figure 1.5. Representation of the two subunits, Large Bit (LgBiT) and Small BiT (SmBiT), of NanoLuc® luciferase. Figure adapted from Dixon *et al.*, 2016 (57).

The developed bioassays are based on the mechanism of GPCR activation combined with the principle of NanoLuc® Binary Technology (NanoBiT®, Promega) (57). NanoBiT® is a structural complementation reporter originally designed to study protein-protein interactions (57). The reporter consists of NanoLuc® luciferase split into two inactive subunits, Large BiT (LgBiT; 18 kDa) and Small BiT (SmBiT; 1.3 kDa), optimised for stabilisation and decreased affinity for each other to prevent recombination of both subunits unless they are brought together closely (**Figure 1.5**). These subunits are fused to two separate proteins of interest that are known or expected to interact with each other. Alternatively, the technology can be used to screen for interacting proteins or to explore protein interactions. Upon interaction of the fusion partners, the subunits are brought into close proximity, resulting in structural complementation and thus restored NanoLuc® luciferase activity. Adding the cell-permeable furimazine substrate results in a bioluminescent signal that can be monitored using a luminometer (30,57). Combining the knowledge of GPCR activation, subsequent desensitisation by β-arrestin 2 (β-arr2) and the described NanoLuc® Binary Technology, the novel bioassays can be used for the activity-based screening of GPCR ligands such as SCRAs, synthetic opioids/opiates and their metabolites.

To allow SCRA screening, constructs were designed consisting of the LgBiT or SmBiT subunits fused to the CB_1 or CB_2 C-terminus and the N- or C-terminus of β-arr2 (**Figure 1.6**). In a first set of experiments, mammalian cells were transiently transfected with the described constructs to find the ideal set-up. Next, the CB_1 and CB_2 bioassays were applied to several SCRAs and SCRA metabolites to determine their *in vitro* activities (30). The transient cannabinoid reporter assays were later improved by generating stable cell systems (8). The advantages of a stable bioassay as compared to the initial transient format, are a reduced workload and a higher reproducibility between independently performed experiments. Moreover, in the stable cell line-based bioassays, the possibility to control the level of receptor and β-arr2 expression was built in. This was

accomplished by coupling the expression of both constructs to that of co-expressed markers, allowing selection by fluorescence-assisted cell sorting. In addition, these co-expressed markers allow easy flow cytometric follow-up of the cell line stability as vector loss and/or overgrowth by non-expressing cells may occur over time (8,54). Applicability of the described cannabinoid reporter assays has been demonstrated in authentic urine, serum and plasma samples (8,53).

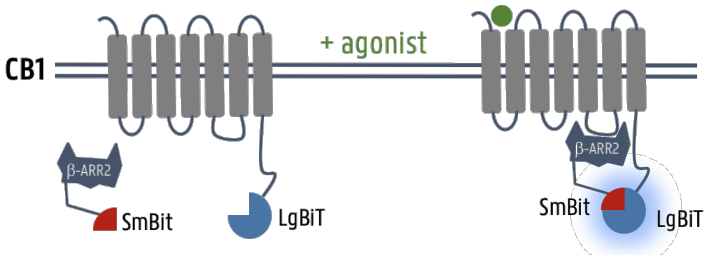

Figure 1.6. Principle of the cannabinoid reporter bioassays. When a ligand exerting cannabinoid activity binds the expressed cannabinoid (CB) receptor, fused to one luciferase subunit (LgBiT/SmBiT), β-arrestin 2 (β-arr2), fused to the other subunit (LgBiT/SmBiT), is recruited and luciferase activity is restored. Example shown for the CB_1 receptor. Figure adapted from Cannaert *et al.*, 2016 (30).

While the detection of SCRA abuse in urine mainly relies on the presence of active metabolites, blood samples primarily contain the SCRA parent compound. However, owing to the high potency of many such compounds, active concentrations in the low- to sub-nanogram per millilitre range are not uncommon. A third research step therefore aimed at improving the sensitivity of the developed bioassays. For this purpose, two C-terminal β-arr2 truncated mutants were evaluated (53). The first mutant (β-$arr2_{TR382}$) lacks the C-terminal amino acids responsible for an intramolecular interaction that keeps β-arr2 in a basal, non-active state (i.e. not bound to the GPCR). Removal of this C-terminus results in a β-arr2 mutant that is constitutively active and can therefore be recruited to the GPCR independent of prior phosphorylation of the receptor (31,53). The design of the second C-terminal β-arr2 truncation mutant (β-$arr2_{TR366}$) is based on the knowledge that the β-arr2 C-terminus is liberated due to a conformational change induced by β-arr2-GPCR interaction. Following this event, the C-terminus interacts with clathrins, components of the endocytic machinery of the cell that drive internalisation of the CB receptor. Thus, when eliminating the predominant clathrin binding site on β-arr2, the internalisation of the CB receptor is reduced and the response of the bioassay becomes more pronounced (32,53,58).

The principle of the developed activity-based cannabinoid bioassays can be applied to other classes of compounds by replacing the CB receptor by any other GPCR (30). Indeed, a similar assay has been developed for the detection of (synthetic) opioids in blood samples by substituting the cannabinoid GPCR by the μ-opioid receptor (56). Here, co-expression of an additional protein, G-protein coupled receptor kinase 2, appeared to be necessary to achieve sufficient sensitivity (54,56).

One important aspect when considering the use of activity-based bioassays for the detection of SCRAs or other (synthetic) drugs, is whether the activity of endogenous or frequently encountered (legal or illegal) substances might interfere with the read-out of the bioassays (54). Natural cannabis, for example, is expected to add to the signal obtained from the bioassays, although it was found that only high concentrations of Δ^9-THC that are consistent with heavy and/or very recent cannabis use, give rise to a positive signal (53). This can be expected from the weak CB agonism of the compound, making Δ^9-THC a less ideal target for the bioassays as compared to the potent SCRAs (53,54).

Quite a few other GPCR activation assays have been used as research tools to determine the *in vitro* activity of SCRAs and to gain insight into receptor signalling (30,54). Examples are the PathHunter® assay (DiscoveRx) or the GTPγS assay (59,60). However, as stated by Cannaert *et al.,* it remains to be evaluated whether these systems can achieve the high sensitivities required to detect activity in biological matrices (54). Furthermore, the bioassays described here offer the advantages of being non-radioactive, homogeneous and relatively easy to perform. CB receptor activation can be monitored in real-time and its measurement proximal to the receptor is known to reduce the incidence of false positives (30).

Overall, it can be concluded that the proposed activity-based bioassays by Cannaert *et al.* offer a promising solution to fill the increasingly palpable void left by the conventional (non-)targeted analytical screening methods for the detection of (novel) abused substances in biological matrices. The bioassays have the potential to be applied in the setting of high-throughput screening to identify suspect samples that require further in-depth analytical investigation (54).

1.3.3 Bioassays for the screening of steroid hormones in biological matrices

Steroid hormones are a class of hormones synthesised from cholesterol. The different functional groups in varying orientations and altering oxidation states that arise during steroidogenesis, make up a large range of lipophilic, relatively low-molecular weight steroids that are biologically active as hormones. According to this biological activity, steroid hormones can be classified into two groups (61). The first group consists of the sex steroids, namely oestrogens, progestogens and androgens. These hormones play a role in reproduction and sex differentiation. Corticosteroids account for the second group, which includes glucocorticosteroids and mineralocorticosteroids. The former contribute in many aspects of immune function and metabolism, whereas the latter influence blood volume and electrolyte balance (61).

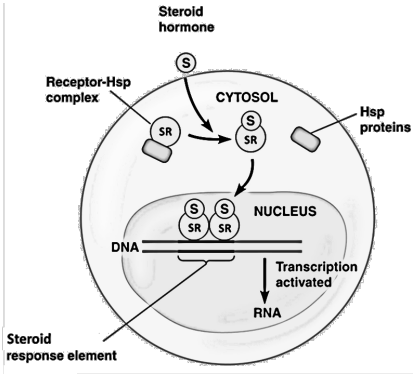

Figure 1.7. Signalling pathway of steroid hormones. S, steroid hormone; SR, steroid receptor; HSP, heat shock proteins. Figure from Cannaert *et al.,* 2018 (54), adapted from Pearson Education Inc., 2012.

Steroid hormones mediate their action via ligand-activated transcription factors, receptors that are members of the large superfamily of nuclear receptors (NRs). NRs can be classified into two major groups, responding to steroidal and non-steroidal ligands (e.g. thyroid hormones), respectively (50,62). Before binding the corresponding ligand, the inactive steroid receptor is initially sequestered in the cytosol of the target cell as a large complex containing chaperone polypeptides such as heat shock proteins (50). When a ligand enters the cell via diffusion, its association with the ligand-binding domain of the receptor induces the dissociation of the receptor away from this complex. This allows the bound receptor to dimerise and after phosphorylation, the now activated hormone-receptor complex translocates to the nucleus (50,62). Here, it recognises a specific DNA sequence known as a steroid response element (SRE). SREs are located within the promoter of the steroid-regulated gene, therefore binding of the occupied receptor activates transcription of DNA, eventually leading to the synthesis of proteins that alter cellular functions (**Figure 1.7**) (50,62,63). Activation of the androgen receptor, for example, leads to both androgenic and anabolic effects. The androgenic effect includes a more profound expression of male secondary sex characteristics. The misuse of androgens, for example in sports, can be attributed mainly to the anabolic effects, such as muscular growth-promotion (64). The mechanism of steroid-induced modulation of target genes is exploited by several assays that can be used for steroid screening (cf. infra).

Steroid hormones are among the most popular performance enhancing drugs abused in both elite and amateur sports (55,65). However, their use has been reported to cause many adverse effects. Anabolic steroid use, for examples, has been associated with infertility, hepatotoxicity and ischemic stroke (66,67). The World Anti-Doping Agency annually publishes a list of non-approved (groups of) substances and methods prohibited both in and out of competition (68). Apart from their continued abuse in sports, steroid hormones are also found as illicitly used growth-promoting agents in meat-producing animals. They are used to stimulate fattening in an attempt to maximise productivity of the animal and profit for the farmer (69). However, due to the growing awareness that remainders of growth-promoters in meat might adversely affect the consumer's health, the use of such growth-promoters in livestock production falls under the European ban published in 1988. Instead of providing a limitative list of forbidden hormones, this ban prohibits all substances having hormonal action (62,69).

The analytical strategies typically used for doping control and residue monitoring are essentially the same techniques as are used in the context of drug control. Screening for hormones can be done by traditional immunoassays, while GC- or LC-MS/MS systems are used for both screening and confirmation procedures (50,55,69). Similar to the emergence of NPS in the drug scene, the black market for hormones makes witty use of the "loopholes" in the detection methods by introducing so-called "designer steroids". Some of these steroids were once synthesised and studied for therapeutic or research purposes, although they are now often produced by clandestine chemists with the aim of being biologically active as hormones, while evading detection owing to their slightly modified chemical structure as compared to known steroids (66,67,70). In addition, both athletes and farmers attempt to avoid getting caught by administering low-dose hormone cocktails. The low concentration of each individual steroid challenges the limits of sensitivity of the current screening methods, although their simultaneous presence may exert additive or even synergistic hormonal effects (50,54,71). Due to the surreptitious nature of the novel substances and methods, minimal or no data regarding their safety is available and these practices are certainly not without risks (66,67).

Following the same rationale as for the screening of NPS, *in vitro* bioassays for steroid hormones allow the detection of even trace amounts of known and designer compounds by monitoring affinity for a given receptor and consequent biological activity of the ligand. Hence, detection of hormonal compounds can be achieved without the need of prior knowledge about their chemical structure. A wide range of *in vitro* bioassays to monitor steroid activity has been proposed. These vary from competitive receptor binding assays, to cell proliferation assays and reporter gene bioassays (RGBAs) (62). RGBAs exploit the natural signalling pathway of steroid hormones, as depicted in **Figure 1.7.** As opposed to receptor binding assays, RGBAs include the transactivation step occurring after binding of the ligand. This results in the assay's ability to differentiate between receptor agonists and antagonists. Furthermore, RGBAs might suffer less from matrix effects as the receptors are located intracellularly, which is in contrast to the receptor preparations used in the binding assays, and not all of the non-specific compounds will be able to enter the cells (62). RGBAs typically allow a more rapid and specific read-out than cell proliferation assays (62,72). They are commonly supported by host cells such as the yeast strain *Saccharomyces cerevisiae* or several mammalian-based cell lines such as osteosarcoma or breast cancer cells (66). Both types of host cells have several advantages and drawbacks, hence, the choice will depend on the intended purpose of the assay. Either way, it is important to keep in mind the different limitations of each cell type when interpreting the results from an RGBA. When assays with both cell types are run in parallel, complementary information can be obtained (62).

1.3.3.1 Yeast-based systems

Yeasts are popular host cells because of their low cost, easy handling and robustness. They do not require complex growth media such as calf serum that may carry steroids and thus risk false positive results (62,73). The robustness of the yeast cells enables them to tolerate and survive extracts from dirty matrices (e.g. urine) without extensive sample clean-up. This can probably be attributed to the presence of the yeast cell wall, making yeasts more tolerant to e.g. salts in urine (74). This cell wall, however, may pose a disadvantage to certain compounds that may be hampered in entering the cell or are pumped out via efflux pumps before reaching the receptor (62).

Another caveat that needs to be addressed when using yeast-based systems, is their non-identical cell machinery as compared to mammalian cells. A lack of steroid metabolising enzymes may hinder the detection of prohormones requiring human metabolism for their activation (66,75). The differences in cell machinery also require attention to be paid to potentially different folding and post-translational modifications (e.g. phosphorylation, glycosylation) of mammalian proteins being expressed in yeast cells. In addition, yeast host cells may not express the appropriate chaperone and co-regulator proteins required for maximal support of steroid mediated transactivation (63,76,77).

An advantage of yeast cells is that they lack endogenous steroid receptors, making them more specific and less susceptible to false positive results than their mammalian analogues (62). Especially for androgen screening, the lack of known endogenous receptors offers a great advantage, as androgen response elements can also be activated by both the progesterone and the glucocorticoid receptor. Mammalian cells containing either of these receptors experience cross-talk between the different steroids, therefore reducing the specificity of the assay (62). Yeasts, on the other hand, always require recombinant expression of the desired human steroid receptors via transformation with the corresponding cDNA. A reporter vector containing an SRE followed by the sequence of a reporter gene, is also introduced (50,54,62). Typically employed reporter genes include β-galactosidase, luciferase and fluorescent proteins such as enhanced green fluorescent protein (EGFP). In contrast to β-galactosidase and luciferase, which require cell lysis or expensive substrates in order to enable read-out, fluorescence can be measured as a function of incubation time, making it an easier and quicker reporter system (62). An additional advantage to the use of fluorescent proteins is that their read-out is not hindered by possible enzyme-inhibiting compounds (66).

An overview of the yeast-based bioassays that have been applied to biological matrices in the context of detecting steroid hormone abuse, can be found in **Table 1.1.**

Table 1.1. Overview of yeast-based reporter bioassays (54).

Matrix	Receptor	Reporter system	Incubation	EC_{50} value	Assay cells	Comments	References
AR assays: yeast-based							
Human urine (78)	hAR^{s}	β-galactosidase^{s}	O/N (79) 24h (78)	3.5 nM DHT (79) 4.73 nM T (79) 3.7 nM DHT (78) 5 nM T (78)	*S. cerevisiae* YPH500	Spiked urine samples only (78). Screening limited to urine samples not containing bioactive endogenous androgens. E_2 and P also respond to some extent.	Gaido *et al.,* 1997 (79) Nielen *et al.,* 2006 (78)
Human urine (80,81)	hAR^{s}	Secreted β-galactosidase^{s}	24h + O/N	-	*S. cerevisiae* PGKhAR	No deconjugation: steroid activity only reflects unconjugated androgens.	Sohoni *et al.,* 1998 (82) Zierau *et al.,* 2008 (80) Wolf *et al.,* 2011 (81)
Human serum (83)	hAR^{s}	*P. pyralis* luciferase^{s}	2.5h	10 nM T (83)	*S. cerevisiae* BMA64-1A	Preliminary screening of human serum samples (84). Potential application for detecting anabolic androgen abuse in athletes and cattle is mentioned (64,84).	Michelini *et al.,* 2005 (83) Michelini *et al.,* 2005 (84) Michelini *et al.,* 2008 (64)
Human urine (85,86) Human serum (86)	hAR^{s}	*P. pyralis* luciferase^{s} *P. pyralis* luciferase^{s} (red emitting mutant)	2h	7.5 nM DHT (85) 15 nM T (85)	*S. cerevisiae* BMA64-1A	Spiked samples only (85). Administration study with T (86). Potential use for detection in athletes is suggested (85,86).	Cevenini *et al.,* 2013 (85) Ekstrom *et al.,* 2013 (86)
Human urine (78) Bovine urine (87,88) Bovine hair (88)	hAR^{s}	Yeast EGFP^{s}	24h (78) 24h (87) 21h (88)	50 nM T (87) 76 nM T (88)	*S. cerevisiae* K20	Spiked samples only. Urine screening limited to samples that do not contain endogenous androgens, such as calf urine and urine from preadolescents (78,87).	Nielen *et al.,* 2006 (78) Bovee *et al.,* 2009 (87) Becue *et al.,* 2012 (88)
Human urine	hAR^{s}	EGFP^{s}	24h	-	*S. cerevisiae* BY4741 *S. cerevisiae* K0110	No hydrolysis: steroid activity only reflects unconjugated androgens. Potential use for detecting abuse in athletes and cattle is suggested.	Wolf *et al.,* 2010 (89)
Bovine urine	hAR^{s}	*Klebsiella* sp. ASR1 phytase^{s} (A-YAS)	6-25h	0.95 nM DHT 0.98 nM T	*A. adeninivorans* G1212	Potential use for detecting abuse in athletes and cattle is suggested.	Gerlach *et al.,* 2014 (90)
ER assays: yeast-based							
Bovine plasma (91)	hER^{s}	β-galactosidase^{s}	18h	-	*S. cerevisiae* BJ3505	Potential use for detecting abuse in cattle is suggested.	Klein *et al.,* 1994 (92) Burdge *et al.,* 1998 (91)
Bovine urine (73–75,93,94) Bovine hair (88)	hERα^{s}	Yeast EGFP	4-24h (74) 24h (73,75,93,94) 21h (88)	0.4 nM E_2 (74) 0.7 nM E_2 (75,88) 0.5 nM E_2 (93)	*S. cerevisiae* K20	Spiked urine only (74,75). Screening limited to urine samples that do not contain bioactive endogenous oestrogens.	Bovee *et al.,* 2004/5 (74,75) Nielen *et al.,* 2004/6 (73,94) Divari *et al.,* 2010 (93) Becue *et al.,* 2012 (88)
AR-ER-PR assay: yeast-based							
Marmoset serum	hAR^{s} hER^{s} hPR^{s}	hAR+GFP hER+DsRed2 hPR+CFP	18h	0.57 nM DHT 0.062 nM E_2 0.467 nM P	*A. adeninivorans* G1212	Simultaneous detection of oestrogens, progestogens and androgens in one experiment.	Chamas *et al.,* 2017 (95)

Abbreviations: **(h)AR**, (human) androgen receptor; **(h)ER**, (human) oestrogen receptor; **CFP**, cyan fluorescent protein; **DsRed2**, discosoma red fluorescent protein; **DHT**, dihydrotestosterone; E_2, 17β-estradiol; EC_{50}, concentration giving a half maximum response (i.e. sensitivity of the assay); **EGFP**, enhanced green fluorescent protein; **O/N**, overnight; **P**, progesterone; **PGK**, phosphoglycerate kinase; **T**, 17β -testosterone. **Superscripts:** ^{s} stable.

1.3.3.2 Mammalian-based systems

Mammalian-based bioassays are characterised by a higher sensitivity and lower specificity as compared to their yeast counterparts. Additionally, they are less robust and more susceptible to the presence of contaminants in complex matrices (62,66). Exposure to such contaminants may induce cytotoxic or apoptotic responses in mammalian cells, potentially and mistakenly resulting in a lower observed steroid activity (96). Important differences between both types of hosts cells are the presence of both endogenous receptors and steroid metabolising enzymes in most mammalian cells.

As biological matrices often contain traces of steroid hormones, nonspecific reactions can occur due to binding to an endogenous receptor whose activation also results in reporter gene transcription. This stresses the importance of the chosen SRE (cf. supra) (62). An additional risk of interference with the read-out of the bioassay is introduced through the need of medium containing serum for mammalian cell growth. Small amounts of steroids present in the serum may indeed give rise to false positive results. This can be avoided by making use of stripped serum (e.g. charcoal-treated) (54,62).

The presence of steroidogenic enzymes in mammalian cells can cause metabolisation of the initial steroid hormone into less or even more potent metabolites. This, of course, results in a different read-out of the activity-based mammalian bioassay as opposed to a parallel yeast screen, where the sensed activity will originate predominantly from the unchanged parent compound. The determination of bioactivity after metabolisation is indeed an important aspect when evaluating the potential physiological activity of (designer) steroids. For example, an *in vitro* androgen bioassay using a human liver cell line has shown that several designer androgens are activated into potent metabolites (66). Mammalian-based systems may thus allow detection of prohormones and evaluation of the full bioactivity *potential* of a substance, while a corresponding yeast screen determines the *intrinsic* activity of compounds, illustrating the complementarity of both assay types (66).

Table 1.2 provides an overview of the mammalian-based bioassays used in the context of detecting steroid hormone abuse in biological samples from athletes and meat-producing animals. Note the lower EC_{50} values for the androgen receptor assays when compared to the values reported for the corresponding yeast-based systems, illustrating the mammalian bioassays' higher sensitivity.

Table 1.2. Overview of mammalian-based reporter bioassays (54).

Matrix	Receptor	Reporter	Incubation	EC_{50}	Assay cells	Comments	References
AR assays: mammalian-cell based							
Bovine urine	hAR[e]	*P. pyralis* luciferase[s] (AR-LUX)	24h	-	T47D	Endogenous expression of ER and PR might reduce specificity of the assay.	Blankvoort *et al.,* 2003 (97)
Human urine (55)	hAR[s]	*P. pyralis* luciferase[s] (AR-CALUX)	24h	0.13 nM DHT (98) 0.63 nM T (98) 0.12 nM DHT (55) 0.87 nM T (55)	U2-OS	The AR-CALUX assay was first described by Sonneveld *et al.* and used to detect endogenous androgenic activity in human and fetal calf serum (98). Houtman *et al.* evaluated spiked urine samples (55). Cross-reactivity with with dexamethasone, E_2 and some synthetic progestins (55).	Sonneveld *et al.,* 2004 (98) Houtman *et al.,* 2009 (55)
Human serum	hAR[t]	*P. pyralis* luciferase[t]	O/N	-	COS-1	No strict doping abuse, but evaluation of topical DHT administration.	Raivio *et al.,* 2002 (99)
Human urine	hAR[s]	CFP-AR-YFP[s]	O/N	0.55 nM DHT 2.04 nM T	HeLa	Only detection of abuse if urine is collected soon after doping event.	Bailey *et al.,* 2016 (100)
GR assays: mammalian-cell based							
Human serum	hGR[t]	Luciferase[t]	O/N	-	COS-1	No strict doping abuse, but evaluation of serum glucocorticoid bioactivity after inhalation of budesonide or fluticasone propionate in asthmatic children.	Raivio *et al.,* 2002 (101)
Bovine urine	hGRα[s]	*P. pyralis* luciferase[s] (GR-CALUX)	24h	1.2 nM DM	U2-OS	The bioassays failed to detect the synthetic prohormone prednisone.	Pitardi *et al.,* 2015 (102)
Bovine liver	hGR[s]	*P. pyralis* luciferase[s] *R.* reniformis[s]	24h	13 nM DM	HeLa	Future experiments should assess if other biological matrices can be tested.	Schumacher *et al.,* 2003 (84)
Bovine urine (103–105) Bovine liver (103,105)	hGRα[s]	*P. pyralis* luciferase[s] (TGRM-Luc)	24h	6.2 nM DM (105) 7.1 nM DM (106) 7.9 nM DM (103) 2.0 nM DM (104)	T47D	Limited assessment of spiked urine samples. Partial validation on spiked liver samples (103). All glucocorticoid-treated animals were detected (104).	Willemsen *et al.,* 2002/4/5 (103,105,106) Connolly *et al.,* 2009 (104)
PR assays: mammalian-cell based							
Bovine urine (105) Bovine liver (103,105)	hPR[e]	*P. pyralis* luciferase[s] (TM-Luc)	24h	1.46 nM P (105) 1.5 nM P (106) 1.1 nM P (103)	T47D	Spiked urine samples only. Highly variable levels of endogenous natural hormones.	Willemsen *et al.,* 2002/4/5 (103,105,106)

Abbreviations: **(h)AR**, (human) androgen receptor; **(h)GR**, (human) glucocorticoid receptor; **(h)PR**, (human) progesterone receptor; **(CA)LUX**, (Chemically Activated) Luciferase eXpression; **CFP**, cyan fluorescent protein; **DHT**, dihydrotestosterone; **DM**, dexamethasone; E_2, 17β-estradiol; EC_{50}, concentration giving a half maximum response (i.e. sensitivity of the assay); **EGFP**, enhanced green fluorescent protein; **O/N**, overnight; **P**, progesterone; **T**, 17β - testosterone; **TGRM**, glucocorticoid-responsive cell line; **TM**, progesterone-responsive cell line; **YFP**, yellow fluorescent protein. **Superscripts:** [e] endogenous; [s] stable; [t] transient.

2 OBJECTIVES

Over the last decade, the detection of SCRAs and other NPS has been the cause of many toxicologists' headaches. The virtually endless number of substances, their chemical diversity and the rapid pace at which they emerge, make these compounds particularly challenging in terms of detection and monitoring. Current approaches mostly make use of targeted, structure-based techniques, such as immunoassays or mass spectrometry-based methods. However, these approaches have important limitations, including a lack of cross-reactivity and the need for prior knowledge of molecular identity. Moreover, the high potency of many SCRAs requires very sensitive detection methods, as low nanogram per millilitre concentrations in biological matrices are not uncommon. These "loopholes" in the current detection techniques emphasise the need for novel, high-throughput-amenable screening tests that aim at identifying suspect samples and may direct further bioanalytical investigation.

Recent research has pinpointed the potential of activity-based bioassays as first-line screening tools for the detection of cannabinoid activity in authentic samples from drug users (8,30,53). The proposed bioassays are based on the functional complementation of a split Nanoluc® luciferase (NanoBiT® Technology) upon activation of the cannabinoid receptors by a (known or novel) agonist. The resulting bioluminescence can be easily measured. This way, the proposed bioassays are capable of detecting (synthetic) cannabinoids purely based on their cannabinoid activity, requiring no prior knowledge about their structure.

In the present dissertation, the previously developed bioassays (8,53) are applied to a large set of serum samples acquired from patients with acute drug-related toxicity treated at the Emergency Department of the Guy's and St. Thomas' Hospital in Westminster (London, UK) from April to December 2016. The main objective of this project is to evaluate the applicability of the developed activity-based cannabinoid bioassays as a screening tool for the detection of SCRAs in a large set of serum samples (n = 471). Both the sensitivity and the specificity of the bioassays will be assessed in two sets of experiments that differ in the amount of sample volume analysed (500, 300 or 100 µL). To do so, the results obtained with the bioassays will be compared to the declared substance use and data acquired through externally performed liquid chromatography coupled to high-resolution tandem mass spectrometry. Secondly, it will be evaluated whether the strength of the CB_1 bioassay's signal can be correlated to the total SCRA concentration present in the sample, hence possibly allowing to formulate a semi-quantitative statement about cannabinoid activity present in the serum samples. Finally, a third objective is to portray the different clinical presentations following SCRA (ab)use to further enhance our understanding of the potential dangers associated with this group of NPS.

3 MATERIALS AND METHODS

3.1 DESIGN OF THE CB REPORTER ASSAYS

The different steps in the development of the CB_1/CB_2 bioassays were previously described by Cannaert *et al.* (8,30,53). In short, the CB reporter assays make use of NanoLuc® Binary Technology (NanoBiT®) (Promega, Madison, WI, USA). The ideal combination of Nanoluc® luciferase subunits coupled to either the C-terminus of the CB_1/CB_2 receptors, or the N-/C-terminus of β-arr2, was previously evaluated (30). It was found that, even in absence of a CB ligand, cells expressing both components of the fusion system (i.e. CB receptor and β-arr2) generated a signal above background. The latter indicates a certain extent of spontaneous recombination of both subunits, possibly caused by some level of constitutive CB-β-arr2 interaction (30). Upon stimulation of all possible combinations with the CB agonist JWH-018 (naphthalen-1-yl-(1-pentyl-1H-indol-3-yl)-methanone), the highest increases in signals were obtained for the following combinations: CB_1-LgBiT/SmBiT-β-arr2 and CB_2-SmBiT/LgBiT-β-arr2 (**Figure 3.1**) (30). Overall, higher absolute signals were generated in the CB_2 bioassay (30). Next, truncation of β-arr2 was evaluated to increase the sensitivity of the bioassays. The first truncation mutant, $\beta\text{-arr2}_{TR382}$, consists of the first 382 amino acids of the total 410 amino acids that make up the wild-type β-arr2. Removal of the C-terminus results in a constitutively active mutant that is recruited to the GPCR independent of prior GPCR phosphorylation (31,53). In the second mutant, $\beta\text{-arr2}_{TR366}$, amino acids 367-385 are eliminated, reducing clathrin binding by approximately 90% (32,53,58). It was found that the combinations CB_1-LgBiT/SmBiT-$\beta\text{-arr2}_{TR366}$ and CB_2-SmBiT/LgBiT-$\beta\text{-arr2}_{TR382}$ yielded significantly higher signals as compared with the wild-type β-arr2 (53). The generation of all above-mentioned constructs was done by means of standard molecular biology techniques and detailed information can be found in the corresponding publications by Cannaert *et al.* (8,30,53).

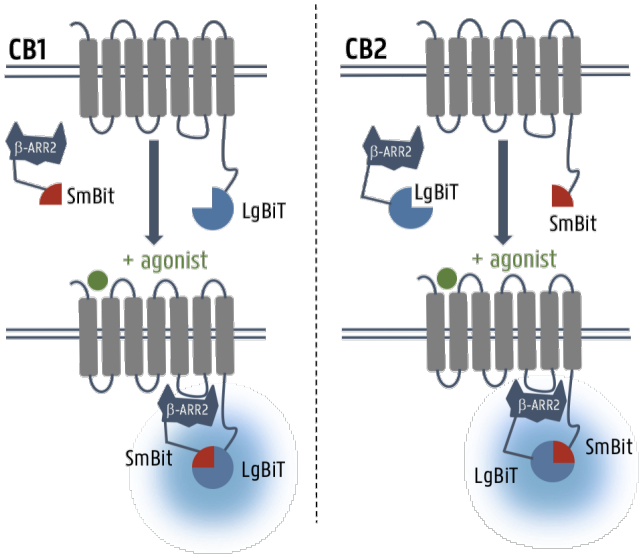

Figure 3.1. Set-up of the CB_1/CB_2 reporter assays: CB_1-LgBiT/SmBiT-β-arr2 and CB_2-SmBiT/LgBiT-β-arr2. β-arr2, β-arrestin 2; CB, cannabinoid; LgBiT/SmBiT, large/small subunit of NanoLuc® luciferase. Figure adapted from Cannaert *et al.,* 2018 (53).

Two stable cell lines expressing either the CB_1-LgBiT/SmBiT-β-$arr2_{TR366}$ or the CB_2-SmBiT/LgBiT-β-$arr2_{TR382}$ fusion constructs were established following retroviral transduction of human embryonic kidney (HEK) 293T cells. The four retroviral constructs were designed with a built-in control of the level of expression of the CB- and β-arr2-constructs. This was established by coupling the expression of the individual CB- and β-arr2-constructs to that of co-expressed markers, EGFP and truncated nerve growth factor receptor (dNGFR) for CB- and β-arr2-constructs, respectively. These markers allow cell sorting and follow-up of cell line stability by means of flow cytometry (8). Cell sorting was done to obtain cells that were positive for both EGFP and dNGFR, which meant that both parts of either one of the fusion combinations (CB_1-LgBiT/SmBiT-β-$arr2_{TR366}$ or CB_2-SmBiT/LgBiT-β-$arr2_{TR382}$) were present. Cells stably expressing the CB reporter systems were readily available at the start of the experiments described in this dissertation.

3.2 CELL CULTURE

The HEK293T cell lines stably expressing the CB_1 or the CB_2 reporter bioassay (CB_1-LgBiT/SmBiT-β-$arr2_{TR366}$ or CB_2SmBiT/LgBiT-β-$arr2_{TR382}$) were cultivated in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Pittsburgh, PA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich, Steinheim, Germany), 2 mM of glutamine, 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B (all Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% CO_2. The cell line was previously found to be stable up to twenty passages. For experiments, 96-well plates were coated with poly-D-lysine (Sigma Aldrich) and the cells were seeded at 5 x 10^4 cells per well one day prior to the experiments (8,53).

3.3 COLLECTION OF SERUM SAMPLES

Serum samples were obtained from 471 patients presenting with acute drug-related toxicity to the Guy's and St. Thomas' Hospital Emergency Department in Westminster (London, UK), from April to December 2016. Ethical approval to perform this study was granted via the National UK Ethics approval reference 14/YH/1293.

3.4 SERUM SAMPLE PREPARATION

The serum samples were stored at -20°C prior to use. Sample preparation for the bioassays was performed following a previously described protocol (53,107). For the majority of samples, 500 µL, or, if not available, 300 µL (i.e. "bioassay per protocol"), was pipetted into a glass tube (16 x 100 mm). An aliquot of 100 µL (i.e. "bioassay with reduced sample volume") was used for the remaining samples that contained less than 300 µL serum. Next, 500 µL of a carbonate buffer (pH 10) was added. The buffer was prepared by dissolving 10.599 g sodium carbonate (Merck, Darmstadt, Germany) in 1 L ultrapure water (Merck) and mixing 534 mL of this solution with 466 mL of sodium hydrogen carbonate solution. The latter was prepared by dissolving 4.201 g sodium hydrogen

carbonate (Merck) in 500 mL ultrapure water. Next, a liquid-liquid extraction was performed by adding 4 mL of n-hexane/ethyl acetate (99:1 V/V) (both from Merck) to the glass tube containing the serum and buffer. The extraction mixture was vortexed for 1 minute and subsequently centrifuged for 10 minutes at 2900 x g. Next, the organic upper layer was transferred to another glass tube (16 x 100 mm) and evaporated at room temperature under a gentle stream of nitrogen (Zymark Ltd., Cheshire, UK) (53,107).

3.5 BIOANALYSIS OF SERUM SAMPLES

Bioanalysis of the serum samples was performed externally by LGC Laboratory and Managed Services (Fordham, UK) using a Thermo XRS Ultra High-Performance Liquid Chromatography system, coupled to a Thermo Q Exactive Focus High Resolution Accurate Mass Spectrometer (LC-HRMS/MS) in heated positive ion electrospray mode. Data were acquired in full scan mode, followed by an additional scan event using "all ion fragmentation". Processing of the data was done using ToxFinder® Software (Thermo Fisher Scientific) against an in-house database containing 918 compounds, primarily SCRAs and SCRA metabolites.

3.6 CANNABINOID REPORTER BIOASSAYS

The CB_1 and CB_2 reporter assays were performed as previously described (8,53). After overnight incubation in the 96-well plates, the cells were washed twice with Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific) to remove any remaining FBS, as synthetic cannabinoids have a tendency of binding to (serum) proteins. Finally, 100 µL of Opti-MEM I was added to the washed wells. Furimazine substrate was added under the form of the non-lytic Nano-Glo® Live Cell detection reagent, which was prepared by diluting the Nano-Glo® Live Cell substrate 20 (CB_1) or 40 (CB_2) times using Nano-Glo® LCS Dilution buffer (all from Promega), and 25 µL was added to the cells in each well. Next, the plate was placed in the Tristar^2 LB 942 luminometer (Berthold Technologies, Bad Wildbad, Germany) and the signal was monitored until stable (10-15 min). During this initial equilibration period, the cell-permeable furimazine substrate had the time to reach and subsequently enter the cells. Meanwhile, the evaporated extracts from Section 3.4 were reconstituted in 100 µL of Opti-MEM I/methanol (50:50 V/V) and, after obtaining a stable signal in the equilibration step, 10 µL of these reconstituted extracts was added to each well. The plate was placed back in the luminometer and bioluminescence was continuously monitored for approximately 6900 seconds (115 minutes). The short-term exposure to a final methanol concentration of 3.7% did not affect the viability of the cells or interfere with the read-out of the bioassays (8,53). All samples were run in duplicate, which is further indicated as "n = 2". One positive and four independent negative controls were run per plate. The positive control consisted of 50 ng/mL of the highly potent CB_1/CB_2 agonist JWH-018 (LGC, Wesel, Germany), which was pipetted directly onto the cells. Blank serum samples of four individuals served as negative controls.

4 RESULTS

A total of 471 serum samples from drug-related cases were analysed for the presence of SCRAs using the CB_1 and CB_2 bioassays and the results were compared to the bioanalytical data acquired through LC-HRMS/MS analysis. The median age of the patients recruited to this study was 32 years (IQR: 26-40 years, range: 17-61 years) and the majority (85.6%) were males.

4.1 BIOANALYSIS

Based on a database containing a total of 918 compounds, LC-HRMS/MS bioanalysis revealed the presence of SCRAs in 70 out of 471 (14.9%) samples. The median age in the SCRA user cohort was 37 years (IQR: 29-43 years, range: 20-57 years) and 66 (94.3%) were males. A complete overview of the analytical data and the corresponding clinical information retrieved upon admission of each patient to the Emergency Department, is shown in **Appendix II.**

A summary of the identified SCRAs together with the detected concentration levels, can be found in **Table 4.1.** The most commonly detected SCRA parent compound was MDMB-CHMICA (n = 22), followed by AMB-CHMICA (n = 17), cumyl-5F-PINACA (n = 10) and 5F-ADB (n = 9). In total, seven different SCRA parent compounds were detected. Two detected metabolites could originate from different parent compounds (**Table 4.1**). Parent compound and corresponding metabolites taken together, the most frequently encountered SCRA was 5F-ADB: traces of this SCRA were detected in a total of 49/70 (70.0%) patients. MDMB-CHMICA or AMB-CHMICA were involved in respectively 31/70 (44.3%) and 23/70 (32.9%) intoxications, whereas traces of cumyl-5F-PINACA were found in 13/70 (18.6%) cases. Intoxication with different SCRAs (i.e. poly-SCRA intoxication) was found in 40/70 (57.1%) cases, with a maximum of five different SCRAs concurrently detected in one patient. SCRA use was self-reported by 57/70 (81.4%) patients. This claimed use was described as “Spice” (n = 55), “Man down” (n = 1) or “Tasmanian weed” (n = 1). Four patients in whom SCRA use was detected, reported the use of an “unknown drug”, which one patient believed to contain cannabis or a SCRA.

Evidence for the use of other drugs of abuse (excluding alcohol and nicotine) was present in 24/70 (34.3%) SCRA users. The detected analytes were (i) cocaine/benzoylecgonine (n = 19); (ii) amphetamine (n = 2); (iii) THC-COOH (n = 2); (iv) gamma-hydroxybutyric acid (GHB) (n = 1); (v) ketamine (n = 1); (vi) methamphetamine (n = 1); and (vii) mexedrone (n = 1). Other commonly detected (prescription) drugs included, but were not restricted to, benzodiazepines (n= 27); opioids/opiates (n = 26), including methadone; and antipsychotics (n = 9). Cotinine was found in all but three SCRA users’ samples. Interestingly, ethyl glucuronide was detected in only 31/70 (44.3%) patients. The median number of analytes co-detected alongside SCRAs, was four. Only one patient’s serum was

clean for all other targeted substances, whereas analysis revealed a maximum of nineteen additional analytes in one SCRA user. An overview of all findings per patient is included in **Appendix II.**

Table 4.1. Overview of the analytically detected SCRAs, the corresponding concentration ranges and number of detections (alone or in combination) (N). Metabolite data are shown between brackets. Chemical names (IUPAC) retrieved from UNODC.

Abbreviated name	Chemical name	Range (pg/mL)	N
5F-ADB (-O-desmethyl; -desfluorohydroxypentyl)	Methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate	**165-8225** (318-913765; 125-7640)	**9** (49; 31)
5F-AKB-48 (-desfluorohydroxypentyl)	N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide	**163-1200** (783-2438)	**4** (4)
AB-CHMINACA (-desamino-carboxylic acid)	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide	**510-11450** (3288-30737)	**3** (3)
AB-, AMB- or EMB-CHMINACA (AB-CHMINACA-desamino-carboxylic acid)	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide Methyl (1-(cyclohexylmethyl)-1H-indazole-3-carbonyl)-valinate Ethyl (1-(cyclohexylmethyl)-1H-indazole-3-carbonyl)-valinate	(171-3288)	**0** (2*)
AB-, AMB- or EMB-FUBINACA (AMB-FUBINACA-O-desmethyl)	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide Methyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate Ethyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate	(1850-226560)	**0** (8**)
AMB-CHMICA (-O-desmethyl)	Methyl (1-(cyclohexylmethyl)-1H-indole-3-carbonyl)-valinate	**110-19038** (1802-163125)	**17** (23)
BB-22 (-carboxy)	Quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate	**1080** (1100)	**1** (1)
Cumyl-5F-PINACA (-desfluorohydroxypentyl)	1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide	**540-24120** (355-3300)	**10** (13)
MDMB-CHMICA (-O-desmethyl, -hydroxy(indole))	Methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate	**355-39940** (168-170575; 350)	**22** (31; 1)

* lone AB-CHMINACA-desamino-carboxylic acid can originate from any of three parent compounds: AB-, AMB- or EMB-CHMINACA.

** lone AMB-FUBINACA-O-desmethyl can originate from any of three parent compounds: AB-, AMB- or EMB-FUBINACA.

4.2 BIOASSAY

Serum extracts were generated as described in Section 3.4 and analysed by means of the previously established CB_1 and CB_2 reporter bioassays (CB_1-LgBiT/SmBiT-β-arr2$_{TR366}$ and CB_2SmBiT/LgBiT-β-arr2$_{TR382}$). In total, 471 serum samples were analysed with both bioassays in two different sets of experiments. Section 4.2.1 describes the results obtained after analysis of 395 serum samples containing minimally 300 µL serum. In a next set of experiments (Section 4.2.2), the remaining 76 samples, for which only 100 µL was available for sample preparation, were analysed. Despite several serum samples being strongly haemolysed, 471 clear extracts were obtained after sample preparation and no interference with the read-out of the bioassays occurred. The scoring of the samples (SCRA-positive/-negative) was done blind-coded and independently by two individuals (the author and Dr. A. Cannaert) based on the raw data, without prior knowledge of the number of positives. Four

independent blanks and a positive control were included in each assay to assess potential positivity of the samples. The presence of SCRAs could be identified by a rise in relative light units (RLU) over time (53). As exemplified in **Figure 4.1**, this rise was either very prominent or more subtle, although still differentiable from the blank signals.

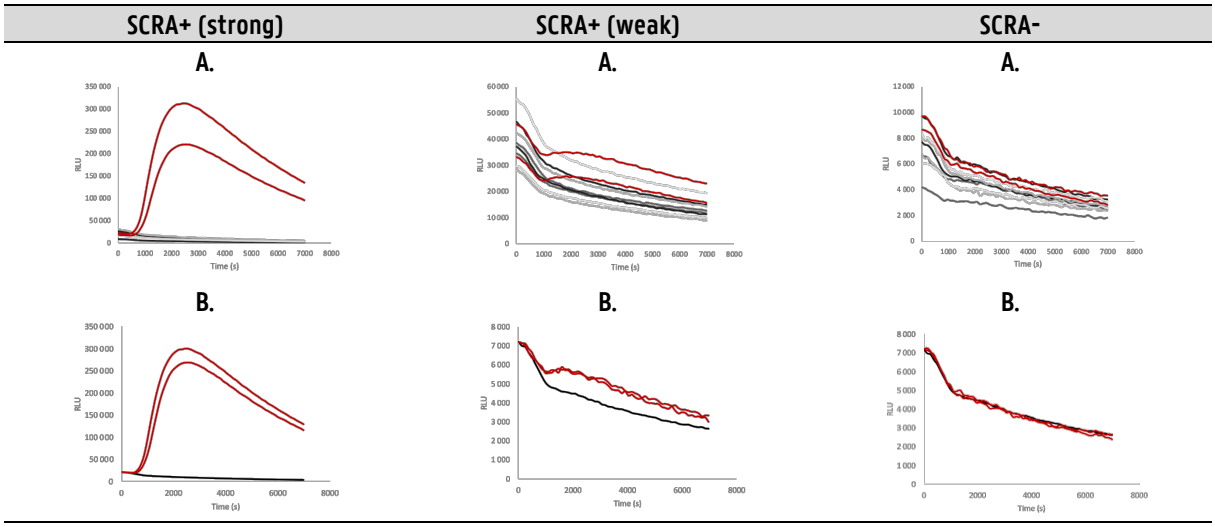

Figure 4.1. Exemplary raw (A) and corrected (B) read-outs of the cannabinoid bioassays for different serum samples (respectively SCRA-positive (strong/weak) and SCRA-negative). Scoring was based upon the raw profiles. The corrected profiles (B) show the average signal of the blanks (black line) and have been corrected for inter-well variability. RLU (y-axis), relative light units; s (x-axis), seconds; red lines, serum samples (n = 2); grey and black lines, four independent blanks (n = 2); single black line, average blank signal.

4.2.1 Bioassay per protocol (≥ 300 µL)

A total of 395 serum samples contained enough sample volume (≥ 300 µL) to perform the sample preparation as described by Cannaert *et al.* (Section 3.4) (53). The starting volume was 500 µL serum for 364 samples, whereas 300 µL serum was used for the remaining 31 samples. When comparing the outcome of the bioassays to bioanalysis, all 52 analytically confirmed SCRA-positive samples were picked up by the bioassays, yielding a sensitivity of 100% (52/52) (**Table 4.2**) (**Appendix II, samples 1-52**). An overview of all positive profiles is provided in **Appendix III-A**. A total of 41 (78.8%) samples showed combined CB_1/CB_2 receptor positivity. The remaining eleven samples showed a rise in RLU in the CB_1 bioassay only. The profiles accompanying the combined CB_1/CB_2-positive samples can be found in **Appendix III-B**. Overall, higher total SCRA concentrations seemed to be present in the combined positive samples compared to the samples causing CB_1 receptor activation only (data not shown).

Table 4.2. Overview and comparison of the results obtained through LC-HRMS/MS analysis and bioassay screening (≥ 300 µL sample volume). LC-HRMS/MS, liquid chromatography high-resolution tandem mass spectrometry.

		HRMS/MS analysis	
		+	-
Bioassay	+	52	13
	-	0	330
Total		**52**	**343**

Of the 343 SCRA-negative samples via LC-HRMS/MS analysis, 330 (96.2%) were correctly scored negative with both the CB_1 and CB_2 bioassays, whereas thirteen (3.8%) scored weakly positive for CB_1 receptor activation only (**Table 4.2**). Bioanalysis, however, showed that six out of thirteen samples contained Δ^9-THC (**Figure 4.2-A**). Consequently, these samples were not considered falsely scored positive as Δ^9-THC is also a (partial) CB agonist, yielding a total of seven false positives. This taken into account, a specificity of 97.9% (330/337) was obtained. The profiles of the positively scored samples containing Δ^9-THC, as well as the false positives, are included in **Appendix III-C** following the corresponding HRMS/MS findings and clinical data. Note that not all samples containing Δ^9-THC were picked up by the bioassays. The lowest level of Δ^9-THC that gave rise to a positive signal in the CB_1 bioassay was 3640 picogram per millilitre. However, other samples containing higher concentrations of Δ^9-THC failed to be detected with the bioassays.

It should be noted that of the seven presumptive false positive samples, one user claimed "Spice" intake, which was not supported by bioanalysis (**Figure 4.2-B**). In this patient, the following compounds were detected: (i) benzoylecgonine, a cocaine metabolite; (ii) clonazepam, diazepam, nordazepam, oxazepam, temazepam; (iii) cotinine; (iv) ethyl glucuronide; (v) methadone/2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP); and (vi) pregabalin. As these compounds were also detected in correctly scored negative samples, no interference with the read-out of the bioassays is expected.

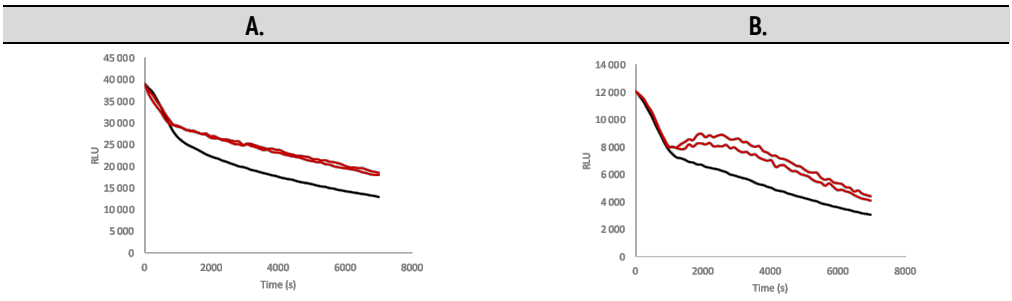

Figure 4.2. CB_1 analysis of (A) a sample containing Δ^9-THC; and (B) the sample where the bioassay supported claimed "Spice" intake despite a lack of analytical confirmation. Both samples were analysed with a starting volume of 500 µL serum and scored positive for CB_1 receptor activation only. Δ^9-THC, (-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol; RLU (y-axis), relative light units; s (x-axis), seconds; red lines, serum samples (n = 2); single black line, average signal of four independent blanks. The profiles represent the data corrected for inter-well variability.

4.2.2 Bioassay with reduced sample volume (100 µL)

Apart from the 395 samples analysed as described in the previous section (≥ 300 µL), an additional 76 serum samples did not contain enough sample volume to perform the bioassays per protocol. For these samples, the sensitivity of the bioassays was put to the test by starting from a reduced volume of 100 µL serum. The results of these analyses are presented in **Table 4.3.**

Table 4.3. Overview and comparison of the results obtained through LC-HRMS/MS analysis and bioassay screening (100 µL sample volume). LC-HRMS/MS, liquid chromatography high-resolution tandem mass spectrometry.

		HRMS/MS analysis	
		+	-
Bioassay	+	16	2
	-	2	56
Total		**18**	**58**

Of the eighteen samples containing SCRAs according to LC-HRMS/MS bioanalysis, sixteen were scored positive with the bioassay screen, yielding a sensitivity of 88.9% (16/18) (**Appendix II, samples 53-70; Appendix IV-A**). Of these, the SCRA(s) present in nine (56.3%) samples activated both the CB_1 and CB_2 receptor and the resulting profiles are shown in **Appendix IV-B.** The remaining seven samples showed activation of the CB_1 receptor only. Two SCRA-containing samples were missed with both bioassays (**Figure 4.3-A**). These samples contained (i) 5F-ADB-O-desmethyl (853 pg/mL), AMB-FUBINACA-O-desmethyl (3287 pg/mL); and (ii) MDMB-CHMICA-O-desmethyl (3376 pg/mL). Compared to the total SCRA concentrations of the other samples in the batch, the SCRA levels of these missed samples were on the lower end, although samples with an even lower total SCRA concentration had been picked up.

A total of 56 samples were correctly scored negative with the bioassays, resulting in a specificity of 96.6% (56/58). Two samples in which bioanalysis failed to detect any cannabimimetic compounds, gave rise to a weak increase in RLU in the CB_1 bioassay. Their corresponding activation profiles can be found in **Figure 4.3-B,C.** Bioanalysis of the first false positive sample (**B**) revealed the presence of (i) cocaine/benzoylecgonine; (ii) codeine and morphine; (iii) cotinine; (iv) diazepam, nordazepam, oxazepam, temazepam; (v) gabapentin; (vi) methadone/EDDP; (vii) paracetamol; and (viii) tetramisole. The user did claim cannabis use, of which no traces were detected via bioanalysis. The second unexpected positive sample (**C**) originated from a user who reported having used "Spice", which would be in accordance with the positive signal as detected by the bioassay but was not supported by any bioanalytical evidence. In this patient, only cotinine was identified. The profiles depicting the raw data of both the missed SCRA-positives, as well as the two (presumed) false positives, are provided in **Appendix IV-C**.

A list of all (prescription) drugs and substances of abuse other than SCRAs that were detected in the correctly scored negative samples from both the per protocol bioassay analysis (343 negatives out of 395

analysed samples), as well as the assays ran with reduced sample volume (56 negatives out of 76 analysed samples), can be found in **Appendix V**.

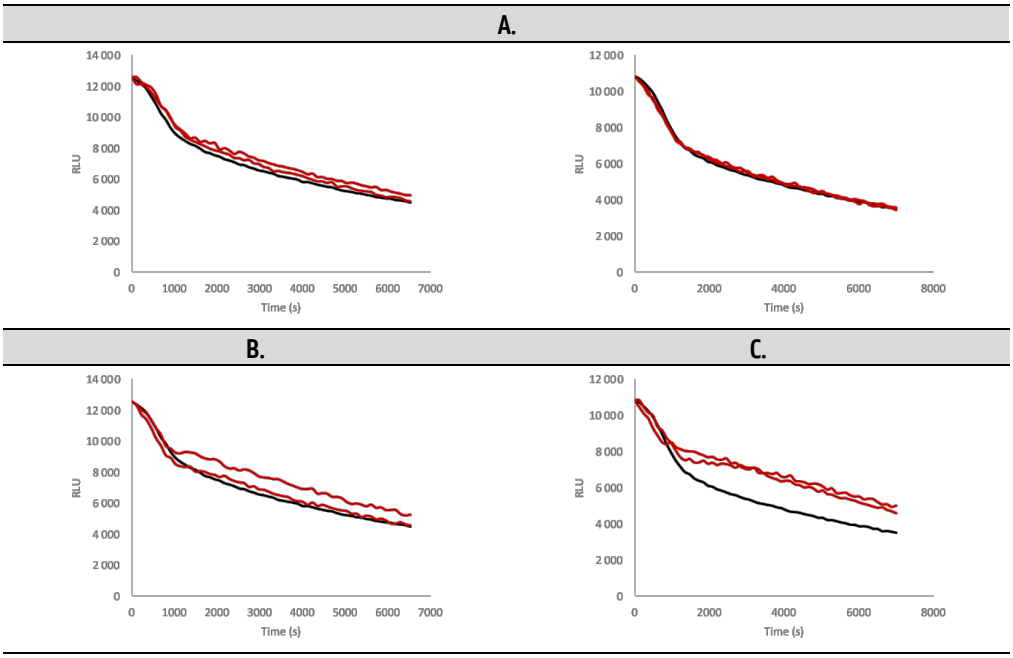

Figure 4.3. CB_1 analysis of the samples (100 µL) that were (A) missed; or (B-C) (currently) falsely scored positive. The positivity found in the bioassay for (C) supported claimed SCRA use that was not bioanalytically evidenced. RLU (y-axis), relative light units; s (x-axis), seconds; red lines, serum samples (n = 2); black line, average signal of four independent blanks. The profiles represent the data corrected for inter-well variability.

4.2.3 Evaluation of a potential correlation between signal strength and SCRA concentrations

To date, the outcome of the cannabinoid bioassays had been used solely to assess presence or absence of a compound with cannabinoid activity or to evaluate the activity of different SCRAs and their metabolites (8,30,53). The available semi-quantitative information in this study allowed a preliminary evaluation of a potential correlation between the extent of receptor activation in the bioassays (i.e. area under the curve, AUC) and semi-quantitative analytical data.

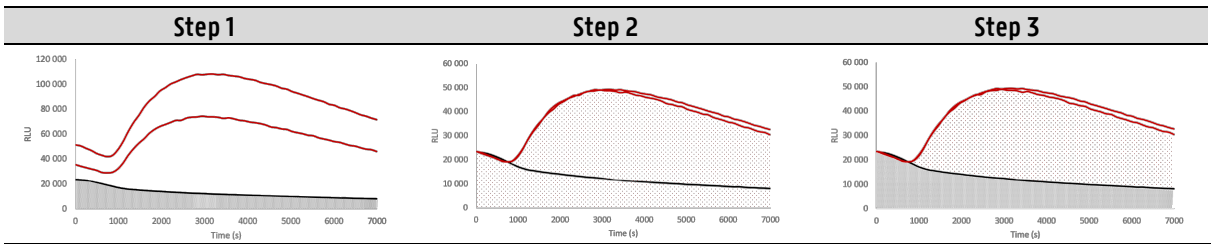

Figure 4.4. Different steps in the calculation of the area under the curve (AUC) of the bioassay signal. RLU (y-axis), relative light units; s (x-axis), seconds; red lines, serum samples (n = 2); black line, average signal of four independent blanks; AUC of the average blank signal; AUC of the signal generated by the serum samples.

The AUC was calculated as follows (**Figure 4.4**). First, the signal generated by the four independent blanks was averaged, after which the AUC of the new average blank signal was calculated via the trapezoidal rule (**Step 1**). Next, the signal originating from the suspected serum sample was forced through the same starting point as the blank signal. This was done by multiplying each value with a correction factor, i.e. the ratio of the average blank RLU to the RLU generated by the serum sample (both at t = 0). The AUC of this signal was calculated (**Step 2**) and the previously obtained AUC of the average blank was subtracted from this value (**Step 3**). This way, the outcome was corrected for both the signal generated by the blanks as for any inter-well variability. Steps 2-3 were performed for both duplicate signals and an average blank-corrected AUC was determined. In a final step, this value was normalised to the AUC of the positive control (50 ng/mL JWH-018) that had been run in the same experiment, which was calculated following the same procedure. The normalised AUC values were then plotted against the total SCRA concentration (parent compound and metabolites), as illustrated below. A full list of the concentrations and obtained AUC values used to plot the graphs in this section, can be found in **Appendix VI**.

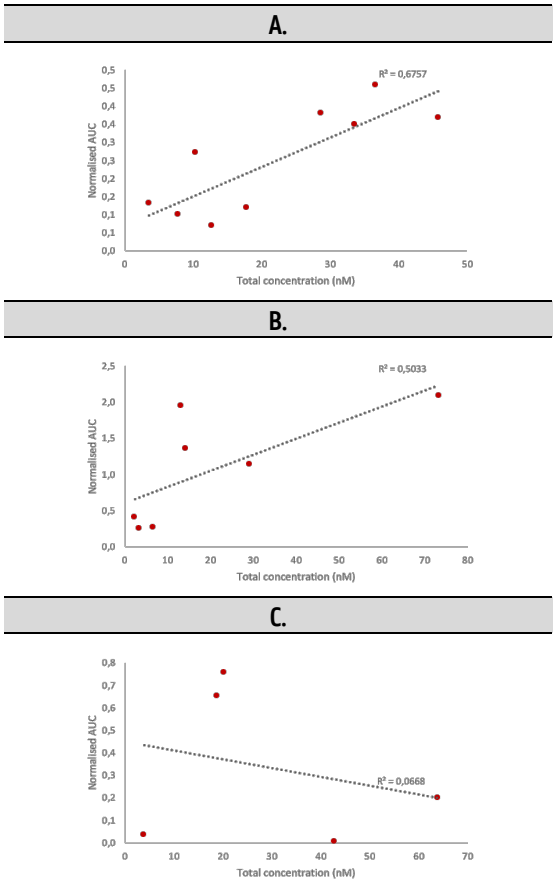

Figure 4.5. Normalised AUC of the bioassay signal (y-axis) generated from intoxications with only one type of SCRA, versus the total concentration (nM, x-axis). (A) 5F-ADB and/or metabolites; (B) Cumyl-5F-PINACA and/or metabolites; (C) MDMB-CHMICA and/or metabolites. AUC, area under the curve.

To evaluate the possibility of a correlation, the AUC versus concentration plots were first generated as described for twenty-two CB_1 profiles resulting from a mono-SCRA intoxication, i.e. samples in which only one type of SCRA, with or without metabolites thereof, were detected bioanalytically and picked up with the CB_1 bioassay (≥ 300 µL sample volume only) (**Figure 4.5**) (**Appendix II, samples 1-22**). In the analysed batch, mono-SCRA intoxication occurred with 5F-ADB (10/52), cumyl-5F-PINACA (7/52) and MDMB-CHMICA (5/52). One sample containing 5F-ADB metabolites together with Δ^9-THC (4360 pg/mL) (**sample 10**) was excluded as it was unknown whether the latter had contributed to the observed signal in the CB_1 receptor activation profile. Note that a more precise evaluation of a correlation would require higher numbers of samples. Assuming linearity, the AUC of two samples of which only 300 µL was used as a starting volume (**samples 11 and 14**), was multiplied by 1.67 (5/3) to obtain an estimate of the extent of receptor activation that would have been caused by 500 µL of these samples. The plots in **Figure 4.5** show

that, while an upward trend can be observed for the samples containing 5F-ADB (**A**) or cumyl-5F-PINACA (**B**), a more random scatter of points arose in the few cases with MDMB-CHMICA (**C**).

It should be kept in mind that while it has been previously reported that many SCRA metabolites retain cannabinoid activity (30,44–47), there is at present a lack of data about the exact potency of many (new) SCRAs and their metabolites. In an attempt to correct for these assumed differing potencies, the concentrations of the metabolites were set at 50%, 75% and 100% of the respective parent compound concentrations and the total concentrations were calculated accordingly. Upon evaluation of the generated plots (**Appendix VII**), it was decided to continue with an assumed metabolite potency of 100% as compared to the parent compound since there were no real grounds for choosing a certain arbitrary potency. As a result, to generate the plots in **Figure 4.5**, no correction was made for any difference in potency between the parent compound and the metabolites.

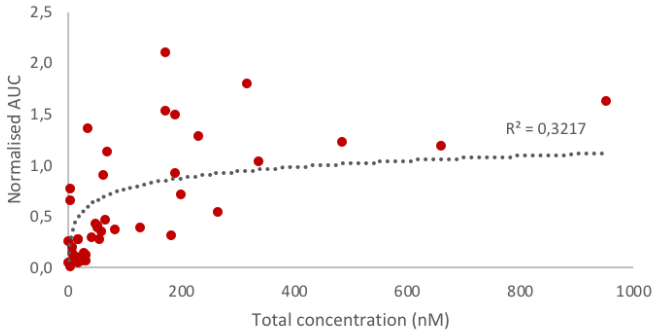

Figure 4.6. Normalised AUC (y-axis) of the bioassay signals versus total concentration (nM, x-axis) in 41 SCRA-positive samples (500 µL). AUC, area under the curve.

Next, it was evaluated whether the upward trend observed for the samples containing 5F-ADB and cumyl-5F-PINACA only, was echoed when the total data set (i.e. including poly-SCRA intoxications) was taken into consideration. The AUC values of the CB_1 bioassay signals of all SCRA-positive samples (500 µL, n = 49) containing SCRAs for which an EC_{50} was available in literature (41/49), were calculated as previously described. The AUC of each profile was plotted against the total SCRA concentration present in the sample (**Figure 4.6**). To do so, the differing potencies of the individual SCRAs were taken into account by dividing the concentration of each SCRA or metabolite by the EC_{50} of the parent compound. The employed EC_{50} values were previously described in literature and are summarised in **Table 4.4**. Again, the same potency was assumed for both parent compound and metabolites due to an apparent lack of literature specifically stating otherwise at present. It should be clear that the resulting "total SCRA concentrations" mentioned in this dissertation should thus be interpreted with caution. **Figure 4.6** shows that there seems to be a tendency of increasing bioassay AUC values, reaching a maximum at higher total SCRA concentrations. Note the difference in plotted concentration range between **Figure 4.5** and **Figure 4.6**, which implies that the upward trend observed in the former corresponds to the outer left (lower) region of the latter graph.

Table 4.4. EC_{50} values of different SCRA parent compounds as reported in literature. The EC_{50} values represent the potency of the SCRAs on the CB_1 receptor. SEM, standard error of the mean.

	pEC_{50} ± SEM (EC_{50}, nM)	Source
5F-ADB	9.23 ± 0.11 (0.59)	Banister *et al.,* 2016 (108)
AB-CHMINACA	8.11 ± 0.32 (7.8)	Longworth *et al.,* 2016 (109)
AMB-CHMICA	8.45 ± 0.08 (3.5)	Banister *et al.*, 2016 (108)
Cumyl-5F-PINACA	9.37 ± 0.06 (0.43)	Longworth *et al.,* 2017 (110)
MDMB-CHMICA	8.00 ± 0.05 (10)	Banister *et al.,* 2016 (108)

4.3 CLINICAL PRESENTATIONS

The clinical data as recorded for each patient upon arrival at and/or discharge from the hospital, are included in **Appendix II.** A summary of the clinical information is provided below.

The median heart rate of the SCRA-positive patients (n = 70) was 89 bpm (IQR: 72-101.8; range: 43-130). Nine (12.9%) patients experienced tachycardia (predefined as a heart rate ≥ 120 bpm) and five (7.1%) cases of bradycardia (predefined as a heart rate ≤ 50 bpm) were reported. The median patient had a body temperature of 36°C (IQR: 36-37, range: 33-38°C) and a Glasgow Coma Score (GCS) of 14 (IQR: 12-15; range: 3-15; one patient reported "pain"). The GCS is a commonly used scale to assess the level of consciousness of a patient, ranging from 3 (i.e. very deep unconsciousness, unresponsiveness) to 15 (normal level of response). A detailed interpretation of the GCS remained difficult as no further breakdown into the different elements used to calculate the final score (eye opening, verbal performance, motor responsiveness) had been provided (111,112). There were no deaths in this patient cohort.

The most frequently observed clinical features among the SCRA user cohort (n = 70) were agitation (n = 25; 35.7%), seizures (n = 14; 20.0%) and hypertension (n = 7; 10.0%) (predefined as a systolic blood pressure ≥ 160 mmHg at any moment throughout admission). In one patient, the reported seizures might have been attributable to alcohol withdrawal. The most often detected SCRAs in agitated patients were 5F-ADB (parent compound, n = 2; metabolites, n =28) and MDMB-CHMICA (parent compound, n = 11; metabolites, n = 13), followed by cumyl-5F-PINACA (parent compound, n = 7; metabolite, n = 8) and AMB-CHMICA (parent compound, n = 5; metabolite, n = 8). Metabolites of 5F-ADB and MDMB-CHMICA were also the most frequent detections in seizing patients (respectively n = 15, n = 5). These observations were in line with the general trend of most often detected SCRAs (**Table 4.1**). Only three patients experienced both agitation and seizure, whereas almost one in two hypertensive patients appeared agitated (42.9%). Hypertension and seizure occurred concurrently in only one patient. Chest pain, psychosis and hallucinations were observed in a minority of the cases (respectively 5.7%; 2.9% and 2.9%). Psychosis and hallucinations occurred only in patients who had taken 5F-ADB and/or AMB-CHMICA, whereas chest

pain was seen only after consumption of 5F-ADB or MDMB-CHMICA. Pyrexia (predefined as a body temperature ≥ 39°C) was reported for only one patient during his stay, whereas no one experienced palpitations.

Thirty-one (44.3%) patients were managed in the Emergency Department and eighteen (25.7%) patients were admitted to the Short Stay Unit of the Emergency Department. One patient self-discharged from the Acute Admissions Ward. Five (7.1%) patients were treated in either the Intensive, Coronary or High Dependency Critical Care Units. One patient with confirmed intake of Spice containing 5F-ADB (metabolites), was admitted to Psychiatry, although the clinical ward notes made no specific mention of psychosis or other mental complications. Eight (11.4%) patients were known to be discharged on medical grounds without specifications on which unit they had been managed in. A total of twelve (17.1%) patients self-discharged from different wards or from the Emergency Department. The median length of stay in the hospital was 5 hours and 29 minutes. The longest stay, lasting 23 hours and 18 minutes, was reported for a hypertensive patient with pyrexia and a GCS of 3. Laboratory analysis of samples obtained from this patient revealed the presence of 5F-ADB (1285 pg/ml), 5F-ADB metabolites (2587-6966 pg/mL) and up to eleven other analytes (cotinine, four benzodiazepines, ethyl glucuronide, ketamine, methadone/EDDP, morphine and pregabalin).

5 DISCUSSION

5.1 APPLICABILITY OF THE PROPOSED BIOASSAYS

The applicability of the proposed activity-based cannabinoid bioassays was evaluated by screening a batch of 471 serum samples from drug-related cases for the presence of (synthetic) cannabinoids. For 395 samples, enough sample volume was available to perform the sample preparation following the protocol as it had been previously described (≥ 300 µL) (8,30,53). In that set-up, the CB_1 bioassay was able to identify all 52 SCRA-positive samples, yielding a sensitivity of 100%. This surpasses the sensitivity of 82% previously reported by Cannaert *et al.*, who applied the bioassays to a batch of 45 serum samples, thereby identifying 18 out of 22 SCRA-positives (53). In both the present, as well as the previous analyses, the detected SCRAs were present in serum samples in concentrations ranging from sub- to hundreds of nanogram per millilitre.

In terms of specificity, the data reported here show a slight decrease as compared to the previously reported value of 100% (53). Here, a specificity of 97.9% was achieved. The decrease in specificity may be partially attributable to the subjectivity accompanying the scoring of the profiles (cf. infra). One user whose sample gave weak CB_1 receptor activation despite bioanalysis being negative for SCRAs, had claimed "Spice" intake. This suggested the need for further in-depth bioanalytical investigation to assess whether the CB_1 bioassay succeeded in picking up the signal of a SCRA that was initially missed via bioanalysis. However, retrospective analysis of the HRMS/MS data did not reveal the presence of any SCRAs.

Of the thirteen samples originally labelled "false positive", bioanalysis revealed that six contained Δ^9-THC, indicating the intake of natural cannabinoids. Consequently, these samples were no longer considered false positives, as the signal picked up by the bioassay could indeed be attributed to the presence of cannabinoid activity. However, not all samples that were bioanalytically shown to contain Δ^9-THC, generated a positive signal in the bioassays. This was not entirely surprising, considering the partial agonism of Δ^9-THC as opposed to the full agonism of most SCRAs (23,53,54). Cannaert *et al.* previously reported the need for relatively high concentrations (> 12 ng/mL) of Δ^9-THC in order to generate a positive signal in the bioassays (53). Here, lower estimated Δ^9-THC levels already seemed capable of generating a signal in the CB_1 bioassay in the absence of any other (detected) SCRAs. However, no further link could be found between the estimated Δ^9-THC concentration levels and the samples in which presence of Δ^9-THC was picked up. Furthermore, the question remains whether other natural cannabinoids were present that may have contributed to the signal observed with the bioassay. In practice, in the case of low positivity in the bioassay screening, the latter could be combined with a conventional immunoassay for the detection of natural cannabinoids after "regular" cannabis use (53). This way, cumbersome analysis to search for SCRAs may be avoided.

For the remaining 76 serum samples, for which only 100 µL sample volume was available in the present study, a sensitivity of 88.9% (16/18) was reached. Potential potency differences aside, it could not be fully explained why two SCRA-containing samples were missed, as four samples containing a lower total SCRA concentration had been picked up. It could be concluded that, as expected, reducing the sample volume by five- or threefold during sample preparation lowered the sensitivity of the bioassays. On the other end, two false positive signals were obtained, resulting in a specificity of 96.6% (56/58). Despite the two corresponding users claiming respectively cannabis or "Spice" intake, which may be supported by the weakly positive signal in the CB_1 bioassay, here, too, this could not be confirmed by any findings through bioanalysis. Furthermore, within this set of samples, no Δ^9-THC-positives were detected with the bioassays. The latter may have been the combined result of the overall low estimated Δ^9-THC concentrations, the decreased sample volume and the partial agonism of Δ^9-THC.

A list of all compounds bioanalytically detected in SCRA-negative samples that were correctly scored as such via the bioassays, is provided in **Appendix V.** The list comprises both other drugs of abuse, as well as "regular" (prescription) drugs. With the caveat that no concentrations were determined, the fact that these compounds did not interfere with the read-out, is yet another argument in favour of the bioassays' specificity. Cannaert *et al.* previously published a list of 288 compounds which, when spiked into urine at certain concentrations, did not influence the bioassays' signal (53). In addition, it has been reported that the presence of endocannabinoids is not expected to interfere with the specificity of the bioassays as, under normal circumstances, their concentration in the blood is very low (53,54). Even in those conditions where endocannabinoid concentration may be increased (eating disorders, obesity, schizophrenia, post-exercise), the read-out of the assays is expected to remain largely unaffected (53,54,113–115).

It is interesting to note that all samples that were scored weakly positive, eventually turned out to be false positives or samples containing Δ^9-THC. This inevitably leads to a discussion on the subjectivity and the required caution accompanying the scoring of such weak signals as SCRA-positive samples. One may argue that, for the purpose of screening, some false positives may be allowed as the subsequent confirmatory analysis is believed to eventually filter these out, leaving only true positive samples in the end. Furthermore, as the SCRA field is known to be very dynamic, the possibility remains that some of the weak "false positive" samples may contain low concentrations of a SCRA or SCRA metabolite that is currently not included in or detected by the employed HRMS/MS database. This is supported by the observation that some of the obtained false positives show a noticeable - although very weak - "arch-like" profile, as is typically seen for SCRAs. The acquired HRMS/MS full scan may enable future retrospective identification of such novel SCRAs in samples that had previously

generated an unexpected positive signal in the bioassays (52). Moreover, theoretically, it might be possible that some false positive samples actually contained (a mixture of) low levels of SCRAs that did not trigger an acquisition through bioanalysis but, owing to the additive bioactivity of the different SCRAs present, did generate a signal in the bioassay.

The proposed bioassays offer the enormous advantage over conventional screening techniques that they are activity-based, which may allow them to more easily keep up with the wide and dynamic drug panorama as shaped by SCRAs and other NPS. Current screening methods comprise immunoassays or chromatographic approaches, which both suffer from some important limitations as they are structure-based methods (cf. supra). Franz *et al.* recently expressed serious concerns over the use of immunoassays for the detection of SCRAs, coining insufficient cross-reactivity and high cut-off values as the main culprits for the assays' often unreliable screening (116). When using a combination of two commonly used immunoassays, the authors reported values for sensitivity and specificity of respectively 2% and 99% at the manufacturers' proposed cut-offs (116). Plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) in a receiver operating characteristic (ROC) curve, the evaluated immunoassays had a predictive power comparable to that of a random distribution (**Figure 5.1-A**) (116). The cannabinoid bioassays, on the other hand, come close to the ROC curve of an ideal assay, as the current values for specificity and sensitivity would generate a value close to the upper left corner of the system of coordinates (**Figure 5.1-B**). The generation of a full ROC curve for the bioassays would require evaluation of the sensitivity and specificity at different cut-off values for assessment of positivity, which remains difficult at present considering the non-instrumental (i.e. non-numerical) way of scoring (117).

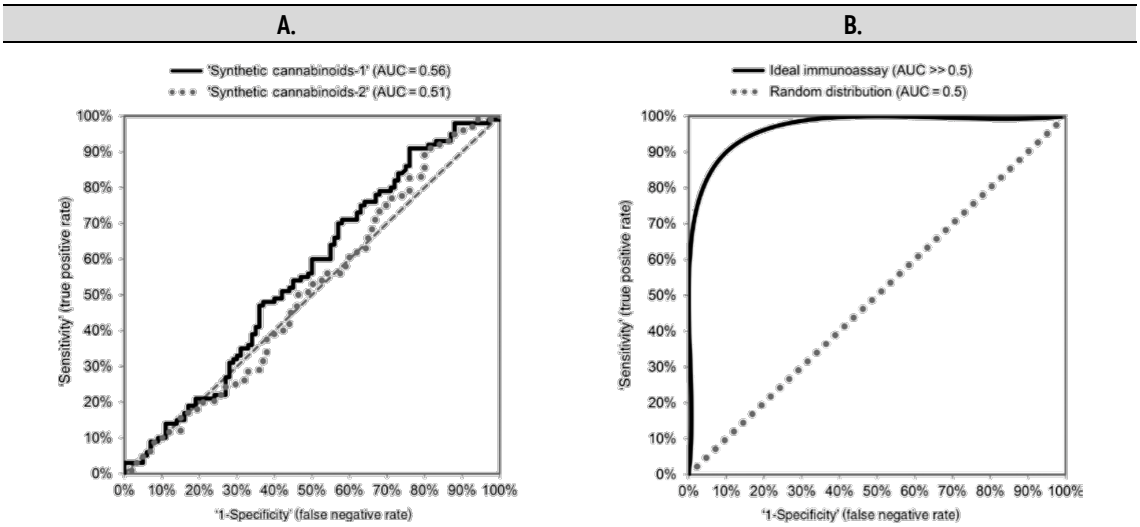

Figure 5.1. Receiver operating characteristic (ROC) curves of two evaluated immunoassays for the screening of SCRAs (A). The plots have an area under the curve (AUC) of 0.51 and 0.56. Exemplary ROC curves corresponding to that of an ideal assay (solid line, AUC >> 0.5) and that of a random distribution (dotted line, AUC = 0.5) are shown for comparison (B). Data were obtained by Franz *et al.* by analysing urine samples (100 negative and 100 positive samples, containing metabolites of a single SCRA). Figure and description from Franz *et al., 2017* (116).

Application of the proposed bioassays is envisaged among various potential settings. For example, in an attempt to keep up with the rapidly expanding spectrum of "legal highs", different countries have started adopting generic NPS legislations, often based on activity rather than the specific identity or chemical structure of a drug (30). In the UK, for example, the Psychoactive Substances Act 2016 schedules substances based on their psychoactive activity, which is defined by the Advisory Council on the Misuse of Drugs as "*... a response in* in vitro *tests qualitatively identical to (that of) substances controlled under the Misuse of Drugs Act 1971...*" (118). Several receptors have been selected as targets for such *in vitro* testing, among which the CB_1 and the μ-opioid receptor, which is where the bioassays may come in (30,118). In addition, any other setting in which regular drug screenings are carried out (e.g. prison, workplace drug testing), may benefit from the use of activity-based bioassays (119). Alternatively, as previously mentioned, the bioassays may also be used for research on structure-activity relationships and to further shed light on the *in vitro* activity of known and novel SCRAs or SCRA metabolites (30). An example of an important parameter to take into consideration when extrapolating the *in vitro* situation to *in vivo* psychoactivity, is whether the compound in question will pass the blood brain barrier and subsequently bind CB_1 receptors with sufficient potency and efficacy to induce psychoactive effects. Furthermore, the quantitative and qualitative nature of the (toxic) effects an individual may experience, depends at least partially on whether or not the consumer has already developed a certain level of tolerance against the effects of cannabinoids (15,120).

5.2 EVALUATION OF A POTENTIAL CORRELATION BETWEEN SIGNAL STRENGTH AND SCRA CONCENTRATIONS

Plotting the AUC of the CB_1 bioassay's signals against the total SCRA concentration present in the samples, resulted in a graph suggesting some level of correlation. Cannaert *et al.* previously showed that a concentration-dependent interaction of CB_1/CB_2 receptor activation with β-arr2 recruitment can be observed upon stimulation with increasingly higher concentrations of different individual SCRAs (8,30). The plots obtained in these studies reach a maximum AUC at high concentration levels, which seems to be reflected by the data plotted here (**Figure 4.6**) (8,30). However, some important limitations must be addressed before definitive conclusions can be drawn and before the plot may be used to semi-quantitatively estimate total cannabinoid activity present in a biological sample.

First, the preliminary assessment assumed equal CB_1 receptor potencies (i.e. EC_{50} values) of each parent compound and its corresponding metabolites. It has indeed been reported earlier that several SCRA metabolites remain active (30,44–47), but data on the newer SCRAs as detected here, are currently lacking. Therefore, the possibility remains that some metabolites may not be active or may have a significantly different potency than

their respective parent compounds, which would of course influence the "total SCRA concentration", as calculated and plotted here.

Second, the employed EC_{50} values were assessed by Banister and Longworth *et al.* using a fluorometric imaging plate reader assay based on CB receptor-dependent membrane hyperpolarisation (108–110). In this assay, activation of human CB receptors, stably expressed in AtT-20 neuroblastoma cells, resulted in the opening of endogenous potassium channels, causing hyperpolarisation of the cells as reflected by a decrease in fluorescence of a membrane potential dye (108–110). Despite being performed by the same research group, the EC_{50} values were obtained from three different publications and, therefore, small differences between experimental set-ups which could have potentially influenced the outcome of the assay, cannot be excluded. Furthermore, it is important to note that, would the EC_{50} values be evaluated with the CB-β-arr2 bioassays, different values may be obtained owing to the different monitored pathway upon activation (8). The possibility that some ligands exhibit biased signalling complicates the extrapolation of EC_{50} values from one assay to another (19,121).

Taking into account the above-mentioned limitations, the preliminary character of the AUC versus concentration plot presented here, must be underlined very clearly. Additional experiments are required to determine the EC_{50} values of the individual SCRAs and SCRA metabolites by means of the CB-β-arr2 bioassays. A broader total concentration must be plotted to more reliably ascertain the observed trend. Only when the above-mentioned issues are adequately tackled, will it be possible to further comment on the potential usefulness of the presumptive correlation as suggested here.

5.3 PATIENT CHARACTERISTICS AND CLINICAL OUTCOME OF SCRA USE

For this study, data were collected from a total of 471 patients presenting with acute recreational drug toxicity to the Emergency Department of the Guy's and St. Thomas' hospital in London in the second half of 2016. Owing to its central location close to many thriving party scenes, hospital admissions linked to drug use occur regularly at the Guy's and St. Thomas' Emergency Department and the detected drugs quite sensitively mirror new trends in substance abuse. Adding to its suitability in evaluating (new) psychoactive substance abuse, Westminster has one of the highest LGBT (lesbian, gay, bisexual, transgender) populations in London, with men having sex with men being a known high-risk group for NPS abuse (122). Bearing this in mind, it should be clear that caution must be exercised when generalising the results discussed below to another setting. In addition, extrapolating the clinical picture of patients being admitted to an Emergency Department to all SCRA users, may give rise to an overestimation of the severity of the symptoms (15,16,123).

SCRAs were detected in 14.9% (70/471) of the patients studied in this cohort. The vast majority (94.3%) of the SCRA users were males, a trend reflected in statistics from previous studies (15,16,42,123–125). Up to 81.4% (57/70) of the patients self-reported their SCRA use, which is a higher percentage than that found in some previous reports, where only half of the SCRA users admitted to or were aware of having consumed SCRAs (15,123). Interestingly, only two patients declared having used a SCRA product named differently than "Spice", whereas in several other studies a more diverse range of product names has been reported (123,124). Despite the high number of declared "Spice" intakes, the exact pattern of detected SCRAs varies markedly between different patients. This illustrates the varying composition between different SCRA products marketed as "Spice". However, it may be possible that some users employ the term "Spice" as a synonym for any SCRA product, irrespective of the actual brand consumed.

The (limited) clinical profile portrayed in the present report appeared to be in line with findings of previous research documenting the symptoms of SCRA users presenting to Emergency Departments (15,16,123,125). The most frequently observed clinical features in the present patient cohort were agitation (35.7%), seizures (20%) and hypertension (10%), whereas reports of chest pain (5.7%), psychosis (2.9%) or hallucinations (2.9%) were less common. Tachy- or bradycardia were experienced by 12.9% and 7.1% of the patients, respectively. Previous research has emphasised an apparent lack of correlation between SCRA concentrations in blood or serum and the severity of poisoning (126–128). Several elements have been proposed as an explanation for this observation, including the potential development of tolerance in experienced users or the presence of certain pre-existing conditions that may make the patient more vulnerable to a particular effect (127).

Of the seven different SCRA parent compounds detected, the most common were MDMB-CHMICA, AMB-CHMICA and cumyl-5F-PINACA. The high number of detected 5F-ADB metabolites, however, indicates the latter parent compound to be the most "popular" product in the studied cohort. The detected SCRAs are known to be very potent, with some online suppliers even indirectly warning for their high potency (e.g. "a very small amount of this product is required for successful research" or "we urge you to use tiny amounts in your studies as to not damage your lab equipment", in addition to the commonly employed phrase "for research purposes only") (129).

Recently, Abouchedid *et al.* published a study in which the presence of SCRAs was analytically confirmed in 10% of patients presenting with acute recreational drug toxicity to the same Emergency Department as reported here (123). Bioanalysis, too, was performed by the same laboratory. The 179 patients included in that study were recruited in the first half of 2015, roughly a year and a half before recruitment of the patients in the light of the present study started (April-December 2016). Illustrating the dynamic character of the SCRA field, the most commonly detected SCRA by Abouchedid *et al.* was 5F-AKB-48 (recovered in 72.2% of the SCRA users) (123). In

the present study, 5F-AKB-48 was detected in only 5.7% of the SCRA-positive patients. The second most prevalent SCRA recovered by Abouchedid *et al.,* 5F-PB-22, was in fact not at all detected in the present user cohort. Vice versa, the most "popular" SCRA in this study, 5F-ADB, was not found in the 2015 cohort despite already being included in the HRMS/MS database at the time (123).

5.4 FUTURE PERSPECTIVES

Notwithstanding the relatively easy application of the cannabinoid bioassays as described here, successful experiments do require the presence of a minimal cell culture facility and mastering of some basic skills and aseptic techniques. A more widespread implementation, however, may be facilitated if the components of the bioassays were to be integrated into a single convenient kit (53). Establishment of a computer-based platform allowing automated (numerical) scoring of the samples, may further reduce the workload and help avoid discussion about the subjectivity associated with the scoring of some weakly positive samples (53).

In order to obtain more information from the bioassays' read-outs, the characterisation of the different encountered SCRA parent compounds and metabolites thereof, is invaluable. A logical next step will thus be the collection of pure reference standards of the different compounds and subsequent evaluation of the signals of increasing concentrations of the obtained standards in the bioassays. This will allow establishment of EC_{50} values based on recruitment of β-arr2 and evaluation of the CB activation potential of the SCRA metabolites. In the end, this information will help to more reliably ascertain the proposed correlation between the AUC of the bioassays' signals and the total SCRA concentration present in the evaluated samples.

An innovative follow-up project that is actively being explored by the Laboratory of Toxicology, is the expansion of the current activity-based bioassays to a novel, refined bioassay designed to allow multiplexing. This way, it may be possible to detect a whole panel of new psychoactive substances (synthetic cannabinoids, psychedelics, opioids/opiates) within a single screening test.

6 CONCLUSIONS

The rise of new psychoactive substances presents significant analytical and clinical challenges to both forensic and clinical toxicologists. With SCRAs currently taking the lead in Europe in number of substances monitored, the high demand for a completely unbiased screening method to detect and monitor this group of compounds, has become undeniably palpable in practice. The experiments described in this dissertation therefore aimed at evaluating the applicability of activity-based cannabinoid bioassays for the detection of SCRAs in serum samples, thereby circumventing the constraints linked to the existing (structure-based) strategies. To do so, the bioassays were applied to a total of 471 serum samples acquired from patients with acute recreational drug toxicity presenting to the Emergency Department of the Guy's and St. Thomas' Hospital in Westminster (London, UK) from April-December 2016. The outcome of the bioassays (SCRA-positive/-negative) was compared to the findings obtained through externally performed LC-HRMS/MS analysis.

With final values for sensitivity and specificity of, respectively, 100% (52/52) and 97.9% (330/337), the applicability of the bioassays (≥ 300 µL sample volume) has been convincingly demonstrated. Lowering the sample volume to 100 µL during sample preparation reduced the sensitivity and specificity of the assay to 88.9% (16/18) and 96.6% (56/58), respectively. The acquired values for specificity may further increase in the future as it may be possible that novel SCRAs are characterised which were not included in the present HRMS/MS database and were therefore not detected bioanalytically. Up until this moment, if such samples gave rise to a positive signal in the bioassays, they were labelled "false positives", thus affecting the specificity of the assay. Especially in two cases where the user had claimed "Spice" intake, which was supported by a (weakly) positive outcome in the CB_1 bioassay despite being negative via bioanalysis, the acquired data may have to be re-evaluated in the future as more SCRAs become known. Theoretically, as the bioassays are activity-based, it is also possible that some false positive samples actually contained (a mixture of) low levels of SCRAs that were not detected bioanalytically but, together, exerted enough activity to generate a signal in the bioassays. However, it is important to stress that any positive result in the bioassays should be analytically confirmed, as the assays remain intended for screening purposes.

A secondary objective of the experiments performed in this dissertation, consisted of evaluating the relationship between the strength (AUC) of the CB_1 bioassay's signals and the total SCRA concentration present in the different samples. The increase in AUC that was observed at lower concentration ranges, appeared to be reaching a maximum at higher concentration levels, suggesting saturation of the system. However, the many uncertainties associated with the calculation of the total SCRA concentration (different parent compounds and metabolites) must be taken into careful consideration upon interpretation of this highly preliminary result.

Finally, patient characteristics and clinical implications of the bioanalytically detected SCRAs were documented and compared to previously published literature. The (limited) clinical profile of the studied cohort appeared to be in line with previous research on the adverse events associated with SCRA use. Based upon the results of a similar study performed roughly one and a half years before recruitment of the present patient cohort, the use of 5F-AKB-48 appeared to have decreased in popularity, whereas 5F-ADB was now the most frequently encountered SCRA. With the caveat that these observations may not be representative for other regions or different settings, they do illustrate the dynamic character of the SCRA drug scene.

In conclusion, the data presented here are in support of a potentially very promising application of the activity-based cannabinoid bioassays developed by the Ghent University Laboratory of Toxicology. The described bioassays have the potential of being applied in the context of high-throughput screening for SCRAs, offering promising perspectives to address the void left by the conventional screening methods. While more time may well be required before a widespread implementation might occur, the results obtained in this dissertation convincingly demonstrate the potential role of activity-based screening in tackling the challenges associated with the detection of NPS.

While this research was ongoing, preliminary results were presented as a poster at the Research Day & Student Research Symposium at the Faculty of Pharmaceutical Sciences (April 19th, 2018) (**Appendix VIII**). This work was also accepted for an oral presentation by the author at the yearly international conference of The International Association of Forensic Toxicologists (TIAFT), to be held in Ghent, August 26-30, 2018 (**Appendix IX**).

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8 APPENDICES

8.1 APPENDIX I: Review manuscript (to be submitted to Critical Reviews in Toxicology)

Looks don't matter, it is what you do that counts: Acitivity-based reporter assays as a new concept for abused substance screening in biological matrices.

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2.1 Introduction

The number of novel designer drugs that is abused is constantly growing. This increase can be seen by the sharp rise of new psychoactive substances (NPS) during the last decade. More than 620 NPS have appeared on the European drug market, as reported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)[1]. These substances are characterized by a high market dynamics and are often not covered by international drug controls and make up a broad range of drugs such as synthetic cannabinoids, stimulants, opioids and benzodiazepines. These NPS are in many cases marketed as 'legal' replacements for illicit drugs (e.g. 'synthetic cannabinoids' for cannabis products)[1].

Steroid hormones are among the most popular performance enhancing drugs abused in both elite and amateur sports[2-3] and their use is prohibited by the World Anti-Doping Agency (WADA) at all times, in and out of competition[4]. Steroids are mainly associated with doping by elite athletes to enhance athletic performance, but since the 1980s, their use by male non-athlete weightlifters to improve appearance by building muscle mass has exceeded their use by competitive athletes[5]. Apart from their continued abuse in sports, steroid hormones are also found as illicitly used growth-promoting agents in meat-producing animals to increase meat production, resulting in higher earnings[6]. The use of such growth promoters in livestock production, however, falls under the European ban published in 1988 (EU directive 96/22/EC). Instead of providing a limitative list of forbidden hormones, the ban prohibits all substances having hormonal actions[7-9].

Previously, drug and doping control focused on 'conventional' drugs of abuse or approved therapeutics. However, drug users and athletes have started to misuse substances that were not tested for and/or were not clinically approved[1, 10-11]. Although steroid hormones have been studied for over 50 years and during that period numerous compounds with a variety of functional groups have been produced, only a small number has been introduced to the pharmaceutical market. In order to try to evade detection, some have resorted to the use of even more dangerous forms, the designer steroids. These steroids are manufactured to closely resemble existing known compounds, but with sufficient chemical diversity to ensure that their detection is more difficult[9, 12].

A worrying common feature of NPS and designer steroids is that no or limited data is available about the safety of these substances. The use of synthetic cannabinoids has been associated with agitation, nausea/vomiting, kidney failure,

cardiovascular problems and psychological disorders as well as death[13-15]. The use of synthetic opioids, due to the high potency and small therapeutic window of these compounds, has been associated with opioid intoxications and numerous deaths[1]. Also anabolic steroid use causes a lot of side effects, such as cardiovascular disease, liver damage, virilization and gynecomastia. Remainders of growth promoting agents in consumer products may have inadvertent effects as well. The health issues posed by these compounds often present serious problems for amateur bodybuilders and recreational athletes misusing steroids, even more so than for professional athletes, because of the insufficient medical attendance and supervision[16].

Amongst the reasons to (ab)use NPS or designer steroids is the lower chance of getting caught, as routine drug or sports doping tests may miss these compounds. Indeed, established gas chromatography mass spectrometry (GC-MS) and liquid chromatography tandem mass-spectrometry (LC-MS/MS) methods are typically set up for the monitoring of selected ions or of particular MS/MS transitions of known substances. Also, other methods, such as immunoassay screenings, might fail to detect novel substances that are being abused, due to the lack of and sometimes unpredictable cross- reactivity[9, 11, 16-18]. In general, the combined immunoreactivity of all compounds that are structurally related to the immunogen will determine the endpoint of these methods[19]. Moreover, immunoassays have a cumbersome developing process and with the high dynamics of the market of new substances, the developed immunoassays might struggle to keep up.

The use of untargeted MS-based screening methods has gained considerable interest to detect and identify novel compounds, although also here the absence of certified reference materials or mass spectral libraries poses a challenge. In this context, high-resolution mass spectrometry (HRMS) has been the method of choice for the broad screening of substances because of its ability to measure a compound's or a fragment's mass with sufficiently high accuracy, allowing its elemental composition to be determined directly[20-22]. However, sensitivity constraints may be present, requiring an analyte to be present at sufficiently high concentration to trigger an acquisition. Moreover, due to the expensive and time-consuming character of this technique, this method is not ideal to function as a screening method and is not routinely implemented in most laboratories.

Given the above, it is clear that there lies potential in novel 'untargeted' screening approaches, which are less expensive, more high-throughput-amenable and more routinely applicable. Activity-based assays, capable of monitoring the biological activity of an abused substance in a biological matrix, have been proposed as such an alternative 'untargeted' screening approach. These biological assays do not require knowledge about a compound's structure and could be used as a screening tool to identify potentially positive samples. In this review, we focus on activity-based reporter bioassays for the detection of NPS -more specifically synthetic cannabinoids and opioids- and steroid hormones in biological matrices.

2.2 Ideal *in vitro* activity-based assay

An ideal *in vitro* bioassay for screening purposes should be rapid, simple, sensitive, selective, reproducible and inexpensive.

- Rapid: As bioassays are to be applied as a screening tool, analysis should be fast and/or multiple analyses should be possible in one run. Shorter test duration allows the analysis of more samples per time frame.
- Simple: The assay should not require a lot of technical experience or highly sophisticated equipment. For cell-based assays, the generation of stable cell lines or the availability of yeast cells improves the simplicity of the assay.

- Sensitivity: Assay sensitivity is primordial as the aim is to detect physiologically relevant concentrations of drugs or hormones in (extracts of) biofluids.
- Selectivity: Although the developed assays should be considered as a screening tool and hence (depending on the context) some level of false positives may be allowed, they should be as selective as possible.
- Reproducible: The results of the screening method should be robust. Independently performed assays should provide consistent (positive and negative) results.
- Inexpensive and high-throughput-amenable: The screening assay should ideally be applicable on large sample sets to identify suspicious samples, which can subsequently be tested with more advanced systems. In addition, as the purpose is to reduce the number of samples that needs to be tested further, the price per analysis should be limited. Automatability and low consumable cost are important in this respect.

2.3 New psychoactive substances: synthetic cannabinoids and opioids

Synthetic cannabinoid receptor agonists (SCRAs) continue to be the largest group of new psychoactive substances (NPS) monitored by the EMCDDA. SCRAs are often marketed as a "safe" and "legal" alternative to marijuana. However, recent reports indicate that many of these compounds may produce serious adverse health effects[13-15]. SCRAs were originally synthesized by research laboratories to investigate the endocannabinoid system or as potential therapeutic drugs because they interact with cannabinoid receptors CB1 and CB2. The last decade, however, they have reappeared via the Internet as designer drugs, being promoted as so-called "legal highs"[1, 23-25].

Although synthetic opioids represent a smaller segment of the illicit drug market, there is an increasing number of reports on the rise of these compounds and on the harms they cause, including non-fatal intoxications and deaths. Synthetic opioids are substances that were initially synthesized to act as agonists for the opioid receptors (μ, δ, and κ subtypes), mainly found in the brain, spinal cord and digestive tract[18, 26-28]. Most act as full agonists, with varying potencies, at the μ-opioid receptor (MOR) and were initially explored by research groups or pharmaceutical companies for potential medicinal use. The last few years, they have found their way to the illicit drug market, being sold as such or in mixtures with other drugs, such as heroin or even cocaine [29]. Both the cannabinoid receptors, CB1 and CB2, and the μ-opioid receptor, MOR, are G-protein coupled receptors (GPCRs). Through the $G_{i/o}$ family of G-proteins, these are coupled to a wide variety of signal transduction pathways. GPCRs are rapidly desensitized by recruitment of the cytosolic protein β-arrestin 2 (βarr2) [30-31].

Our research group recently reported on live cell-based reporter assays for activity-based detection of SCRAs (as well as their metabolites) and (synthetic) opioids in biofluids[32-35]. The developed assays utilize a structural complementation-based approach, designed to monitor protein interactions within living cells (NanoLuc Binary Technology)[36]. More particularly, fusion constructs were generated between one of two inactive subunits of NanoLuc luciferase and either a GPCR (CB1 or CB2 or MOR) or βarr2. Upon GPCR activation, the cytosolic βarr2 protein, fused to one part of NanoLuc, will interact with the GPCR, fused to the other part of NanoLuc, leading to structural complementation of the NanoLuc luciferase subunits. This results in a restoration of luciferase activity, which generates a bioluminescent signal in the presence of the furimazine substrate (Figure 2.1).

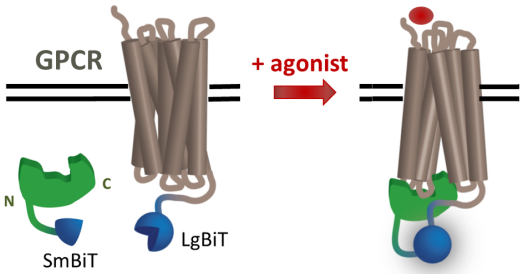

Figure 2.1. Example of the set-up of the GPCR activation assay (NanoBiT Technology).

The applicability of the cannabinoid reporter assay has been demonstrated in transient and stable mammalian cell systems for the detection of SCRAs (as well as their active metabolites) in authentic urine, serum and plasma samples[32-34]. The advantages of using stable bioassays, as compared to a transient format, are a reduced workload and higher reproducibility within experiments. Moreover, in the stable cell line-based bioassays we designed, we built in the possibility to control the level of expression of the GPCR or βarr2. This was achieved by coupling the expression of the CB- and βarr2-constructs to that of co-expressed markers, which can be followed by flow cytometric analysis. Interestingly, as overexpression of the GPCR and βarr2 fusion proteins might lead to a counter selection of overexpressing cells, these co-expressed markers also allow to follow up the stability of the cell lines over time and, if needed, may allow cell sorting, to select for cells with a certain level of expression.

For the activity-based screening of SCRAs in urine samples, the sensitivity largely depends upon the presence of active (type I) metabolites, as SCRAs are typically heavily metabolized, with hardly -if any- main compound being detectable in urine. Good sensitivities were obtained from urine samples for UR-144/XLR-11 users (94.4%; 17/18) and ADB-CHMINACA users (81.8%; 9/11). Surprisingly, in urine from users of the related AB-CHMINACA only a sensitivity of 33.3% (4/12) was found[33].

In contrast to urine, application of SCRA screening on extracts from blood (or plasma or serum derived thereof) will primarily rely on the presence of the parent compound. However, the highly potent nature of some compounds makes that in some instances active concentrations are in the low-to sub-ng/ml range, thus requiring highly sensitive detection. To improve the sensitivity of the existing SCRA bioassay, a modified assay was set up, in which a truncated rather than a full-length βarr2 protein was combined with either CB1 or CB2[34]. Application of this improved bioassay on a set of 45 serum samples resulted in a positive scoring of 18/22 SCRA positive samples, some with sub-ng/ml concentrations, corresponding with an analytical sensitivity of 82%. All SCRA negative samples were correctly scored negative in the CB1 and CB2 bioassays, leading to a specificity of 100% (23/23). The presence of other common drugs of abuse and/or low concentrations of Δ^9-tetrahydrocannabinol (THC; < 1 ng/ml) did not lead to a positive result. Only extracts from samples in which high concentrations of THC (> 12 ng/ml) were present gave rise to a positive result in 16/18 (89%) of cases, which is somewhat expected as the assay screens for cannabinoid activity[34].

A similar bioassay has been developed for the detection of opiates and synthetic opioids[35]. Here, in addition to overexpression of MOR and βarr2, overexpression of an additional protein, G-protein coupled receptor kinase 2, was necessary to achieve sufficient sensitivity. This protein promotes βarr2 recruitment to the activated MOR. Sensitivity and specificity of the MOR reporter assay were evaluated using 107 authentic postmortem blood samples with known presence or absence of the synthetic opioids U-47700 or furanyl fentanyl, as determined by LC-MS/MS and quadrupole-time of flight (Q-TOF) analysis[35]. A first finding was that in 8 synthetic opioid positive samples no positive signal was obtained. In these

samples, Q-TOF analysis revealed the MOR antagonist naloxone, which can obviously also prevent MOR activation induced by opioid agonists. Hence, evaluation was further based on non-naloxone containing samples. For U-47700 positive samples (74.5 – 547 ng/mL), sensitivity was 100% (8/8). For furanyl fentanyl containing samples (<1 – 38.8 ng/mL), 21 out of 22 samples (95%) were screened positive; it was not possible to test whether the missed sample contained naloxone. A specificity of 93% (55/59) was obtained for the opioid negatives. An additional 5 samples (found to contain opioids codeine, (nor)buprenorphine or loperamide) were correctly scored positive. In 5 negatively scored samples, Q-TOF analysis revealed the presence of alfentanil or sufentanil (both < 1 ng/ml) or dextromethorphan/levomethorphan or dextrorphan/levorphanol. For the latter two, as the LC-MS/MS method could not distinguish between the enantiomers (inactive dextro- and active levoform), it was not known what form was (mainly) present. The absence of detection of activity in these samples could be explained by the presence of the inactive enantiomer (dextroform)[35].

This MOR reporter bioassay was also applied on several biological matrices in a case report involving a fatal intoxication with carfentanil, an extremely potent opioid[37]. The extracts of the urine, vitreous and blood revealed a very potent opioid signal, even upon dilution of the sample. In this case, even the application of 1 µL of pure urine (without any sample preparation) in the bioassay generated a clearly positive signal.

There is a multitude of other GPCR activation assays available, several of which have been applied as research tools for studying CB and MOR receptor signaling.[38-50] However, as far as we are aware, only the reporter assays we developed have currently been applied on biological matrices as an untargeted screening strategy. It remains to be evaluated whether other, commercially available systems, can achieve the very high sensitivity that is required to screen biofluids for activity.

2.4 Steroid hormones

The parent compound from which all steroids are derived is cholesterol (Figure 2.2). During steroidogenesis different functional groups in varying orientations and oxidation states arise, resulting in a wide range of lipophilic, low-molecular weight, biologically active compounds. These serve as hormones, meaning that they may act as chemical messengers to regulate different cellular functions[51]. According to their biological activity and pharmacological effects, steroid hormones can be divided in two important groups. A first group includes the sex steroids, estrogens, progestogens and androgens, which produce sex differences and support reproduction. The second group includes the glucocorticoids, which regulate many aspects of metabolism and immune function, and the mineralocorticoids, which regulate blood volume and electrolyte content[51].

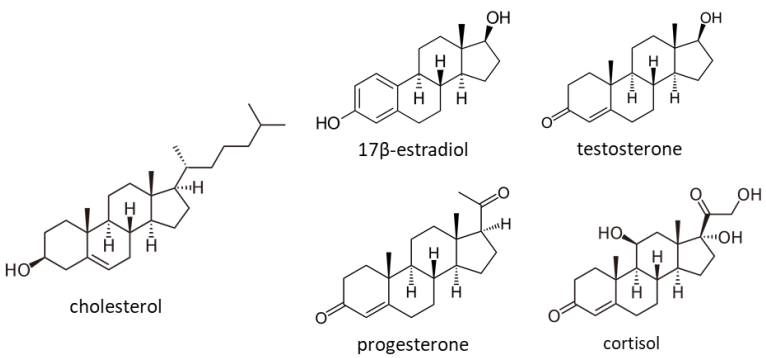

Figure 2.2. Chemical structures of cholesterol, 17β-estradiol (estrogen), testosterone (androgen), progesterone and cortisol (glucocorticoid).

The steroid hormone receptors (SR) are members of the large superfamily of ligand-activated transcription factors (Figure 2.3)[7, 52]. In its inactive form, the receptor is initially sequestered, in the cytosol of the target cell, under the form of large protein complexes containing Heat Shock Proteins (HSPs). Ligand binding induces dissociation of the receptor from these complexes. This allows the ligand-bound receptor to dimerize and, after phosphorylation, the now activated hormone-receptor complex translocates to the nucleus, where it recognizes a specific DNA sequence, the steroid response element (SRE), in the promoter of a steroid-regulated gene. The principle of this ligand-induced modulation of target genes forms the basis of test systems which can be used for steroid hormone screening (reporter gene bioassays, cfr. infra).

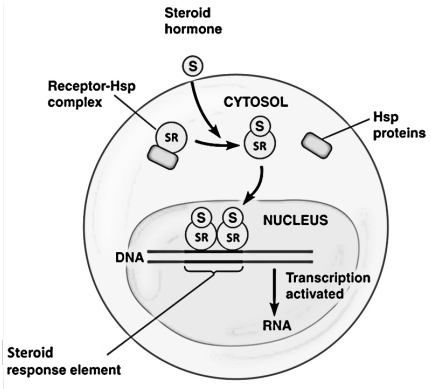

Figure 2.3. Signaling pathway of steroid hormones.
Picture altered from Pearson Education Inc. 2012.

A major limitation of the methods that are routinely used in doping control is that they most often cannot identify compounds of unknown structure and rely on prior knowledge of the structure of a steroid[10, 20, 52-53]. The black market for hormones makes witty use of the 'loopholes' in detection methods by introducing so-called "designer steroids". These novel steroids are synthesized with the aim of being biologically active as hormones, while evading detection owing to their slightly modified chemical structure compared to known steroids[12]. The fact that they are often synthesized in clandestine laboratories without appropriate quality controls adds up to the potential health risks these substances pose to abusers and doped-meat consumers. In addition, both athletes and farmers attempt to evade detection by administering low-dose hormone cocktails. In this approach, each substance will be present at very low concentrations, challenging the limits of sensitivity of the screening assays used to detect hormonal substances (both immunoassays as MS-based methods). Yet, owing to their additive effect, considerable biological activity may be exerted[54].

In this context, also here, bioactivity-based tools (bioassays) may be applied for the detection of steroids, by focusing on common mechanisms of action. A wide range of *in vitro* bioassays to monitor the steroid activity of compounds, comprising receptor binding assays, cell proliferation assays and receptor-dependent gene-expression assays (the so-called reporter gene bioassays)[8]. As the focus of this review lies on activity-based reporter bioassays, the former two will only be addressed shortly.

2.4.1 Receptor binding assays

Receptor binding assays can be used to detect all compounds having affinity for a given receptor. The principle is based upon competition of a ligand in an unknown sample with a labeled (usually radiolabeled) ligand for binding to a receptor. The extent to which the unknown sample replaces receptor binding of the labeled hormone correlates with its bioactivity. The principle of these radio-receptor assays is similar to that of conventional radio-immunoassays, in which antibodies are used instead of receptors. These assays thus monitor one steroid feature, i.e. binding to the steroid

receptor, but cannot distinguish between receptor agonists and antagonists because only the strength of the binding of a substance to the receptor is determined and not the activation or deactivation of the receptor[7, 52, 55].

2.4.2 Cell proliferation assays

Cell proliferation is a process further down the pathway than binding and transcription. The E-screen was one of the first *in vitro* bioassays used to determine the estrogenic activity of compounds and extracts. In parallel to the E-screen, the A-screen was developed for detecting androgenic activity. Although the simplicity of these assays is attractive, many factors have been shown to affect the outcome of the assay, reducing the reproducibility of these assays. These factors include, but are not restricted to, other compounds that may have an impact on cell growth, as well as differences in cell line clones, cell culture conditions and serum lots, thus complicating standardization of the assay to ensure inter-laboratory reproducibility[7, 56]. Furthermore, proliferative responses can only be determined after a number of days, resulting in a test that is not very rapid.

2.4.3 Reporter gene bioassays

Reporter gene bioassays exploit the natural signaling pathway of steroid hormones (Figure 2.3). In contrast to receptor binding assays, they also include the transactivation step and, consequently, can distinguish between receptor agonists and antagonists. Moreover, they might suffer less from matrix effects, as not all of the non-specific compounds will be able to enter the cells and reach the steroid receptors, as is the case with receptor preparations when using receptor binding assays. Many reporter gene assays have been developed, using both yeast and mammalian cells[7]. These are capable of amplifying and measuring biological activity, can be sensitive and provide information on the presence of steroid receptor activating compounds, independently of knowing the structure[10]. Both types of host cells have several advantages and drawbacks and the choice will depend on the intended purpose of the assay. Either way, it is important to keep in mind the different limitations while interpreting the results from these bioassays. When assays with both cell types are run in parallel, complementary information can be obtained.

I. Yeast

The first bioassays were developed in yeast because they grow easily and are economical, due to their rapid growth and easy attainment of stable transformants (compared to mammalian cells)[11, 57]. Typically, yeast cells are transformed with steroid receptor cDNA and a reporter vector containing an SRE, driving expression of a reporter gene, such as luciferase, β-galactosidase or a fluorescent protein. Fluorescence is associated with typical limitations, such as potentially high background and photobleaching, resulting in a lower sensitivity. However, it offers the advantage that the signal can be followed as a function of incubation time. Moreover, measurement of fluorescence is easier, quicker and cheaper than the measurement of β-galactosidase or luciferase activity, which may require cell wall disruption and/or the addition of expensive substrates. An additional advantage associated with the use of fluorescent proteins is that their read-out is not hindered by possible enzyme-inhibiting compounds[7, 58].

Because yeast cells are steroid independent for their growth and lack endogenous steroid receptors, yeast-based assays have a high specificity for hormones[9, 59]. Especially for androgens, the lack of known endogenous receptors in yeast is a great advantage compared with mammalian cell lines, as androgen responsive elements (AREs) can also be activated by the progesterone and glucocorticoid receptor (PR and GR). Mammalian cells containing either of these receptors experience cross-talk between the different steroids[7]. Many efforts to construct an ARE that is specific and no longer

inducible by the PR or GR have remained without success. It is doubtful whether a specific ARE can be found, as the consensus progesterone and glucocorticoid responsive elements (PRE/GRE) are equal to the consensus ARE[58, 60-62].
Yeast-based assays have been demonstrated to be robust, being more resistant to environmental contaminants than mammalian cells. This is an important advantage when measuring complex samples[63-64] and can probably be attributed to the presence of the yeast cell wall, making the cell more tolerant to dirty matrices or extracts. This cell wall, however, may pose a disadvantage for certain compounds that may be hampered to enter the cell or are pumped out via efflux pumps before reaching the receptor[7, 65].

Amongst the challenges when setting up new yeast systems is that expression of mammalian proteins may pose problems such as incorrect folding, phosphorylation, glycosylation or other post-translational modification. Additionally, yeast systems lack the appropriate chaperone and co-regulator proteins (e.g. HSPs) which are necessary for proper steroid mediated transactivation[10-11, 66].

Table 2.1 provides an overview of the yeast-based assays that have been used in the context of detecting abuse of steroid hormones in athletes and meat-producing animals[i]. These assays are briefly described below.

A yeast-based androgen screening assay with a secreted form of β-galactosidase was developed[67] and applied on authentic human urine samples from anabolic steroid abusers[16] and following the administration of methyltestosterone (MT)[68]. The yeast androgen assay was able to detect MT use in urine of volunteers after a single ingestion of MT for up to 307 h. In contrast, the detection limit of the GC–MS/MS method, which was used in comparison was about 118 h after exposure. This difference can be explained by the fact that detection of MT abuse by GC–MS/MS is dependent on tracing specifically known metabolites, whereas the yeast androgen assays detects the sum of the remnants of primary substance as well as of all known and unknown metabolites via their combined activity[68].

Another yeast-based androgen screening assay, using luciferase as reporter protein, was developed[69] and evaluated on human serum by Michelini and coworkers[58, 70]. The utilized *P. pyralis* luciferase was truncated to abolish peroxisomal targeting, thus allowing measurement of luciferase expression in intact living cells. An advantage of both systems is that they do not require cell lysis prior to read-out. A further improvement in robustness of the latter assay included the introduction of an internal viability control based on a constitutively expressed red-emitting *P. pyralis* mutant luciferase. Applicability of the improved bioassay was demonstrated using urine and serum samples[71-72]. A major limitation in the study of Cevenini *et al.* is that prior to spiking the urine was first pretreated with charcoal to remove any endogenous steroids[71]. This charcoal-treated urine, which also served as a blank, does not reflect the real situation. Ekstrom *et al.* successfully monitored androgen activity following administration of testosterone to healthy males for 4 days in urine (not deconjugated) and up to 15 days in serum[72].

Wolf *et al.* developed two androgen yeast-based assays with yeast Enhanced Green Fluorescence Protein (yEGFP) as a reporter[73]. As the two yeast strains that were used were phylogenetically very different, the combination of both assays allows detection of the activity of a wide range of androgenic substances, as some androgens do not respond in one yeast, but do in the other.

[i] Table 2.1 is included in the introduction of this dissertation (**Table 1.1. Overview of yeast-based reporter bioassays**).

The most-sensitive yeast-based androgen screen (A-YAS; *Arxula*-Yeast Androgen Screen) is based on transgenic *Arxula adeninivorans* yeast cells, engineered to express the human androgen receptor (hAR) gene, which may induce expression of a phytase reporter gene, resulting in conversion of *p*-nitrophenylphosphate to *p*-nitrophenol, allowing colorimetric read-out. A (limited) assessment in (deconjugated) cattle urine showed a correlation in androgen equivalence compared to the results obtained by GC-MS analysis[74].

Of the different yeast-based assays that were developed by the group of M. Nielen[63, 75], yEGFP appeared to be best suited as a reporter protein for high-throughput screening and was used to evaluate spiked urine samples[63]. The developed yeast-based estrogen and androgen assays were validated for qualitative screening for the presence of estrogenic activity in calf urine in accordance with EC decision 2002/657/EC[61, 76]. Applicability of the estrogen bioassay was demonstrated using a panel of more than 120 authentic calf urine samples[9]. When compared with the results obtained by GC-MS/MS, yeast-based screening yielded 5.6% false positives and only 1 (0.8%) false negative[9]. Also the urine of 17β-estradiol-treated veal calves was tested, resulting in a sensitivity of 94.3% and a specificity of 100%[77]. A further expansion of the estrogen and androgen bioassay applications involved the set-up of a system via which extracts of calf urine could be analyzed by gradient LC with, in parallel, bioactivity and mass spectrometric detection. This was achieved via effluent splitting toward a 96-well fraction collector and an electrospray quadrupole time-of-flight mass spectrometer (Q-TOF-MS). The result was an estrogen/androgen biogram (see Figure 2.4)[56, 75, 78]. Next to urine samples, also hair was evaluated as a matrix for both androgens and estrogens, revealing that it was possible to detect the presence of androgens up to at least 14 days after treatment with 60 mg testosterone cypionate and 60 mg testosterone decanoate and to detect the presence of estrogens up to at least 56 days after a single pour-on treatment with 25 mg of estradiol benzoate[56].

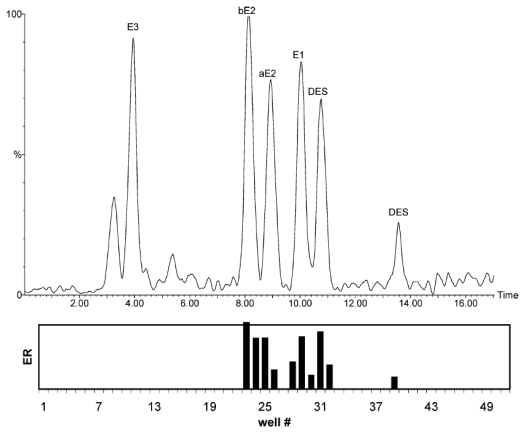

Figure 2.4: Example of a reconstructed LC/Q-TOF-MS chromatogram and reconstructed estrogen biogram of a standard mixture of estrogens (E3, estriol; bE2, 17β-estradiol; aE2, 17R-estradiol; E1, estrone and DES, diethylstilbestrol; 1 ng each). Reprinted (adapted) with permission from Nielen *et al.*[78]. Copyright 2004 American Chemical Society.

Burdge *et al.* used a yeast-based assay with β-galactosidose as a reporter (readily developed by Klein *et al.* in 1994[79]) to measure endogenous estrogen activity in bovine plasma[80]. Deconjugation was found to be a key step as estrogen conjugates (17β-oestradiol-3-glucuronide and 17β-oestradiol-3-sulfate) only produced a negligible response in the assay[79]. The authors demonstrated assay applicability by monitoring the signal generated by endogenous levels of estrogens during the reproductive cycle of females, suggesting potential application of this assay for surveillance of exogenous estrogens in cattle (especially in males, as in those the level of endogenous estrogens is very low)[80].

As a follow-up of the above-mentioned *Arxula adeninivorans* yeast assay, engineered to express a human steroid receptor that drives expression of a phytase reporter gene, Chamas *et al.* developed a yeast-based assay capable of detecting estrogens, progestogens, and androgens in a single step. This was achieved by combining three yeast strains, each expressing a different human receptor, driving the expression of different fluorescent reporter proteins. Application of the combined assay on marmoset (a monkey species) serum and comparison with an immunoassay for progestogens revealed a good correlation between both types of assays[81].

I. Mammalian cells

Mammalian cell-based bioassays are typically developed in immortalized cell lines, which are relatively easy to culture. The SRE-reporter gene vectors are either transiently or stably introduced into these cells[10]. Stable cell lines have the possibility of vector loss and degradation over time, if they do not contain a selection marker. The use of transient transfection methods does not risk this vector loss and degradation, but brings along a heavier workload, as every new run requires a new transfection, and might lead to more variable results. Variation can be minimized, though, by using co-transfected control vectors that may serve as an internal control for transfection efficiency.

Cell choice is an important consideration for mammalian cell-based assays, because the cellular responsiveness will be determined by its environment, including the composition of cofactors and receptor expression levels, which varies between different types of mammalian cells[64].

Mammalian cell-based bioassays have been reported to have a higher sensitivity than yeast assays[7, 11, 53]. This can also be seen in Table 2.1 and 2.2, where the EC_{50} values from the androgen assays in mammalian cells are lower than those from the yeast-based assays. However, endogenous expression of steroid hormone receptors by mammalian cells (e.g. GRs in CHO cells[60]) can lead to nonspecific reactions[64]. More particularly, if a sample also contains other steroid hormones, as biological matrices typically do, these can bind to the endogenous receptors, also resulting in reporter gene transcription. Hence, this reduces the specificity of the measurement of steroid bioactivity. This makes the choice of the SRE particularly important[7]. Additionally, as the growth of mammalian cells requires the presence of serum, which contains small amounts of steroids and other growth factors, stripped serum should be used[7, 82]. For example, charcoal treatment of serum is an effective process for removing any interfering steroid compounds.

Below we discuss mammalian cell-based assays that have been used in the context of detecting abuse of steroid hormones in athletes and meat-producing animals. An overview is provided in Table 2.2[ii].

Mammalian-cell based assays for the detection of androgens, using *P. pyralis* luciferase and yellow fluorescent protein as reporters, have been developed and evaluated on urine[2, 6, 83] and serum[84] samples. As mentioned above, a major drawback associated with the use of mammalian cells to screen for androgens is the cross-reactivity with other steroid hormones[2, 6], although Bailey *et al.* report this is less an issue with the androgen assay they developed.[83].

Pitardi *et al.* evaluated the GR-CALUX (Chemical-Activated LUciferase gene eXpression) bioassay on spiked and incurred bovine urine samples[86], while Schumacher *et al.* developed and applied a dual luciferase reporter screening assay for the detection of synthetic glucocorticoids in calf liver samples[87]. The latter assay used a second luciferase as an internal control to correct for assay variability and matrix effects. Oral administration of dexamethasone (0.4 mg/day for 20 days)

[ii] Table 2.2 is included in the introduction of this dissertation (**Table 1.2. Overview of mammalian-based reporter bioassays**).

was picked up by the bioassay from day 2 until day 20. In some samples, glucocorticoid activity could still be detected on day 21-23.[86] Analysis of liver samples from non-treated animals could be distinguished from those who had received an injection with dexamethasone or flumethasone[87]. Willemsen *et al.* developed mammalian-cell based assays for estrogens, androgens, glucocorticoids and progestogens[90], although only the glucocorticoid and progesterone reporter assay were (limitedly) assessed on spiked urine and liver samples[82, 88]. A partial validation (recovery, repeatability, capability of detection) for the detection of glucocorticoids on liver samples was performed, although the amount of samples was below 20, which is stated to be a minimum according to EU directive 2002/657/CE (concerning the performance of analytical methods and the interpretation of results)[88]. In the study of Connolly *et al.*, all animals with glucocorticoids could be distinguished from those who did not receive treatment[89].

Apart from the above-described assays, many more reporter gene assays for steroid hormones are available. Several of these have been applied on a wide variety of matrices, such as feed, dietary supplements, wastewater etc. Discussion of these is beyond the scope of this review. Also for some clinical applications and for the determination of exposure to endocrine disrupting chemicals, steroid reporter assays have been used. However, although potentially of use, the utility of these assays for detecting steroid abuse or misuse has not been formally demonstrated[19, 91-95]. Only the assays listed in Table 2.1 and 2.2 have currently been applied on biological matrices for the purpose of screening for the (ab)use of steroid hormones.

2.5 Concluding Remarks

Bioactivity-based screening may be an effective tool to detect the presence in a biological matrix of unknown or new compounds (synthetic cannabinoids, opioids or steroids) that are not monitored via established mass spectrometry-based methods, which often work in MRM (multiple reaction monitoring) mode and/or apply (commercially) available libraries. Inherent to these activity-based assays is that they can only serve as a screening and eventually still require analytical methodology to establish a compound's presence or identity. When applied in large-scale screening programs, these bioassays may have the potential to serve as a cost-effective tool to identify those samples that require further in-depth bioanalytical investigation[7].

An important aspect when considering the use of reporter assays for the detection of drugs and doping is whether the activity of endogenous or frequently encountered (legal or illegal) substances might interfere with the assay. In the case of monitoring cannabinoid activity in biological matrices, the presence of endogenous cannabinoids (endocannabinoids) is not expected to interfere with the read-out as these are only present at very low concentrations in blood (in the range of low pmol/mL). Although in some conditions (eating disorders, obesity, schizophrenia, post-exercise) the endocannabinoid concentrations can rise, this will never be to an extent that this would lead to interference (< 10 pmol/mL)[96-98]. On the other hand, the presence of natural cannabinoids (e.g. THC) may result in a positive read-out of the SCRA bioassay. This co-detection of the use of cannabis or cannabis-derived products is expected since the bioassays screen for all cannabinoid activity. It should be noted, though, that THC is overall only a weak agonist at the CB receptor, making it a less ideal target for the bioassays compared to the potent SCRAs. The observation that only a positive signal was obtained when high THC concentrations (> 12 ng/mL THC) were present in plasma -indicating heavy cannabis use- is consistent with this[34]. For the opioid screening assay, not only the new synthetic opioids will be detected when using the activity-based assay, but also opiates (e.g. morphine) and opioids (e.g. fentanyl), which are clinically used as analgesic drugs. Positivity of these assays is quite easily picked up by routinely applied immunoassays or existing GC-MS or LC-MS/MS-based procedures. Yet, application of the bioassay in an early screening stage could be used to rule out a relevant

presence of any (legal or illegal) opiate or opioid, which might render some 'targeted' immunoassay-based screening procedures superfluous. As mentioned above, the presence of naloxone might interfere with the read-out of the MOR bioassay. As we noted elsewhere, it remains to be evaluated whether the incorporation of a minimal amount of agonist already at the start of the assay may help to cope with this intrinsic limitation. If naloxone would be present, this would result in a decrease in the assay, which would also indicate that further testing of that sample is required.

For steroid screening purposes, activity-based screening assays are susceptible to substances that may be naturally present in the sample matrix, such as natural hormones: 17β-estradiol, testosterone, progesterone, cortisol and other endogenous analogues of these hormones[52]. This is an important and inherent limitation. As a consequence, the utility of activity-based screening for steroids is limited to those samples that do not (or only to a limited extent) contain bioactive endogenous hormones. Examples include urine from preadolescents or from calves for androgen activity assays[75]. Another way to cope with the presence of endogenous compounds in the sample matrix is to use biological passport programs, as has been implemented for athletes. This allows comparison against an individual's prior personal measurements[99-101]. A potential additional problem with steroid screening is that there is an interplay between the commonly (ab)used cannabinoids and steroids. It has been reported that cannabis, and in particular its constituents THC and cannabinol, could interact with the AR in rat prostate cytosol[102] and that marijuana smokers show decreased fertility[103-104]. Cevenini *et al.* observed a strong anti-androgenic activity of the natural cannabinoids[71]. To what extent this might pose problems associated with steroid detection in real samples is unknown.

If activity-based bioassays are to be performed on urine samples, their capability to detect drug use depends upon the presence of active drug or drug metabolite in urine, and will often require deconjugation of inactive metabolite conjugates (via e.g. β-glucuronidase). E.g. for several SCRAs, it is known that almost no parent compound can be found in urine. However, the fact that many SCRA phase I metabolites are still active[32-33, 42-44, 46, 105] still allows the detection of SCRA use *via* urine. Also for synthetic opioids, urine-based screening does not pose a problem, as the active parent compounds are found in urine. For steroid hormones, however, it was reported that most of the known long-lasting androgen steroid phase I metabolites, known to be prevalent in urine following intake, are functionally inactive in an androgen bioassay[83]. Thus, the androgen assay would only detect abuse if a urine sample would be accidentally collected soon after the steroid doping[83]. Again, this is an important limitation.

Although the applicability of activity-based screening tools for steroid hormones has been evaluated since the early 2000s, it is clear from the above that these assays suffer from some inherent limitations, which may be one of the reasons why they are currently not routinely employed in doping control laboratories[106]. The cannabinoid and opioid reporter assays on the other hand do not seem to suffer from the problem of endogenous background. However, these were only very recently developed (the first report only dating from 2016)[32, 35] and, despite the fact that the first applications seem successful[33-35], there appears to be room for further improvement. Furthermore, it remains an open question whether broad dissemination will happen.

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8.2 APPENDIX II: Analytical findings and clinical presentation of the SCRA user cohort upon admission to the Emergency Department

Samples 1-52: ≥ 300 µL. Samples 53-70: 100 µL.

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	Age	M/F	Claimed use	Detected SCRA	Concentration (pg/mL)	Other findings	HR (bpm)	BP (mmHg)	T (°C)	GCS	Seizures	Agitation	Other	LoS (h:min)	Discharge
1	49	M	Unknown drug (cannabis or SCRA)	5F-ADB-O-desmethyl	1358		76	128/83	36.6	15				04:35	ED
2	44	M	Crack Heroin	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	2538 428	Clonazepam, chlordiazepoxide, cotinine, demoxapam, ethyl glucuronide, methadone/EDDP, mirtazapine, nefopam	86	115/68	36.9	15			Psychosis	17:57	EMU
3	40	M	Cannabis Ethanol	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	387 2610 910	Cotinine, diazepam, ethyl glucuronide, nordazepam, oxazepam, temazepam	97	139/91	35.6	12				01:34	ED
4	43	M	Cocaine Heroin Spice	5F-ADB-O-desmethyl	4830	Benzoylecgonine, cotinine, ethyl glucuronide, tetramisole	82	136/95	35.8	14				17:36	EMU
5	44	M	Ethanol "Man Down" (SCRA)	5F-ADB-O-desmethyl	6732	Cotinine, ethyl glucuronide, mirtazapine, paracetamol	88	113/76	36.4	15			Chest pain	03:17	ED
6	42	M	Ethanol Methadone Pregabalin Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	1285 6966 2587	Clonazepam, cotinine, diazepam, ethyl glucuronide, ketamine, methadone/EDDP, midazolam, morphine, nordazepam, pregabalin	94	128/74	36.2	3			HTN Pyrexia	23:18	Critical care (ICU/DHU/CCU) (Self)
7	31	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	11850 850	Cotinine	70	109/50	36.5	15	*Yes*			02:37	ED
8	36	F	Cannabis Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	13490 360	Cotinine	72	103/58	36.2	15				05:15	Psychiatry
9	43	M	Cannabis Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	2047 13697 1573	Cotinine, cocaine/benzoylecgonine, dihydrocodeine, morphine, paracetamol, tetramisole	90	145/95	36	15	*Yes*		Chest pain	12:52	AAW (Self)
10	29	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl	28646 6544	Cotinine, ethyl glucuronide, **Δ^9-THC (4360 pg/mL)**	125	150/100	35	14	*Yes*	*Yes*		02:22	Medical
11	36	M	Ethanol Spice	Cumyl-5F-PINACA-desfluorohydroxypentyl	871	Cotinine, ethyl glucuronide, quinine	92	131/87	35.6	12			HTN	11:20	Medical
12	36	M	Ethanol Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	1340 1053	Cotinine, ethyl glucuronide	124	93/73	38.1	15				06:10	EMU
13	31	M	Cocaine Ethanol	Cumyl-5F-PINACA-desfluorohydroxypentyl	1253	Cotinine, ethyl glucuronide, THC-COOH	92	102/70	36.6	15		*Yes*		02:28	ED
14	26	M	Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	3560 1270	Cotinine, paracetamol	80	125/88	35.3	15		*Yes*	HTN	03:10	Self
15	26	M	Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	4350 827	Cotinine, paracetamol, dihydrocodeine	84	123/84	36.5	15		*Yes*		03:17	ED
16	27	M	Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA -desfluoro hydroxypentyl	7430 3300	Cotinine, flupenthixol, procyclidine, **Δ^9-THC (890 pg/mL)**	71	143/61	36	8				11:52	EMU
17	38	M	Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	24120 2817	Buprenorphine, clonazepam, cotinine, diazepam, gabapentin, nordazepam, oxazepam, pregabalin	91	140/85	37	15		*Yes*		03:41	ED
18	47	M	Crack Ethanol Heroin	MDMB-CHMICA-O-desmethyl	1510	Benzoylecgonine, buprenorphine, chlordiazepoxide, clonazepam, cotinine, diazepam, ethyl glucuronide, etifoxine, gabapentin, nordazepam, omeprazole, oxazepam, tetramisole	96	128/80	37.3	14			Chest pain	03:55	Self

Analytical findings and clinical presentation of the SCRA user cohort upon admission to the Emergency Department *(continued)*. **Samples 1-52, ≥ 300 µL; Samples 53-70, 100 µL.**

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	**Age**	**M/F**	**Claimed use**	**Detected SCRA**	**Concentration (pg/mL)**	**Other findings**	**HR (bpm)**	**BP (mmHg)**	**T (°C)**	**GCS**	**Seizures**	**Agitation**	**Other**	**LoS (h:min)**	**Discharge**
19	30	M	Ethanol Spice	MDMB-CHMICA-O-desmethyl	7270	Benzoylecgonine, codeine, diazepam, EDDP, ethyl glucuronide, morphine, nordazepam, noscapine metabolite, olanzapine, oxazepam, tetramisole	90	108/62	34.5	15	*Yes*			02:34	ED
20	27	M	Unknown drug	MDMB-CHMICA MDMB-CHMICA-O-desmethyl	3976 20560	Cotinine, diazepam, etifoxine, haloperidol, lorazepam, nordazepam, oxazepam, temazepam	96	128/70	36.6	14				03:03	ED
21	35	M	Ethanol Spice	MDMB-CHMICA MDMB-CHMICA-O-desmethyl MDMB-CHMICA-hydroxy(indole)	3880 12200 350	Cotinine, lorazepam, THC-COOH, **Δ^9-THC (1474 pg/mL)**	89	176/157	37.3	14		*Yes*	HTN	11:14	Medical
22	34	F	Spice	MDMB-CHMICA MDMB-CHMICA-O-desmethyl	3210 4600	Cotinine, ethyl glucuronide	72	101/54	35.5	9	*Yes*	*Yes*		07:00	EMU
23	20	M	Ethanol MDMA	5F-ADB-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl	1158 110 1802	Cotinine, ethyl glucuronide, paracetamol	121	139/79	35.1	14		*Yes*		03:50	ED
24	42	M	Crack Heroin Spice	5F-ADB-O-desmethyl AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	3070 3825 425 380	Benzoylecgonine, carbamazepine, codeine, cotinine, GHB, methadone/EDDP, morphine, tetramisole, zuclopenthixol	43	85/68	36.4	8		*Yes*		00:18	Critical care (ICU/HDU/CCU)
25	40	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl MDMB-CHMICA-O-desmethyl	5388 498 168	Cocaine/benzoylecgonine, codeine, cotinine, diazepam, ethyl glucuronide, etifoxine, haloperidol, methadone/EDDP, midazolam, morphine, nordazepam, noscapine metabolite, oxazepam, oxycodone, papaverine, temazepam, tetramisole	100	111/52	35.9	8		*Yes*		08:04	EMU (Self)
26	30	M	Ethanol Unknown drug	5F-ADB-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	3020 520 57588	Cotinine, ethyl glucuronide	56	110/59	34.7	14	*Yes*			16:45	EMU
27	43	M	Crack Heroin	5F-ADB-O-desmethyl AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	6353 6728 780 21413	Benzoylecgonine, codeine, cotinine, methadone/EDDP, morphine, noscapine metabolite, nordazepam	118	141/98	37.1	14		*Yes*		18:30	EMU
28	29	M	Ethanol Spice	5F-ADB-O-desmethyl Cumyl-5F-PINACA Cumyl-5F-PINACA -desfluorohydroxypentyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	318 958 1532 3596 108116	Ethyl glucuronide	121	130/80	35.9	14		*Yes*		03:50	ED
29	42	M	Ethanol Spice	5F-ADB-O-desmethyl MDMB-CHMICA-O-desmethyl	10110 18655	Cotinine	79	107/63	36.6	15	*Yes*			05:30	ED (Self)
30	46	M	Ethanol Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	8747 2822 885 1285	Cotinine, demoxapam, ethyl glucuronide, lorazepam, nordazepam, pregabaline, sertraline	110	176/127	35.6	4		*Yes*	HTN	17:01	EMU
31	26	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	1250 667 6397 1613	Cotinine, paracetamol	110	152/87	35.6	15		*Yes*		04:43	ED

Analytical findings and clinical presentation of the SCRA user cohort upon admission to the Emergency Department *(continued)*. **Samples 1-52, ≥ 300 µL; Samples 53-70, 100 µL.**

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	**Age**	**M/F**	**Claimed use**	**Detected SCRA**	**Concentration (pg/mL)**	**Other findings**	**HR (bpm)**	**BP (mmHg)**	**T (°C)**	**GCS**	**Seizures**	**Agitation**	**Other**	**LoS (h:min)**	**Discharge**
32	27	M	Spice	5F-ADB-O-desmethyl AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	23258 15318 968 17116	Cotinine	95	105/55	37	15		*Yes*		02:41	ED (Self)
33	37	M	Crack Ethanol Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	31747 1247 1490 1333	Benzoylecgonine, diazepam, lidocaine, lorazepam, methadone/EDDP, morphine, nordazepam, paracetamol, pregabalin, tramadol	72	126/63	37.3	9	*Yes*	*Yes*		11:54	Critical care (ICU/HDU/CCU)
34	43	M	Ethanol Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AB-CHMINACA AB-CHMINACA-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl	30949 1511 743 30737 1123 21091	Cotinine, diazepam, ethyl glucuronide, etifoxine, methadone/EDDP	100	125/85	37.2	14				05:42	ED
35	42	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	30523 2609 6426 34606	Benzoylecgonine, carbamazepine, cotinine, methadone/EDDP, oxycodone, pregabalin, zuclopenthixol	64	95/55	36.4	14		*Yes*		05:59	ED
36	23	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	38938 1183 735 7778	Cotinine, diazepam, lorazepam, temazepam	116	156/74	36	14		*Yes*		11:36	Critical care (ICU/HDU/CCU)
37	21	M	Spice	5F-ADB 5F-ADB-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	550 40375 355 32150	Cotinine, lorazepam	83	135/78	36.1	14		*Yes*		22:22	ED (Self)
38	40	M	Ethanol Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	1210 43324 1452 15766 5436	Chlordiazepoxide, clonazepam, cotinine, demoxapam	71	138/82	36.1	14				03:40	ED
39	36	M	Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	8225 28745 4420 19038 72658	Cocaine/benzoylecgonine, codeine, cotinine, methadone/EDDP, morphine, paracetamol, tetramisole	110	114/70	37.1	15		*Yes*	Hallucinations	20:15	Medical
40	42	M	Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	2290 59800 3730 1800 9790	Cotinine, methadone/EDDP, zuclopenthixol	82	93/49	37.1	14			HTN Psychosis Hallucinations	17:03	EMU
41	37	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	69485 2180 495 9490	Cotinine	57	107/76	35.4	15				02:55	ED

Analytical findings and clinical presentation of the SCRA user cohort upon admission to the Emergency Department *(continued)*. **Samples 1-52, ≥ 300 µL; Samples 53-70, 100 µL.**

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	**Age**	**M/F**	**Claimed use**	**Detected SCRA**	**Concentration (pg/mL)**	**Other findings**	**HR (bpm)**	**BP (mmHg)**	**T (°C)**	**GCS**	**Seizures**	**Agitation**	**Other**	**LoS (h:min)**	**Discharge**
42	29	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	92220 6730 990 28550	Benzoylecgonine, codeine, cotinine, diazepam, midazolam, morphine, paracetamol, tetramisole	130	130/70	37.2	14		*Yes*		09:01	EMU
43	28	F	Cocaine Ethanol Heroin Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	121224 6616 2432 75404	Benzoylecgonine, cotinine, morphine, tetramisole	72	107/50	37	3				03:05	ED
44	46	M	Ethanol Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	145555 1905 1590 28385	Cotinine, ethyl glucuronide	110	150/80	37	15		*Yes*		14:06	EMU
45	41	M	Ethanol Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	191550 7640 940 26720 640 7520	Cotinine	69	88/53	34.5	15				03:04	ED
46	26	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	913765 6490 9505 8250 170575	Cotinine	65	120/80	36.4	15				04:50	ED
47	51	M	Ethanol Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl BB-22 BB-22-carboxy	1297 28550 3703 1080 1100	Cotinine, ethyl glucuronide	94	113/82	36.5	15	*Yes*			08:26	EMU
48	22	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AB-CHMINACA-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl	20975 1030 3288 915 13958	Cotinine, risperidone	44	121/73	36.2	14	*Yes*			03:53	Self
49	30	M	Spice	5F-ADB 5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl 5F-AKB-48 5F-AKB-48-desfluorohydroxypentyl AMB-FUBINACA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	165 1250 125 163 783 23298 10020 15340	Citalopram, cotinine	61	123/75	35.5	15				01:10	EMU

Analytical findings and clinical presentation of the SCRA user cohort upon admission to the Emergency Department *(continued)*. **Samples 1-52, ≥ 300 µL; Samples 53-70, 100 µL.**

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	**Age**	**M/F**	**Claimed use**	**Detected SCRA**	**Concentration (pg/mL)**	**Other findings**	**HR (bpm)**	**BP (mmHg)**	**T (°C)**	**GCS**	**Seizures**	**Agitation**	**Other**	**LoS (h:min)**	**Discharge**
50	43	M	Ethanol Unknown drug	5F-ADB-O-desmethyl 5F-AKB-48 5F-AKB-48-desfluorohydroxypentyl AMB-FUBINACA-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	334 758 2438 4778 990 3778 39940 14432	Cotinine, ethyl glucuronide	94	107/42	36.1	7				02:20	ED
51	34	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-FUBINACA-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	52840 888 23790 1493 61053 12265 68065	Cotinine, methadone/EDDP, mirtazapine, oxycodone, pregabalin	44	102/65	36.6	PAIN				02:50	ED
52	35	M	Methadone Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl 5F-AKB-48 5F-AKB-48-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	99525 2390 835 2415 10340 163125 13895 82885	Benzoylecgonine, Cotinine, diazepam, etifoxine, lorazepam, morphine, methadone/EDDP, nordazepam, olanzapine, oxazepam, pregabalin, temazepam	52	106/67	37.1	8		*Yes*		04:45	ED
53	57	M	Butane Cocaine Ethanol Spice	MDMB-CHMICA-O-desmethyl	275	Cotinine, diazepam, ethyl-glucuronide, nordazepam	130	146/88	34.9	14			Chest pain	03:55	ED
54	50	M	Heroin	Cumyl-5F-PINACA-desfluorohydroxypentyl	766	Benzoylecgonine, carbamzepine, clonazepam, codeine, demoxapam, ethyl-glucuronide, methadone/EDDP, morphine, noscapine, oxazepam, pregabalin, quinine, tetramisole	82	125/85	33.4	10	*Yes*			06:29	Critical care (ICU/HDU/CCU) (Self)
55	25	M	Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	540 355	Cotinine, warfarin	84	147/88	36.8	14				03:48	ED
56	35	M	GHB	MDMB-CHMICA-O-desmethyl	3376	Codeine, cotinine, diazepam, etifoxine, morphine, nordazepam, oxazepam, pregabalin, quetiapine, risperidone, temazepam	109	119/91	36.7	15				08:30	EMU
57	40	M	Crystal meth Spice	MDMB-CHMICA-O-desmethyl	3770	Benzoylecgonine, cotinine, demoxepam, diazepam, nordazepam, oxazepam, tetramisole, zuclopenthixol	59	119	35.8	13				02:29	Self
58	34	M	Cannabis Cocaine MDMA Spice	5F-ADB-O-desmethyl AMB-FUBINACA-O-desmethyl	853 3287	Benzoylecgonine, cotinine, ethyl-glucuronide, quinine, tetramisole	75	125/82	33.3	15	*Yes*			05:46	EMU
59	39	M	Ethanol Spice	5F-ADB-O-desmethyl AMB-FUBINACA-O-desmethyl	2945 1850	Cotinine, ethyl-glucuronide	101	139/87	36.6	15	*Yes (EtOH w/d?)*			16:00	Medical
60	35	M	Ethanol "Dinosaur" Spice	Cumyl-5F-PINACA Cumyl-5F-PINACA-desfluorohydroxypentyl	4556 1386	Amphetamine, amitriptyline, cotinine, cyclizine, ethyl-glucuronide, lidocaine, lorazepam, methamphetamine, mexedrone, zolpidem and metabolites	122	124/69	35.6	7		*Yes*		10:28	EMU

Analytical findings and clinical presentation of the SCRA user cohort upon admission to the Emergency Department *(continued).* **Samples 1-52, ≥ 300 µL; Samples 53-70, 100 µL.**

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	**Age**	**M/F**	**Claimed use**	**Detected SCRA**	**Concentration (pg/mL)**	**Other findings**	**HR (bpm)**	**BP (mmHg)**	**T (°C)**	**GCS**	**Seizures**	**Agitation**	**Other**	**LoS (h:min)**	**Discharge**
61	34	M	Ethanol Heroin Spice	5F-ADB-O-desmethyl AMB-FUBINACA-O-desmethyl	5670 2625	Benzoylecgonine, codeine, cotinine, EDDP, morphine, papaverine, tetramisole	79	136/98	36.3	15				03:37	ED
62	40	F	Ethanol Heroin	5F-ADB-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl	4640 640 8350	Cocaine/benzoylecgonine, cotinine, demoxapam, diazepam, ethyl-glucuronide, nordazepam, paracetamol, tetramisole	95	128/76	35.9	13				05:56	Medical
63	42	M	Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl AMB-CHMINACA-O-desmethyl	22014 6943 1209 2894 171	Carbamazepine, cotinine, dihydrocodeine, methadone/EDDP, zuclopenthixol	44	84/74	35.8	14				02:35	ED
64	49	M	Crack Ethanol Heroin Pregabalin Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl AMB-CHMICA AMB-CHMICA-O-desmethyl	6280 1200 1207 27533	Amphetamine, codeine, cotinine, diazepam, etifoxine, methadone/EDDP, nordazepam, oxazepam, pregabalin, temazepam	105	104/63	37	14				03:55	ED
65	45	M	Ethanol Spice	AB-CHMINACA AB-CHMINACA-O-desmethyl	11450 25680	Cotinine, ethyl-glucuronide	125	90/50	36	9		*Yes*		17:44	EMU
66	47	M	Tasmanian weed (SCRA)	MDMB-CHMICA MDMB-CHMICA-O-desmethyl	5920 34280	Cotinine, ethyl-glucuronide	129	131/82	36.6	8	*Yes*		HTN	13:38	Medical
67	27	M	Cannabis Spice	5F-ADB-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	1665 16835 40270	Cotinine	102	112/67	35.8	14				03:36	Self
68	43	M	Ethanol Spice	AMB-CHMICA-O-desmethyl AMB-FUBINACA-O-desmethyl MDMB-CHMICA-O-desmethyl	24840 37470 10470	Cotinine, EDDP, ethyl-glucuronide, pregabalin	89	152/91	37.1	15				05:28	Self
69	38	M	Methadone Spice	5F-ADB-O-desmethyl 5F-ADB-desfluorohydroxypentyl 5F-AKB-48 5F-AKB-48-desfluorohydroxypentyl AB-CHMINACA (very weak, probably present) AB-CHMINACA-O-desmethyl AMB-CHMICA AMB-CHMICA-O-desmethyl MDMB-CHMICA MDMB-CHMICA-O-desmethyl	19745 2595 1200 2095 510 11030 4050 27355 17910 9410	Codeine, cotinine, cyclizine, diazepam, dihydrocodeine, etifoxine, methadone/EDDP, mirtazapine, nordazepam, oxazepam, oxycodone, paracetamol, pregabalin, temazepam	48	89/48	36.2	6				03:05	ED
70	28	M	Spice	5F-ADB-O-desmethyl AMB-CHMICA-O-desmethyl AMB-FUBINACA-O-desmethyl MDMB-CHMICA-O-desmethyl	8410 96160 226560 13650	Benzoylecgonine, cotinine, diazepam, ethyl-glucuronide, nordazepam, temazepam, tetramisole	89	108/67	35.9	9				11:01	Medical

Abbreviations: Δ^9-**THC**, (-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol; **AAW**, Acute Admissions Ward; **BP**, blood pressure; **CCU**, Coronary Care Unit; **ED**, Emergency Department; **EDDP**, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone; **EMU**, Emergency Medical Unit (Emergency Department Short Stay Unit); **F**, female; **GCS**, Glasgow Coma Score; **GHB**, gamma-hydroxybutyric acid; **HDU**, High Dependency Unit; **HR**, heart rate; **HTN**, hypertension; **HRMS/MS**, high-resolution tandem mass spectrometry; **ICU**, Intensive Care Unit; **LoS**, length of stay; **M**, male; **MDMA**, 3,4-methylenedioxymethamphetamine; **T**, body temperature; **THC-COOH**, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol.

SCRAs: **5F-ADB,** methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate; **5F-AKB-48,** N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide; **AB-CHMINACA,** N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide; **AMB-FUBINACA,** methyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate; **AMB-CHMICA,** methyl (1-(cyclohexylmethyl)-1H-indole-3-carbonyl)-valinate; **BB-22,** quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate; **Cumyl-5F-PINACA,** 1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide; **MDMB-CHMICA,** Methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate.

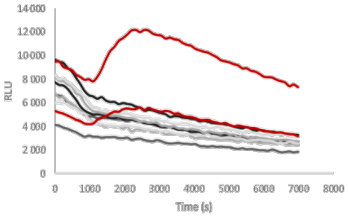
Definitions: **hypertension,** systolic blood pressure ≥ 160 mmHg at any moment throughout admission; **pyrexia,** body temperature ≥ 39°C.

8.3 APPENDIX III: Bioassay per protocol (≥ 300 µL)

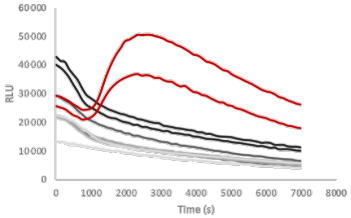
8.3.1 Appendix III-A: Overview of all SCRA-positive profiles (≥ 300 µL)

CB_1-positive samples

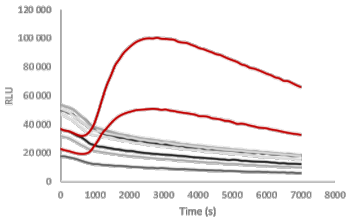
Sample 1



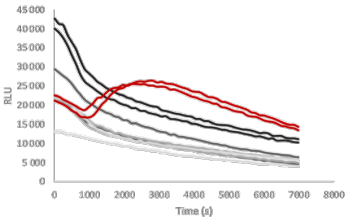
Sample 2



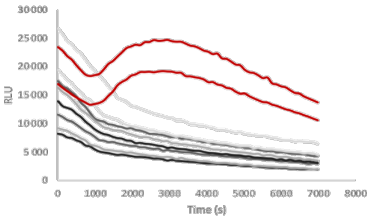
Sample 3



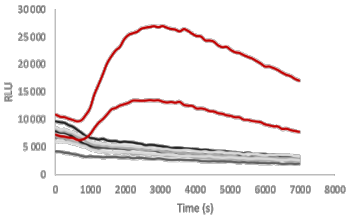
Sample 4



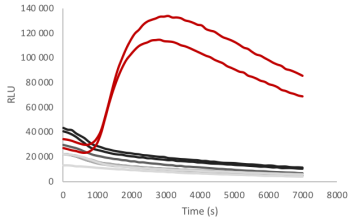
Sample 5



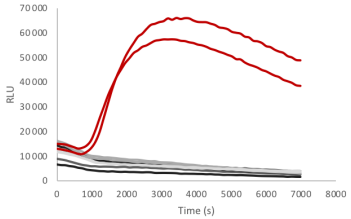
Sample 6



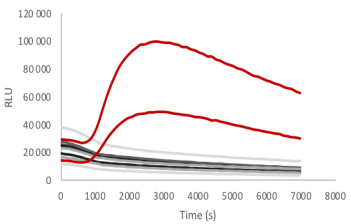
Sample 7



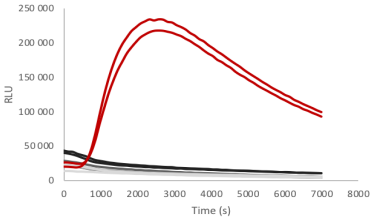
Sample 8



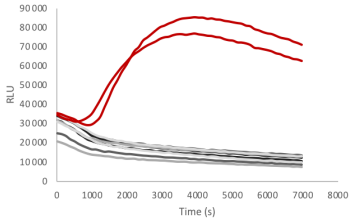
Sample 9



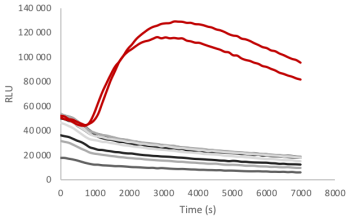
Sample 10



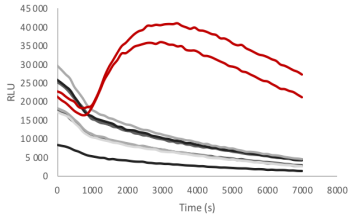
Sample 11*



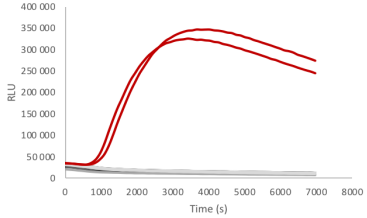
Sample 12



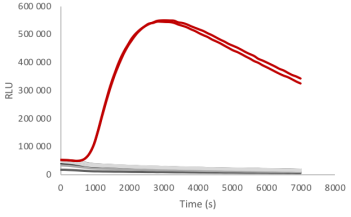
Sample 13



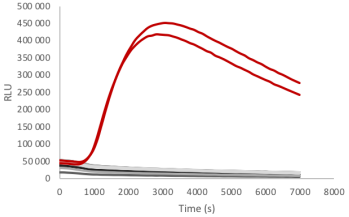
Sample 14*



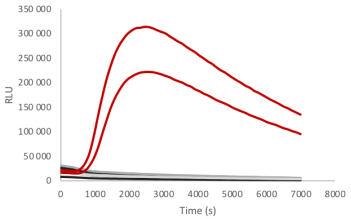
Sample 15



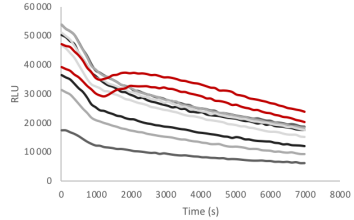
Sample 16



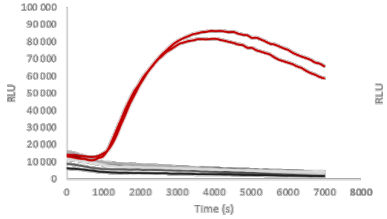
Sample 17



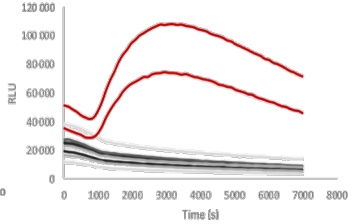
Sample 18



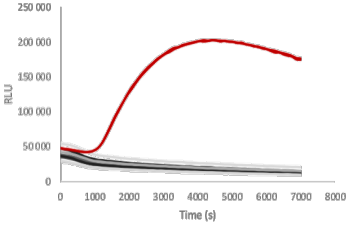
Sample 19



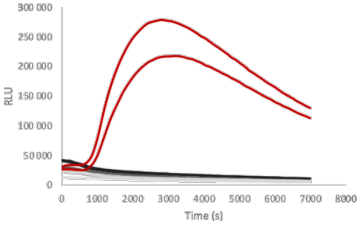
Sample 20



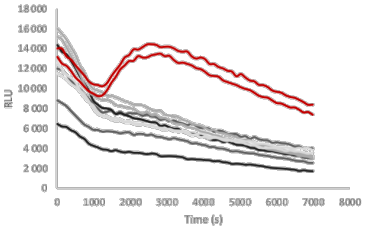
Sample 21



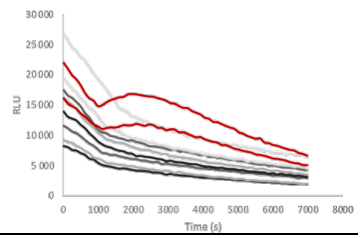
Sample 22



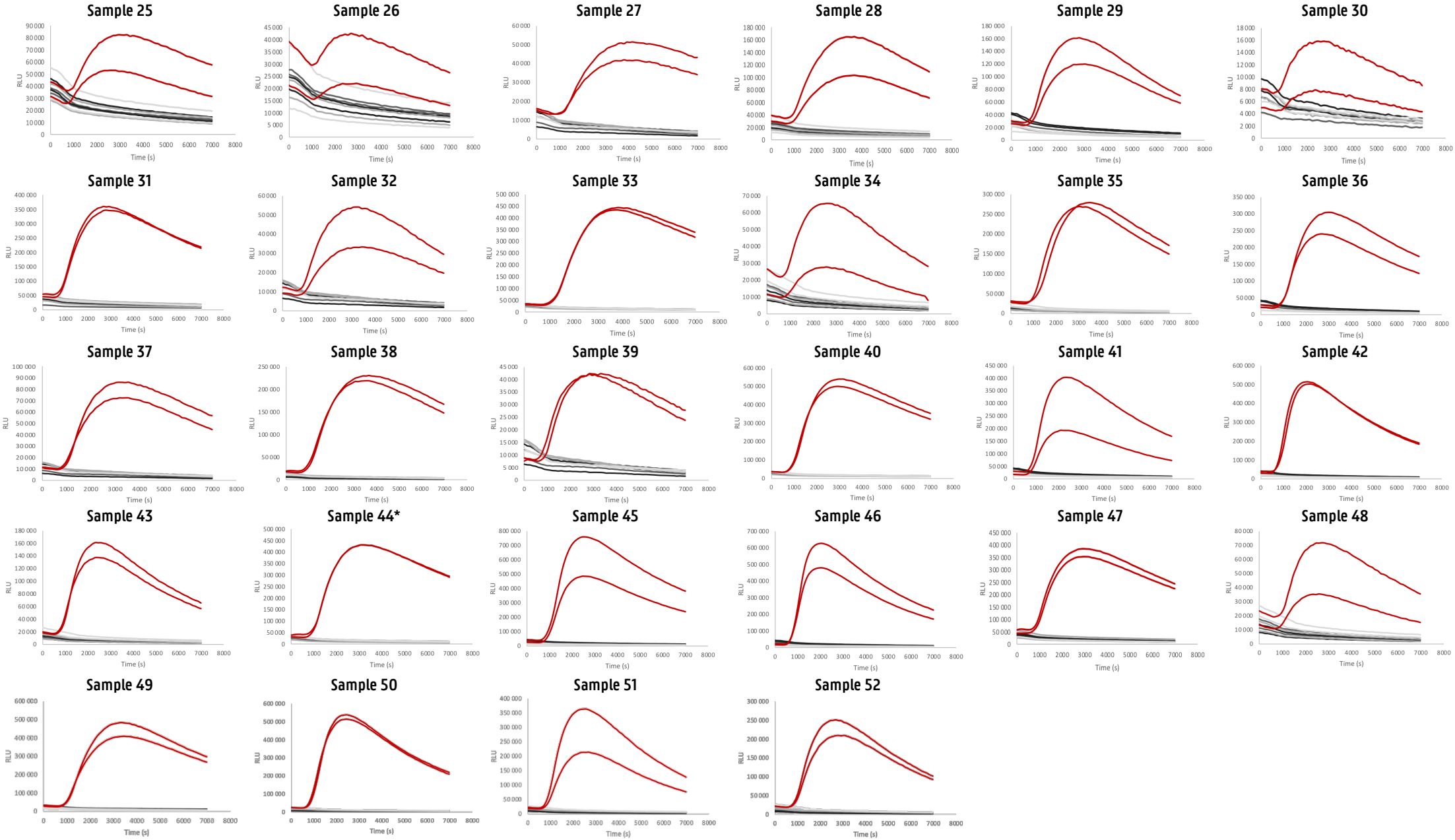
Sample 23



Sample 24



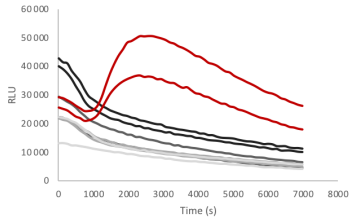
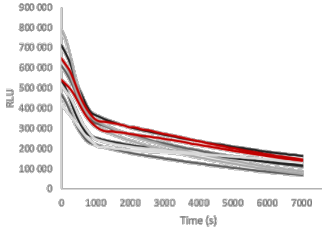
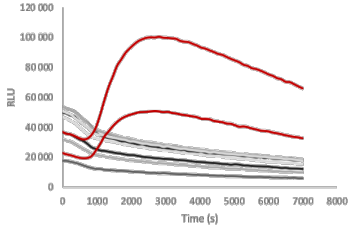
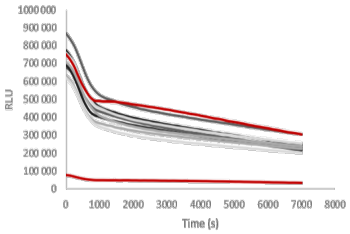
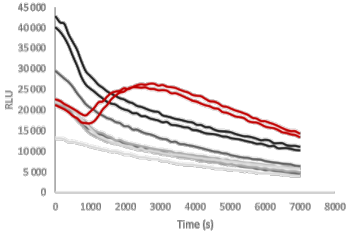
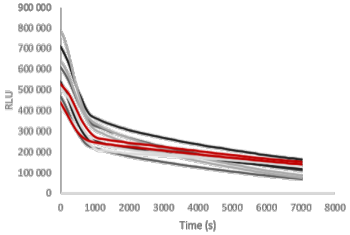
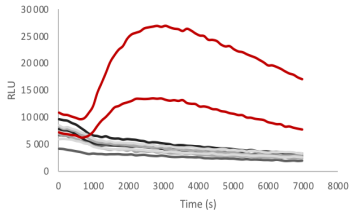
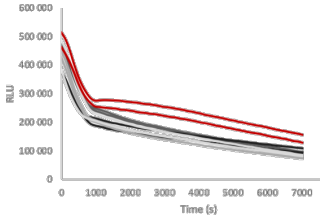
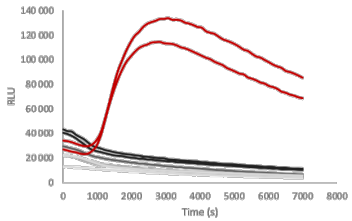
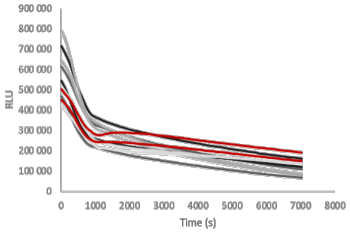
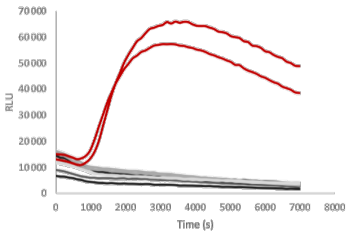
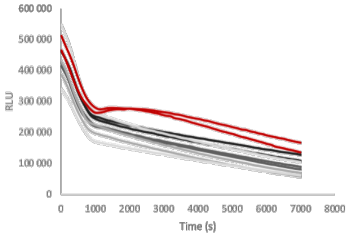
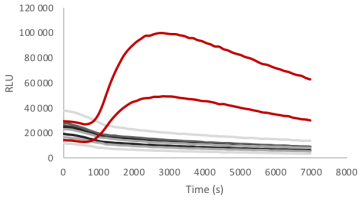
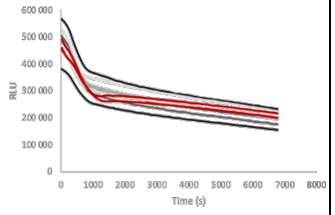
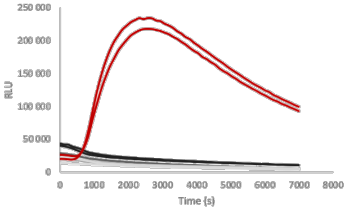
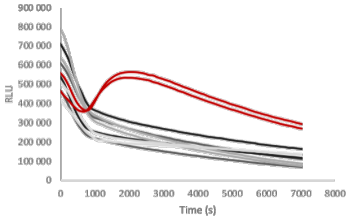
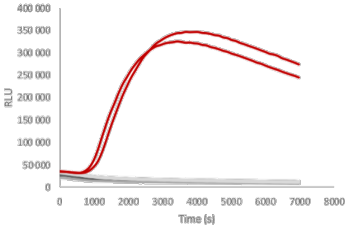
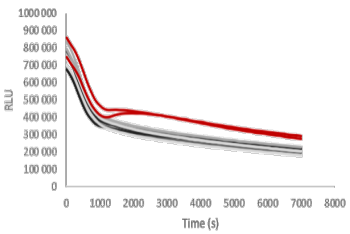
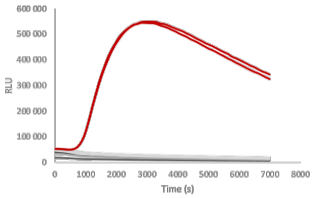
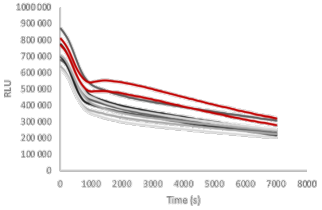
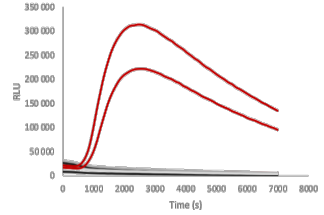
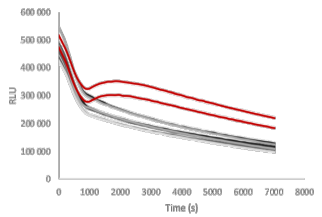
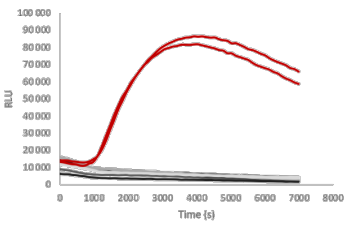
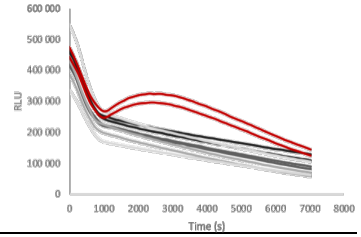
CB_1-positive samples *(continued)*



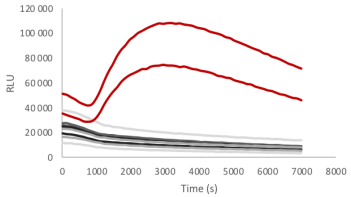
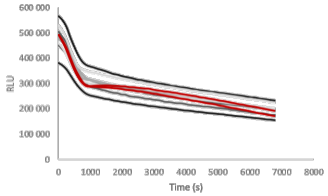
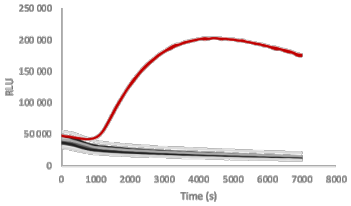
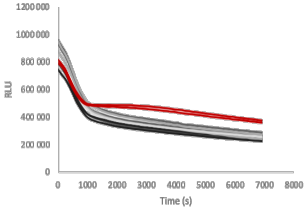
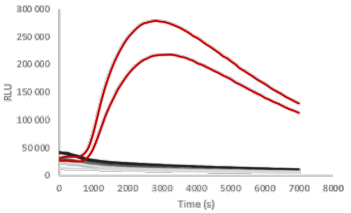
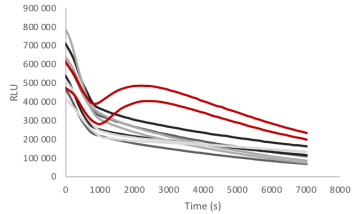
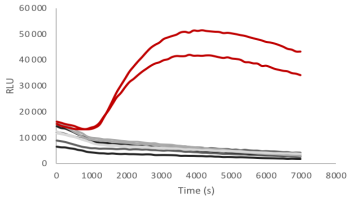
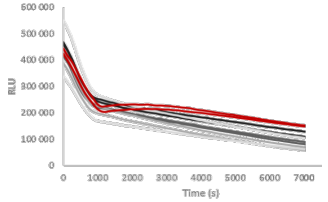
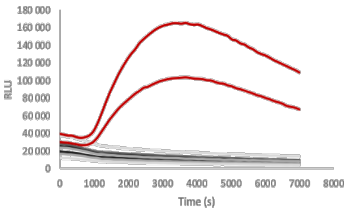
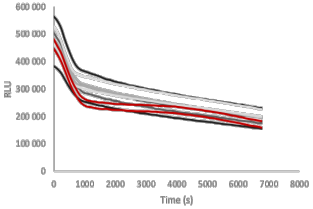
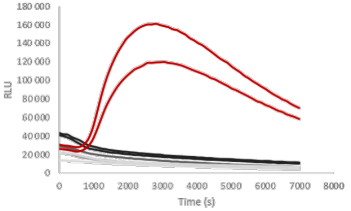
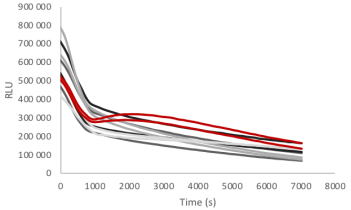
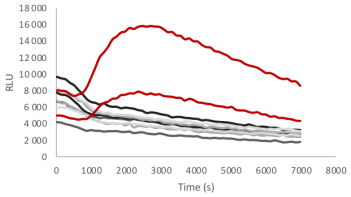
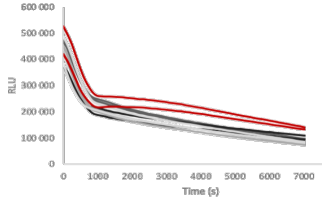
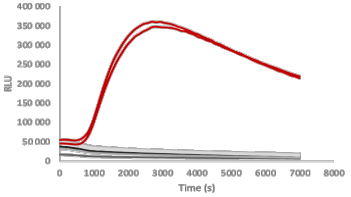
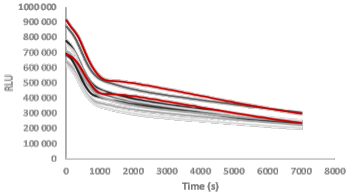
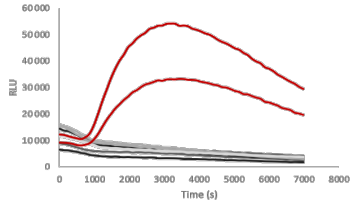
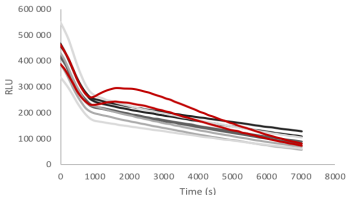
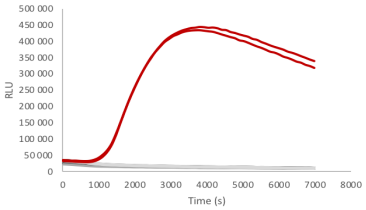
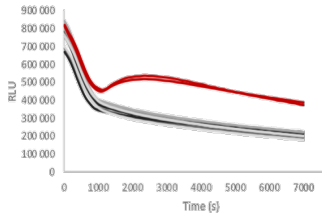
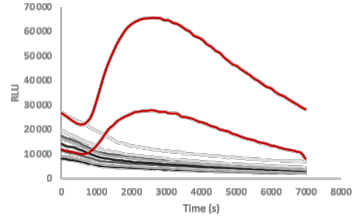
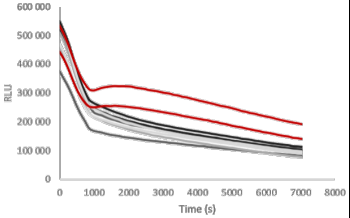
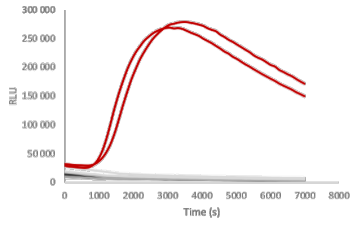
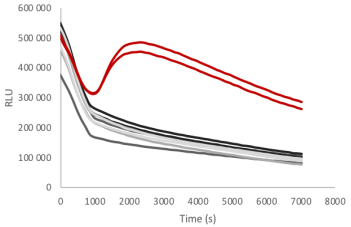
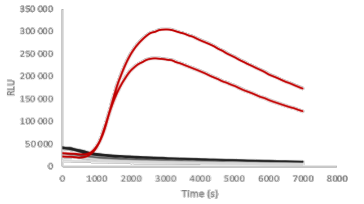
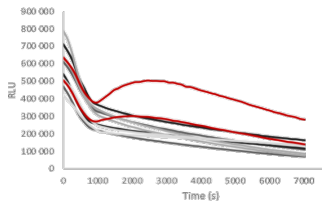
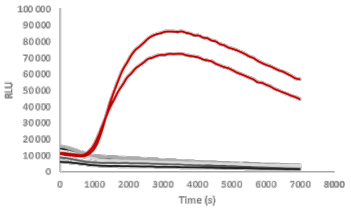
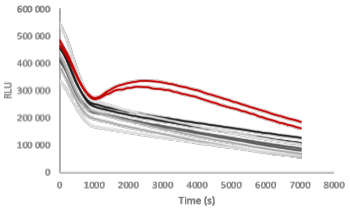
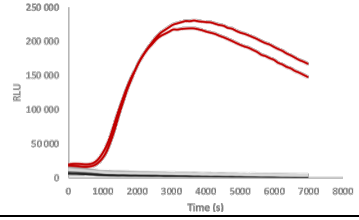
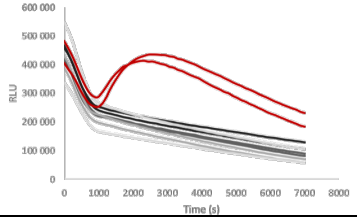
The profiles represent the raw data from the CB_1 bioassay. Samples for which 300 µL serum was used as a starting volume, are indicated with an asterisk (*). For all other samples, 500 µL serum was used. Legend: Red lines, serum samples (n = 2); **grey and black lines**, four independent blanks (n = 2). **Abbreviations:** RLU (y-axis), relative light units; s (x-axis), seconds.

8.3.2 Appendix III-B: Combined CB_1/CB_2-positives (≥ 300 µL)

Combined CB_1/CB_2-positive samples

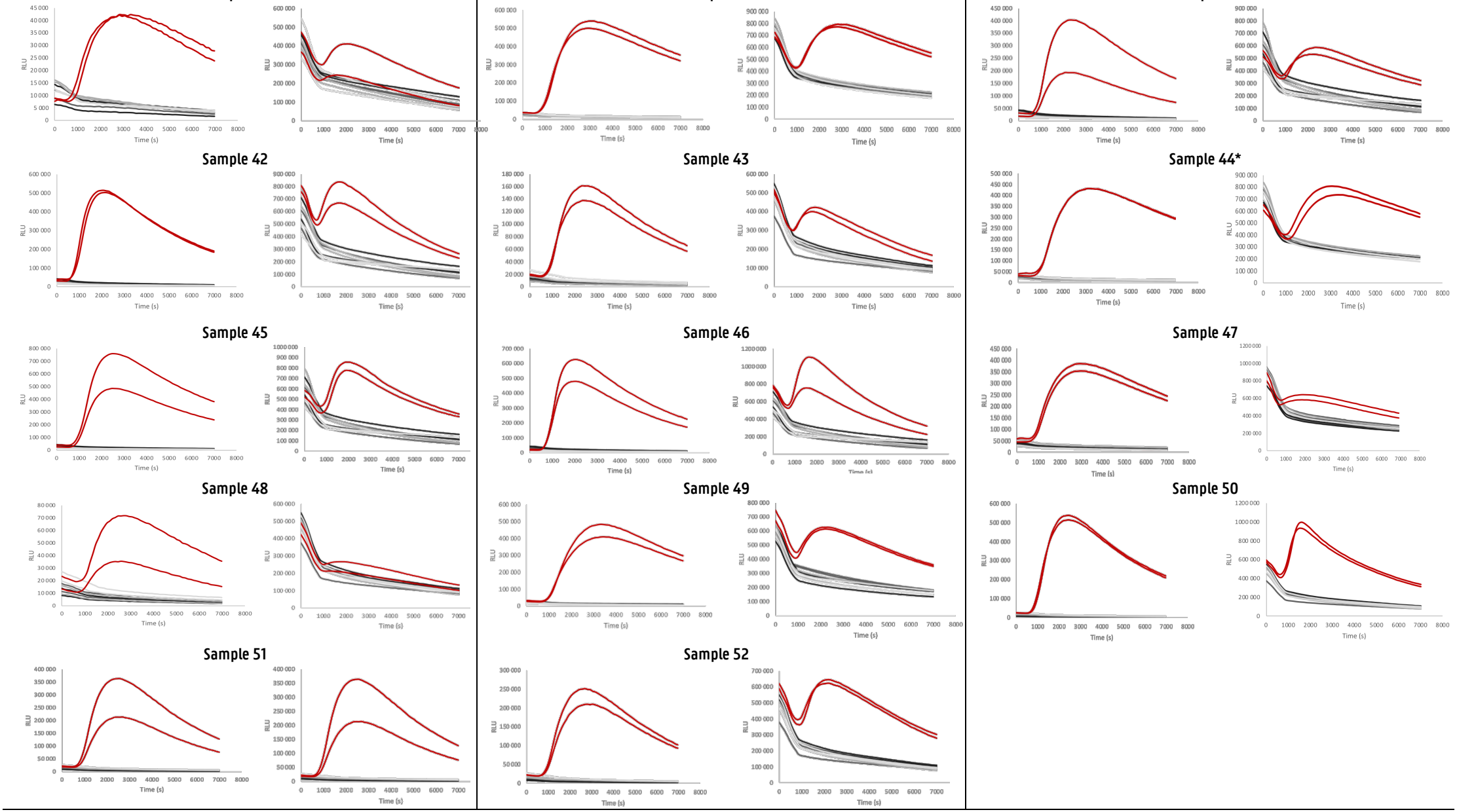
CB_1	CB_2	CB_1	CB_2	CB_1	CB_2
Sample 2		Sample 3		Sample 4	
					
Sample 6		Sample 7		Sample 8	
					
Sample 9		Sample 10		Sample 14*	
					
Sample 15		Sample 17		Sample 19	
					

Combined CB_1/CB_2-positive samples *(continued)*

CB_1	CB_2	CB_1	CB_2	CB_1	CB_2
Sample 20		Sample 21		Sample 22	
					
Sample 27		Sample 28		Sample 29	
					
Sample 30		Sample 31		Sample 32	
					
Sample 33		Sample 34		Sample 35	
					
Sample 36		Sample 37		Sample 38	
					

Combined CB_1/CB_2-positive samples *(continued)*

CB_1	CB_2	CB_1	CB_2	CB_1	CB_2
Sample 39		Sample 40		Sample 41	
Sample 42		Sample 43		Sample 44*	
Sample 45		Sample 46		Sample 47	
Sample 48		Sample 49		Sample 50	
Sample 51		Sample 52			



The profiles represent the raw data from the CB_1 and CB_2 bioassay. Samples for which 300 µL serum was used as a starting volume, are indicated with an asterisk (*). For all other samples, 500 µL serum was used. Legend: **Red lines**, serum samples (n = 2); **grey and black lines**, four independent blanks (n = 2). Abbreviations: **RLU (y-axis)**, relative light units; **s (x-axis)**, seconds.

8.3.3 Appendix III-C: Data on Δ^9-THC-containing positive and false positive samples (≥ 300 µL)

a) HRMS/MS findings and clinical characteristics.

Samples 71-76: Δ^9-THC-containing samples.
Samples 77-83: false positive samples.
Sample 83: self-reported "Spice" use as supported by the bioassay without bioanalytical evidence.

Samples for which 300 µL serum was used as a starting volume, are indicated with an asterisk (*). For all other samples, 500 µL serum was used.

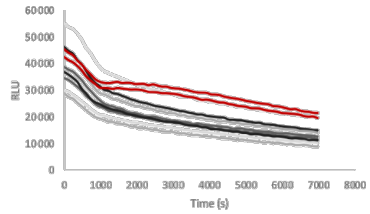
Patient information				HRMS/MS analysis	Clinical measurements				Clinical features				
Nr	Age	M/F	Claimed use	Findings	HR (bpm)	BP (mmHg)	T (°C)	GCS	Seizures	Agitation	Other	LoS (h:min)	Discharge
71	33	M	Cocaine Ethanol Heroin	Cotinine, ethyl glucuronide, THC-COOH, **Δ^9-THC (3640 pg/mL)**	88	121/95	37	15			Chest pain Palpitations	01:32	-
72	34	M	Cannabis	Oxazepam, sertraline, THC-COOH, **Δ^9-THC (4188 pg/mL)**, temazepam	77	164/118	36.7	15	*Yes*	*Yes*	HTN Hallucinations	02:38	-
73	32	F	Cocaine Ethanol Marijuana MDMA	Amphetamine, cocaine/benzoylecgonine, cotinine, ethyl glucuronide, MDMA, tetramisole, THC-COOH, **Δ^9-THC (4833 pg/mL)**	-	-	-	-	-	-	-	05:20	-
74	31	M	Ethanol GHB	Cotinine, ethyl glucuronide, quinine, THC-COOH, **Δ^9-THC (5264 pg/mL)**	66	104/53	35.2	15		*Yes*		00:54	-
75	19	M	Cannabis Ethanol Ecstasy	Bisoprolol, chlorpheniramine, cotinine, ethyl glucuronide, ketamine, MDMA, **Δ^9-THC (6801 pg/mL)**	95	164/109	36.4	15			Chest pain HTN Palpitations	02:36	-
76	26	M	Cannabis	Diazepam, ketamine, paracetamol, quinine, THC-COOH, **Δ^9-THC (13050 pg/mL)**	130	150/67	36.6	15				14:24	-
77 *	24	M	Cannabis Ethanol Ketamine Unknown drug	Cotinine, ethyl glucuronide, ketamine, MDMA, THC-COOH	120	130	38.7	15			Chest pain	14:28	-
78	38	M	Heroin	Benzoylecgonine, codeine, cotinine, diazepam, ethyl glucuronide, lidocaine, methadone, morphine, nordazepam, noscapine metabolite, pregabalin, tetramisole	88	90/53	36.9	10		*Yes*		05:12	-
79	61	M	Crystal meth	Amphetamine, cotinine, domperidone, lorazepam, methamphetamine, zoplicone	109	153/101	37.9	15				16:27	-
80	41	M	Ethanol Unknown drug	Cocaine/benzoylecgonine, cotinine, ephylone, ethyl glucuronide, paracetamol, tetramisole	113	160/92	37.9	14		*Yes*	HTN	07:18	-
81	17	M	Cannabis Ethanol Unknown drug	Cotinine, MDMA, methiopropamine	127	148/48	36.5	14	*Yes*	*Yes*		16:02	-
82	26	M	Crystal meth	Amphetamine, cotinine, diazepam, methamphetamine, nordazepam, oxazepam, paracetamol	100	129/89	35.9	15		*Yes*	Chest pain Palpitations	18:31	-
83	51	M	Heroin Methadone Pregabalin **Spice**	Benzoylecgonine, clonazepam, cotinine, diazepam, ethyl glucuronide, methadone/EDDP, nordazepam, oxazepam, pregabalin, temazepam	80	129/92	35.4	15				12:16	-

Abbreviations: Δ^9-THC, (-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol; **BP**, blood pressure; **F**, female; **GCS**, Glasgow Coma Score; **GHB**, gamma-hydroxybutyric acid; **HR**, heart rate; **HRMS/MS**, high-resolution tandem mass spectrometry; **HTN**, hypertension; **LoS**, length of stay; **M**, male; **MDMA**, 3,4-methylenedioxymethamphetamine; **T**, body temperature; **THC-COOH**, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol.

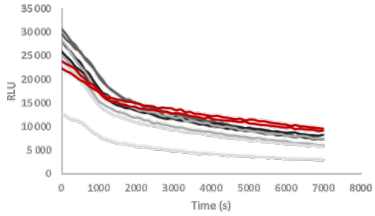
b) CB_1 bioassay read-out.

Δ^9-THC-containing samples picked up by the bioassay

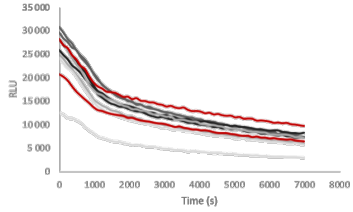
Sample 71



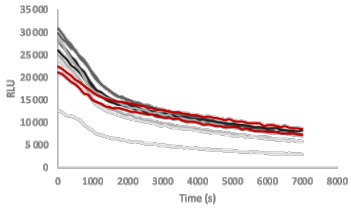
Sample 72



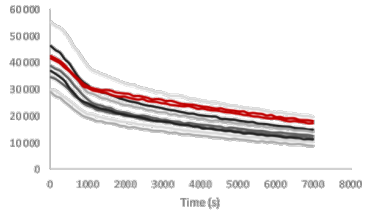
Sample 73



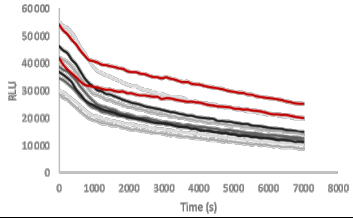
Sample 74



Sample 75

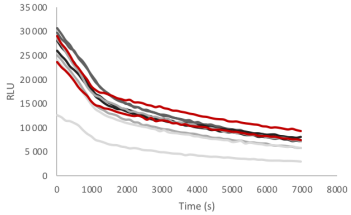


Sample 76

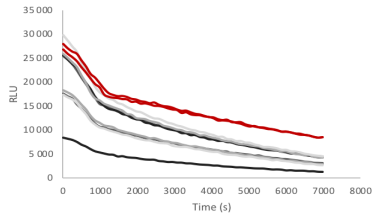


False positive samples

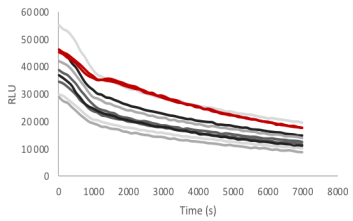
Sample 77*



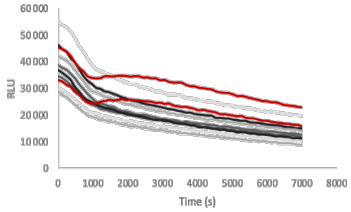
Sample 78



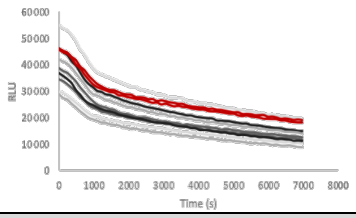
Sample 79



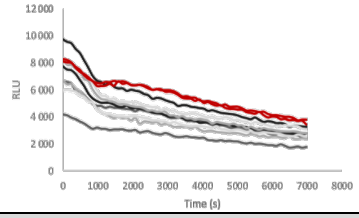
Sample 80



Sample 81

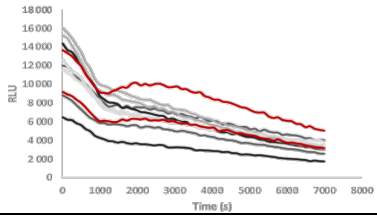


Sample 82



Self-reported "Spice" intake as supported by the bioassay read-out but not by bioanalysis

Sample 83

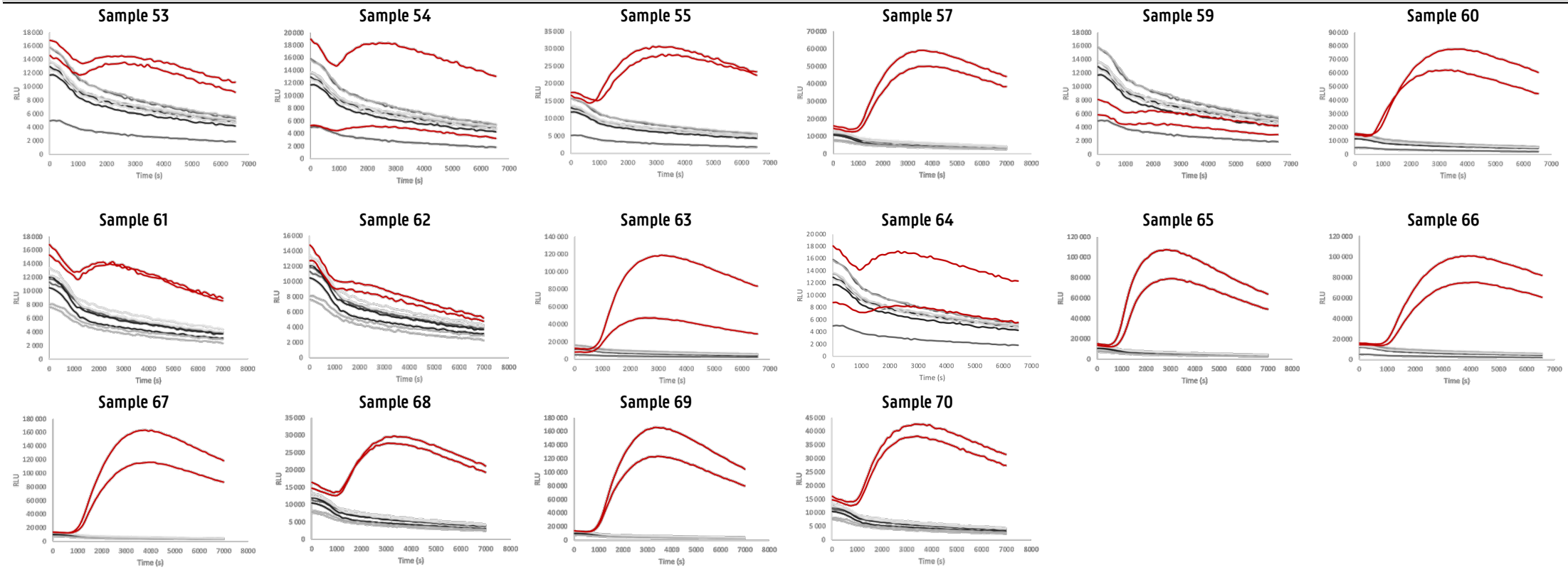


The profiles represent the raw data from the CB_1 bioassay. Samples for which 300 µL serum was used as a starting volume, are indicated with an asterisk (*). For all other samples, 500 µL serum was used. **Legend:** Red lines, serum samples (n = 2); grey and black lines, four independent blanks (n = 2). **Abbreviations:** RLU (y-axis), relative light units; s (x-axis), seconds; Δ^9-THC, (-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol.

8.4 APPENDIX IV: Bioassay with reduced sample volume (100 µL)

8.4.1 Appendix IV-A: Overview of all SCRA-positive profiles (100 µL)

CB_1-positive samples

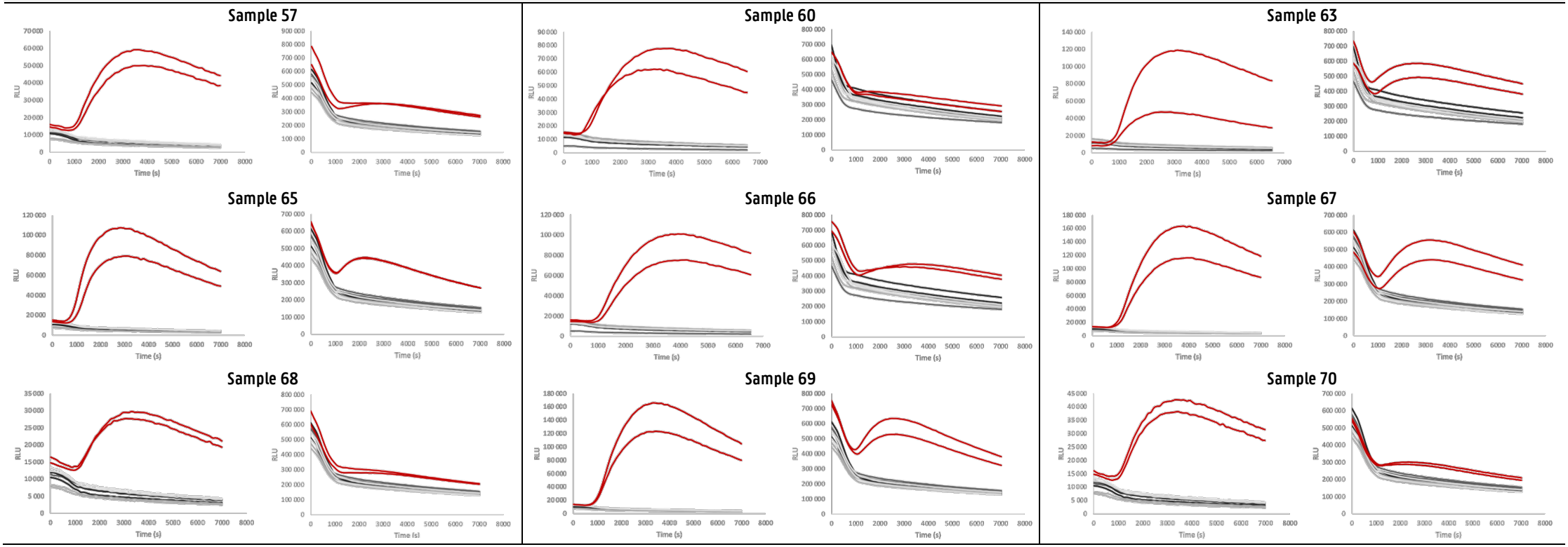


The profiles represent the raw data from the CB_1 bioassay. **Legend:** Red lines, serum samples (n = 2); **grey and black lines**, four independent blanks (n = 2). **Abbreviations:** **RLU (y-axis)**, relative light units; **s (x-axis)**, seconds.

8.4.2 Appendix IV-B: Combined CB_1/CB_2-positive profiles (100 µL)

Combined CB_1/CB_2-positive samples

CB_1	CB_2	CB_1	CB_2	CB_1	CB_2
Sample 57		Sample 60		Sample 63	
Sample 65		Sample 66		Sample 67	
Sample 68		Sample 69		Sample 70	



The profiles represent the raw data from the CB_1 and CB_2 bioassay. Legend: Red lines, serum samples (n = 2); grey and black lines, four independent blanks (n = 2). **Abbreviations:** RLU (y-axis), relative light units; s (x-axis), seconds.

8.4.3 Appendix IV-C: Data on the missed and false positive samples (100 µL)

a) HRMS/MS findings and clinical characteristics.

Patient information				HRMS/MS analysis			Clinical measurements				Clinical features				
Nr	**Age**	**M/F**	**Claimed use**	**Detected SCRA**	**Concentration (pg/mL)**	**Other findings**	**HR (bpm)**	**BP (mmHg)**	**T (°C)**	**GCS**	**Seizures**	**Agitation**	**Other**	**LoS (h:min)**	**Discharge**
56	35	M	GHB	MDMB-CHMICA-O-desmethyl **(MISSED)**	3376	Codeine, cotinine, diazepam, etifoxine, morphine, nordazepam, oxazepam, pregabalin, quetiapine, risperidone, temazepam	109	119/91	36.7	15				08:30	EMU
58	34	M	Cannabis Cocaine MDMA Spice	5F-ADB-O-desmethyl **(MISSED)** AMB-FUBINACA-O-desmethyl **(MISSED)**	853 3287	Benzoylecgonine, cotinine, ethyl-glucuronide, quinine, tetramisole	75	125/82	33.3	15	*Yes*			05:46	EMU
84	37	M	Cannabis Heroin Methadone			Cocaine/benzoylecgonine, codeine, cotinine, diazepam, gabapentin, methadone/EDDP, morphine, nordazepam, oxazepam, paracetamol, temazepam, tetramisole	96	106/74	37.1	15				04:43	ED (Self)
85	44	M	**Spice**			Cotinine	71	143/98	35.8	15				10:26	EMU

Abbreviations: **BP**, blood pressure; **EDDP**, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone; **F**, female; **GCS**, Glasgow Coma Score; **HR**, heart rate; **HRMS/MS**, high-resolution tandem mass spectrometry; **HTN**, hypertension; **LoS**, length of stay; **M**, male; **MDMA**, 3,4-methylenedioxymethamphetamine; **T**, body temperature.

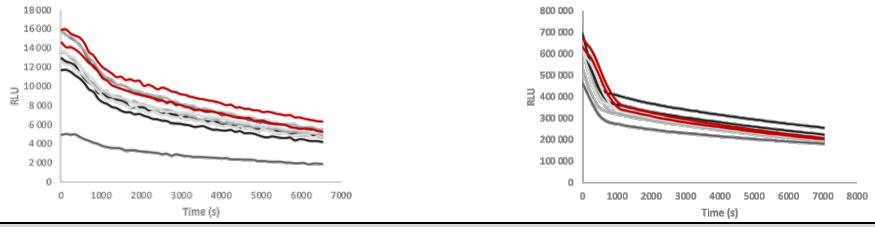
SCRAs: **5F-ADB**, methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate; **AMB-FUBINACA**, methyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate; **MDMB-CHMICA**, Methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate.

b) Bioassay read-out.

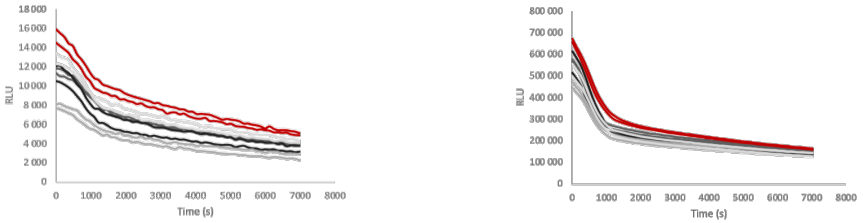
Missed samples

CB_1 **CB_2** **CB_1** **CB_2**

Sample 56

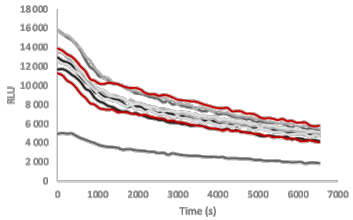


Sample 58



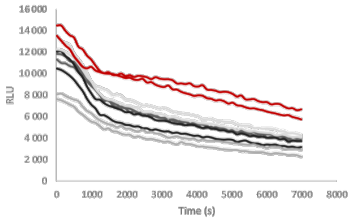
False positive sample

Sample 84



Self-reported "Spice" intake as supported by the bioassay read-out but not by bioanalysis

Sample 85



The profiles represent the raw data from the CB_1 (and CB_2) bioassay. Legend: **Red lines**, serum samples (n = 2); **grey and black lines**, four independent blanks (n = 2). **Abbreviations:** **RLU (y-axis)**, relative light units; **s (x-axis)**, seconds; **Δ^9-THC**, (-)-Δ^9-6a,10a-*trans*-tetrahydrocannabinol.

8.5 APPENDIX V: List of compounds detected in correctly scored negative samples.

Anabolic-androgenic steroids	
• Stanozolol • Trenbolone	
Antibiotics	
• Metronidazole • Trimethoprim	
Anti-epileptics	
• Carbamazepine • Gabapentin • Lamotrigine • Levetiracetam • Phenytoin • Pregabalin	
Antidepressants	
Selective serotonin reuptake inhibitors	**Others**
• Citalopram • Dapoxetine (treatment of premature ejaculation) • Fluoxetine • Paroxetine • Sertraline • Trazodone (antagonist and reuptake inhibitor) • Venlafaxine	• Amitriptyline (tricyclic antidepressant) • Mirtazapine
Antihistaminics	
• Cetirizine • Chlorpheniramine • Diphenhydramine • Hydroxyzine • Promethazine	
Antipsychotics	
• Aripiprazole • Flupenthixol • Haloperidol • Olanzapine	• Quetiapine • Risperidone • Zuclopenthixol
Nonsteroidal anti-inflammatory drugs	
• Benzydamine • Piroxicam	
Opioids/opiates	
• 6-monoacetylcodeine (6-MAC) • 6-monoacetylmorphine (6-MAM) • Acetylcodeine • Acetylmorphine • Buprenorphine (partial agonist) • Codeine • Dextromethorphan • Fentanyl	• Methadone/2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone (EDDP) • Morphine • Naloxone (antagonist) • Noscapine • Oripavine • Papaverine • Tramadol

List of compounds detected in correctly scored negative samples *(continued)*.

Phosphodiesterase-5 inhibitors

- Sildenafil
- Tadalafil
- Vardenafil

Sedatives/hypnotics

Benzodiazepines

- Alprazolam
- Clonazepam
- Chlordiazepoxide
- Demoxepam
- Diazepam
- Diclazepam
- Etizolam
- Flubromazepam
- Flurazepam
- Lorazepam
- Lormetazepam
- Midazolam
- Nordazepam
- Oxazepam
- Prazepam
- Temazepam

Z-products

- Zolpidem
- Zoplicone

Stimulants

Phenylethylamines

- 3,4-methylenedioxy-N-ethylamphetamine (MDEA)
- 3,4-methylenedioxymethamphetamine (MDMA)
- 4F-methylphenidate
- Amphetamine
- Dimethamphetamine
- Dimethoxychloroamphetamine
- Methamphetamine

Cathinones

- 3-methylmethcathinone (3-mephedrone)
- 4-chloro-methcathinone (clephedrone)
- 4-chloro-N-ethylcathinone
- 4-chloro-α-pyrrolidinopropiophenone (4-chloro-α-PPP)
- 4-methyl-ephedrone (mephedrone)
- 4-methyl-ethcathinone
- 4-methyl-N-ethyl-pentedrone
- 4-methyl-N,N-dimethylcathinone
- 4-methylpentedrone
- Dibutylone
- N-ethylpentylone (ephylone)
- α -methoxy-mephedrone (mexedrone)
- α-pyrrolidinovalerophenone (α-PVP)

Others

- Cocaine/benzoylecgonine
- Ephedrine (antihypotensive)
- Mazindol
- Modafinil

Others

- Baclofen (skeletal muscle relaxant)
- Chlorthenoxazine (analgesic)
- Clomiphene (selective oestrogen receptor modulator)
- Cotinine (nicotine metabolite)
- Diethyltoluamide (DEET)
- Domperidone (antiemetic, dopamine (D_2) antagonist)
- Ethyl glucuronide (ethanol metabolite)
- Etifoxine (anxiolytic, anticonvulsant)
- Gamma-hydroxybutyric acid (GHB) (depressant)
- Ketamine (dissociative, anaesthetic)
- Lidocaine (local anaesthetic)
- Lysergic acid diethylamide (LSD) (psychedelic)
- Minoxidil (potassium channel opener, vasodilator)
- Nevirapine (antiretroviral)
- Omeprazole (proton pump inhibitor)
- Paracetamol (analgesic, antipyretic)
- Procyclidine (anticholinergic)
- Quinine (component of tonic, antimalarial)
- Ranitidine (histamine (H_2) antagonist)
- Salbutamol (β_2-receptor agonist)
- Tetramisole (anthelmintic)

8.6 APPENDIX VI: List of SCRA concentrations and estimated normalised AUC values (≥ 300 µL)

Samples for which 300 µL serum was used as a starting volume, are indicated with an asterisk (*). For all other samples, 500 µL serum was used.

Nr	SCRA	Concentration (nM)	Potency corrected concentration (nM)	Total concentration (nM)	Normalised AUC
1	5F-ADB-O-desmethyl	3.735	6.331	6.331	0.1291
2	5F-ADB-O-desmethyl	6.984	11.84	13.77	0.09823
	5F-ADB-desfluorohydroxypentyl	1.140	1.932		
3	5F-ADB	1.024	655.4	18.01	0.2709
	5F-ADB-O-desmethyl	7.182	4424		
	5F-ADB-desfluorohydroxypentyl	2.424	1542		
4	5F-ADB-O-desmethyl	13.29	22.53	22.53	0.06745
5	5F-ADB-O-desmethyl	18.52	31.40	31.40	0.1172
6	5F-ADB	3.404	5.770	49.94	0.3783
	5F-ADB-O-desmethyl	19.17	32.49		
	5F-ADB-desfluorohydroxypentyl	6.890	11.68		
7	5F-ADB-O-desmethyl	32.61	55.27	59.10	0.3486
	5F-ADB-desfluorohydroxypentyl	2.264	3.837		
8	5F-ADB-O-desmethyl	37.12	62.91	64.54	0.4561
	5F-ADB-desfluorohydroxypentyl	0.9588	1.625		
9	5F-ADB	5.422	3469	80.17	0.3681
	5F-ADB-O-desmethyl	37.69	2322 x 10		
	5F-ADB-desfluorohydroxypentyl	4.190	2667		
11 *	Cumyl-5F-PINACA-desfluorohydroxypentyl	2.384	5.545	5.545	0.2401
12	Cumyl-5F-PINACA	3.647	3116	15.18	0.2631
	Cumyl-5F-PINACA-desfluorohydroxypentyl	2.882	2450		
13	Cumyl-5F-PINACA-desfluorohydroxypentyl	3.430	0.02182	3.430	0.2439
14	Cumyl-5F-PINACA	9.688	8279	30.61	1.167
*	Cumyl-5F-PINACA-desfluorohydroxypentyl	3.475	2953		
15	Cumyl-5F-PINACA	11.84	1012 x 10	32.79	1.353
	Cumyl-5F-PINACA-desfluorohydroxypentyl	2.262	1922		
16	Cumyl-5F-PINACA	20.22	1729 x 10	68.02	1.126
	Cumyl-5F-PINACA -desfluorohydroxypentyl	9.030	7674		
17	Cumyl-5F-PINACA	65.64	152.7	170.6	2.087
	Cumyl-5F-PINACA-desfluorohydroxypentyl	7.707	17.92		
18	MDMB-CHMICA-O-desmethyl	4.076	151.0	0.4076	0.03626
19	MDMB-CHMICA-O-desmethyl	19.62	1.962	1.962	0.6543
20	MDMB-CHMICA	10.34	397.6	6.584	0.2012
	MDMB-CHMICA-O-desmethyl	55.49	2056		
21	MDMB-CHMICA	10.09	1.009	4.389	0.007719
	MDMB-CHMICA-O-desmethyl	32.93	3.293		
	MDMB-CHMICA-hydroxy(indole)	0.8739	0.08739		
22	MDMB-CHMICA	8.348	0.8348	2.076	0.7592
	MDMB-CHMICA-O-desmethyl	12.42	1.242		
23	5F-ADB-O-desmethyl	3.186	5.401	6.930	0.05668
	AMB-CHMICA	0.2969	0.08483		
	AMB-CHMICA-O-desmethyl	5.055	1.444		
24	5F-ADB-O-desmethyl	8.447	14.32	17.60	0.04051
	AMB-CHMICA-O-desmethyl	10.73	3.066		
	MDMB-CHMICA	1.105	0.1105		
	MDMB-CHMICA-O-desmethyl	1.026	0.1026		
25	5F-ADB-O-desmethyl	14.83	25.13	27.42	0.1467
	5F-ADB-desfluorohydroxypentyl	1.326	2.248		
	MDMB-CHMICA-O-desmethyl	0.4534	0.04535		
26	5F-ADB-O-desmethyl	8.310	5119	29.76	0.06382
	MDMB-CHMICA	1.352	52.00		
	MDMB-CHMICA-O-desmethyl	155.4	5759		
27	5F-ADB-O-desmethyl	17.48	29.63	41.00	0.2887
	AMB-CHMICA-O-desmethyl	18.87	5.392		
	MDMB-CHMICA	2.029	0.2029		
	MDMB-CHMICA-O-desmethyl	57.80	5.780		
28	5F-ADB-O-desmethyl	0.8750	539.0	47.41	0.4144
	Cumyl-5F-PINACA	2.607	2228		
	Cumyl-5F-PINACA -desfluorohydroxypentyl	4.192	3563		
	MDMB-CHMICA	9.352	359.6		
	MDMB-CHMICA-O-desmethyl	291.8	1081 x 10		
29	5F-ADB-O-desmethyl	27.82	47.15	52.19	0.4021
	MDMB-CHMICA-O-desmethyl	50.35	5.035		

List of SCRA concentrations and estimated normalised AUC values (≥ 300 µL) *(continued)*.

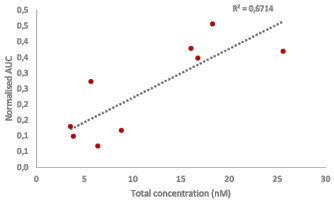
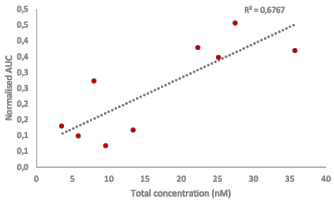
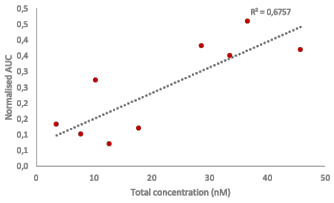
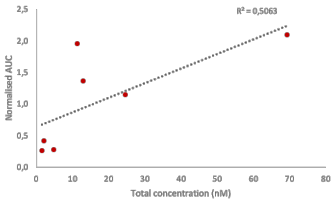
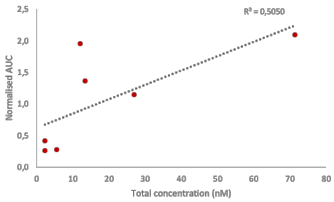
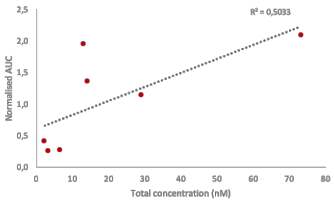
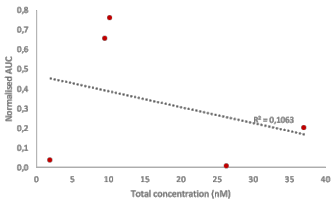
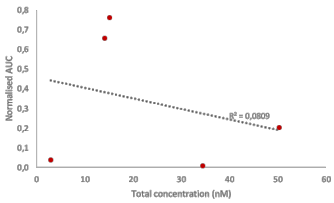
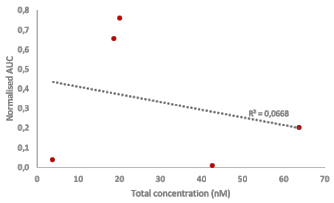
Nr	SCRA	Concentration (nM)	Potency corrected concentration (nM)	Total concentration (nM)	Normalised AUC
30	5F-ADB-O-desmethyl	24.07	40.79	54.11	0.2677
	5F-ADB-desfluorohydroxypentyl	7.5161	12.74		
	MDMB-CHMICA	2.302	0.2302		
	MDMB-CHMICA-O-desmethyl	3.468	0.3468		
31	5F-ADB-O-desmethyl	3.440	2119	59.59	0.9064
	5F-ADB-desfluorohydroxypentyl	1.776	1130		
	Cumyl-5F-PINACA	17.41	1488 x 10		
	Cumyl-5F-PINACA-desfluorohydroxypentyl	4.414	3752		
32	5F-ADB-O-desmethyl	64.00	108.5	125.6	0.3871
	AMB-CHMICA-O-desmethyl	42.97	12.28		
	MDMB-CHMICA	2.517	0.2517		
	MDMB-CHMICA-O-desmethyl	46.20	4.620		
33	5F-ADB-O-desmethyl	87.35	5381 x 10	171.6	1.533
	5F-ADB-desfluorohydroxypentyl	3.320	2113		
	Cumyl-5F-PINACA	4.055	3465		
	Cumyl-5F-PINACA-desfluorohydroxypentyl	3.648	3101		
34	5F-ADB-O-desmethyl	85.16	144.3	180.7	0.3165
	5F-ADB-desfluorohydroxypentyl	4.026	6.823		
	AB-CHMINACA	2.084	0.2672		
	AB-CHMINACA-O-desmethyl	89.76	11.51		
	AMB-CHMICA	3.031	0.8659		
	AMB-CHMICA-O-desmethyl	59.17	16.91		
35	5F-ADB-O-desmethyl	83.99	142.3	186.8	1.484
	5F-ADB-desfluorohydroxypentyl	6.948	11.78		
	AMB-CHMICA	17.34	4.955		
	AMB-CHMICA-O-desmethyl	97.08	27.74		
36	5F-ADB-O-desmethyl	107.1	181.6	189.2	0.9245
	5F-ADB-desfluorohydroxypentyl	3.149	5.338		
	MDMB-CHMICA	1.911	0.1912		
	MDMB-CHMICA-O-desmethyl	20.99	2.099		
37	5F-ADB	1.457	2.470	199.5	0.7166
	5F-ADB-O-desmethyl	111.1	188.3		
	MDMB-CHMICA	0.9232	0.09232		
	MDMB-CHMICA-O-desmethyl	86.78	8.678		
38	5F-ADB	3.206	5.433	230.6	1.273
	5F-ADB-O-desmethyl	119.2	202.1		
	5F-ADB-desfluorohydroxypentyl	3.867	6.555		
	AMB-CHMICA	42.56	12.16		
	AMB-CHMICA-O-desmethyl	15.25	4.357		
39	5F-ADB	21.79	36.93	263.9	0.5405
	5F-ADB-O-desmethyl	79.09	134.1		
	5F-ADB-desfluorohydroxypentyl	11.77	19.95		
	AMB-CHMICA	51.39	14.68		
	AMB-CHMICA-O-desmethyl	203.8	58.24		
40	5F-ADB	6.067	10.28	315.2	1.783
	5F-ADB-O-desmethyl	164.5	278.9		
	5F-ADB-desfluorohydroxypentyl	9.934	16.84		
	AMB-CHMICA	4.858	1.388		
	AMB-CHMICA-O-desmethyl	27.46	7.847		
41	5F-ADB-O-desmethyl	191.2	324.1	336.6	1.035
	5F-ADB-desfluorohydroxypentyl	5.806	9.841		
	MDMB-CHMICA	1.287	0.1287		
	MDMB-CHMICA-O-desmethyl	25.62	2.562		
42	5F-ADB-O-desmethyl	253.8	430.1	484.1	1.225
	5F-ADB-desfluorohydroxypentyl	17.92	30.38		
	AMB-CHMICA	2.672	0.7635		
	AMB-CHMICA-O-desmethyl	80.09	22.88		
43	5F-ADB-O-desmethyl	333.6	565.4	657.5	1.175
	5F-ADB-desfluorohydroxypentyl	17.62	29.87		
	AMB-CHMICA	6.564	1.876		
	AMB-CHMICA-O-desmethyl	211.5	60.44		
44 *	5F-ADB-O-desmethyl	400.5	678.8	695.5	1.502
	5F-ADB-desfluorohydroxypentyl	5.074	8.600		
	MDMB-CHMICA	4.135	0.4135		
	MDMB-CHMICA-O-desmethyl	76.62	7.662		
45	5F-ADB-O-desmethyl	527.1	893.3	952.2	1.628
	5F-ADB-desfluorohydroxypentyl	20.35	34.49		
	AMB-CHMICA	2.537	0.7249		
	AMB-CHMICA-O-desmethyl	74.96	21.42		
	MDMB-CHMICA	1.664	0.1664		
	MDMB-CHMICA-O-desmethyl	20.30	2.030		

SCRAs: **5F-ADB**, methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate; **5F-AKB-48**, N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide; **AB-CHMINACA**, N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide; **AMB-FUBINACA**, methyl (1-(4-fluorobenzyl)-1H-indazole-3-carbonyl)-valinate; **AMB-CHMICA**, methyl (1-(cyclohexylmethyl)-1H-indole-3-carbonyl)-valinate; **BB-22**, quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate; **Cumyl-5F-PINACA**, 1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide; **MDMB-CHMICA**, Methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate.

8.7 APPENDIX VII: Different AUC-concentration plots evaluating an arbitrary potency of the SCRA metabolites.

Calculation example for 50% metabolite potency:

Total SCRA concentration = 0.50*(concentration M1) + 0.50*(concentration M2) + (concentration PC)
(M1-2, metabolites 1-2; PC, parent compound)

50%	75%	100%
5F-ADB		
		
R^2 = 0.6714	R^2 = 0.6767	R^2 = 0.6757
Cumyl-5F-PINACA		
		
R^2 = 0.5063	R^2 = 0.5050	R^2 = 0.5033
MDMB-CHMICA		
		
R^2 = 0.1063	R^2 = 0.0809	R^2 = 0.0668

SCRAs: **5F-ADB**, methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate; **Cumyl-5F-PINACA**, 1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide; **MDMB-CHMICA**, Methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate.

8.8 APPENDIX VIII: Poster Research Day & Student Research Symposium

GHENT UNIVERSITY

It doesn't matter what you look like, it's what you do that counts:
Activity-based Bioassays for the Detection of Synthetic Cannabinoid Receptor Agonists in Serum

FACULTY OF PHARMACEUTICAL SCIENCES

Marthe Vandeputte, Annelies Cannaert, Christophe P. Stove (Christophe.Stove@UGent.be)
Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University, Belgium

Introduction

Synthetic cannabinoid receptor agonists (SCRAs) are the largest group of compounds currently monitored in Europe by the EU Early Warning System on new psychoactive substances. Their rapid rate of emergence and chemical diversity make detection and monitoring of this group of compounds particularly challenging. Current **structure-based approaches** (immunoassays and mass spectrometry-based methods) are flawed by e.g. lack of cross-reactivity and a required prior knowledge about their structure. Also, **sensitivity** is an important aspect as the high potency of many SCRAs results in concentrations in the low- to sub-ng/mL range in biological matrices. In this study, we evaluated the applicability of recently developed **activity-based bioassays** to serve as a screening tool for the detection of SCRAs in a large set (n = 396) of serum samples collected from drug-related cases. Bioassay results and bioanalytical results were compared.

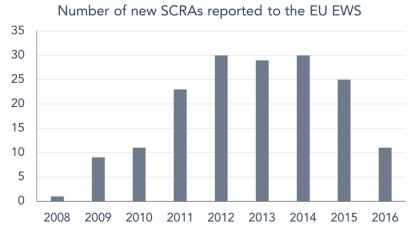
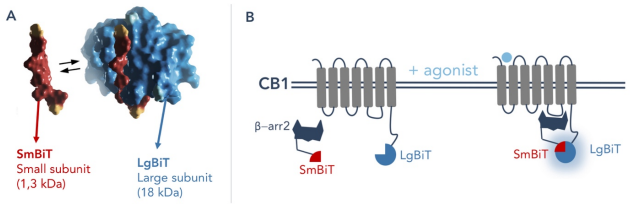


Figure 1. Number of new SCRAs reported to the EU Early Warning System.

Bioassay

Figure 2. (A) The two subunits of the Nanoluciferase. (B) Principle of the cannabinoid bioassay.



The developed bio-assays are based on the **NanoBiT® Technology (Promega)**, which uses structural complementation of Nanoluciferase (NLuc) to detect protein-protein interactions (Fig. 2A). Here, two inactive **NLuc subunits (SmBiT/LgBiT)** are fused to either the cannabinoid receptors (CB1/CB2) or the β-arrestin 2 (β-arr2) protein. Activation of the receptor (in this case, by one or more SCRAs present in serum extracts) results in recruitment of β-arr2, resulting in **structural complementation** of NLuc and restoration of its activity. Following addition of a luciferase substrate, a **bioluminescent signal** is elicited that can be easily monitored (Fig. 2B).

Protocol

BIOASSAY

Seed HEK293T cells stably transduced with the cannabinoid reporter system.

↓ O/N incubation

- Wash cells (2x) with OptiMEM I
- Add 100 µL OptiMEM I
- Add 25 µL of luciferase substrate

↓ 10 min equilibration

- Add 10 µL of extract

↓ + 4 independent blanks
+ 1 positive control (50 ng/mL JWH-018)

SAMPLE PREP

0.5 mL serum
+ 0.5 mL carbonate buffer (pH 10)
+ 4 mL n-hexane/ethyl acetate (99:1 V/V)

↓ Evaporate organic layer

↓ Reconstitute in 100 µL MeOH/OptiMEM I (50:50 V/V)

2h read-out on TriStar² luminometer (Berthold Technologies)

Results

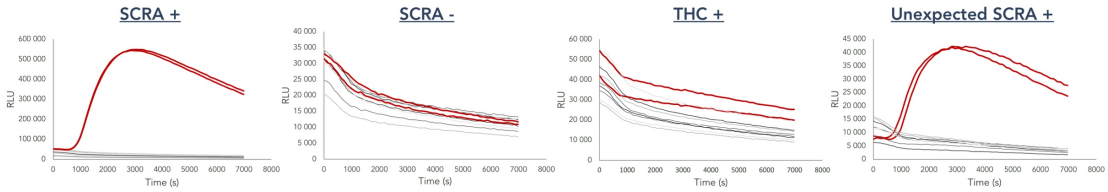


Figure 3. Bioluminescence obtained from the CB_1 bioassay of four different serum samples. Abbreviations: THC = 11-nor-9-carboxy-tetrahydrocannabinol; RLU = relative light units; s = seconds. Legend: grey and black lines = 4 independent blanks (n = 2); red lines = serum samples (n = 2).

Hospital lab results			Bioassay results	
Claimed use +	Bioanalysis +	43		43 +
Claimed use -	Bioanalysis +	9		9 +
Claimed use -	Bioanalysis -	332	320 -	12 +
Claimed use +	Bioanalysis -	11	10 -	1+
Total		395	330 -	65 +

→ **Sensitivity 100%** (52/52)

→ Including **6 THC positives** → **Specificity 97.9%** (330/337)

→ Further **in-depth analytical investigation required**

Conclusion

The **applicability and added value** of the proposed bioassays for the **activity-based detection of SCRAs in serum** was convincingly demonstrated. The bioassays have the potential to be applied in the setting of **high-throughput screening** of suspect samples, offering a promising solution to fill the void left by conventional screening methods.

8.9 APPENDIX IX: Accepted abstract TIAFT2018 (oral presentation)

It doesn't matter what you look like, it's what you do that counts:

Activity-based bioassays for the detection of synthetic cannabinoid receptor agonists in serum.

Marthe Vandeputte[1], Annelies Cannaert[1,2], Simon Hudson[3], Joanna C. White[4], Matthew Bundell[4], John R. Archer[4,5], David M. Wood[4,5], Paul I. Dargan[4,5], Christophe P. Stove[1]

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[2] Laboratory of Toxicology, National Institute of Criminalistics and Criminology, Brussels, Belgium.

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[4] Clinical Toxicology, Guy's and St Thomas' NHS Foundation Trust, London, UK.

[5] Faculty of Life Sciences and Medicine, King's College London, London, UK.

Introduction: Synthetic cannabinoid receptor agonists (SCRAs) are the largest group of new psychoactive substances worldwide. Their rapid emergence and chemical diversity seriously hamper detection. Current approaches for detection of these substances (typically only present at low ng/mL concentrations in biological matrices) are based on targeted, structure-based methods, such as immunoassays or mass spectrometry-based methods. However, both these approaches have important limitations (e.g. lack of cross-reactivity and prior knowledge of structure required).

Aim: By application on a large set of serum samples (n = 471) from cases with acute drug toxicity, we aimed at evaluating a new activity-based screening concept (bioassay) as an alternative to conventional structure-based SCRA screening approaches.

Methods: In the bioassay, 10 µL serum extract is applied onto stable cell lines, in which activation of a cannabinoid receptor (CB1 or CB2), fused to one part of luciferase, leads to recruitment of β-arrestin 2, fused to the other part. The resulting functional complementation leads to measurable bioluminescence. The extracts are prepared by an alkaline liquid-liquid extraction of 0.1 to 0.5 mL serum with 0.5 mL carbonate buffer (pH 10) and 4 mL n-hexane/ethylacetate (99:1 v/v). The supernatant is evaporated and reconstituted in 100 µL Opti-MEM/methanol (50:50 v/v). The outcome of the bioassays is compared to the results of the 'gold standard' high resolution accurate mass (HRAM) analysis.

Results: When starting from 300 or 500 µL serum (n = 395), all 52 analytically confirmed SCRA-positive samples were picked up by the bioassays, yielding a sensitivity of 100% (52/52). Of the 343 SCRA-negative samples (via HRAM), 330 were correctly scored negative, whereas 13 scored positive in the bioassay. 6/13 could be explained by the presence of THC, eventually yielding 7 'false positives' and a specificity of 97.9% (330/337). Application on another 76 samples with a starting volume of only 100 µL resulted in a sensitivity and specificity of respectively 88.9% (16/18) and 96.6% (56/58). As in a total of 2 'false positives' there was a patient history of SCRA use -though not analytically confirmed- specificity is even likely higher. Further in-depth analytical investigation is required to explain the cannabinoid activity found in these samples.

Conclusion: Here, we have convincingly demonstrated the potential of bioassay-based screening for SCRAs via the successful application on a large set of authentic serum samples, containing a variety of SCRAs. This approach offers the opportunity to serve as an alternative first-line screening tool for the presence of SCRAs in biological matrices.

8.10 APPENDIX X: Summaries of the lecture series "Internationalisation @ Home"

8.10.1 Appendix X-A: The pharmacist is a key stakeholder in measuring and managing patients' adherence to medications (Dr. Bernard Vrijens)

"Drugs don't work in patients who don't take them." (C. Everett Koop, 1985). Dr. Bernard Vrijens, much-celebrated author and General Manager-Chief Science Officer at the AARDEX Group, was invited to our faculty to explain the ongoing relevance of this statement and the pharmacist's crucial role in this context.

Poor medication adherence remains a significant, although often neglected problem that may result in suboptimal treatment outcomes and high added health care costs. Nonadherence occurs when a patient fails to *initiate*, *implement* and/or *persist* the prescribed medication regimen. Depending on the level at which the patient fails to adhere, different forms of nonadherence can be described with different consequences. However, combined with the observation that compliance behaviour can change over time, it becomes very difficult to classify a patient as (non)adherent. When plotting various important outcomes through the course of drug development, a palpable "adherence gap" can be observed. Regulators focus mostly on efficacy, while, in the end, patients are interested in real-life effectiveness. During clinical trials a gradual decline in adherence occurs, but since the medication compliance drops to even lower levels in real-life, our estimate of efficacy lies somewhere in between intrinsic efficacy and actual effectiveness. Potential consequences of this gap are a poor estimation of toxicity, inappropriate dosing regimens and treatment failure due to lack of effectiveness. An important aspect when considering the consequences of nonadherence, is '*drug forgiveness*', or the amount of implementation necessary to achieve optimal treatment outcome (e.g. once versus twice daily intake of NOACs). It should thus be clear that potential nonadherence remains a scarcely addressed issue both during development as in clinical practice. Disruptive innovations (big data analysis) will increasingly uncover this problem. Prediction of adherence is hampered by the myriad of associated factors and the biggest bottleneck continues to lie with the physician. Electronic prescriptions, refill databases and electronic monitoring are probably the most important tools to monitor and enhance medication adherence, and a key role is played by pharmacists. However, the "golden solution" to prevent every type of nonadherence does not exist: the whole system needs change (e.g. "six sigma" approach). Patient-tailored and measurement-guided intervention will be required in order to retain our key role as both developers and pharmacists above artificial intelligence.

8.10.2 Appendix X-B: The uses of Hippocratic epidemiology (Professor Albert Hofman)

In the light of the Research Day & Student Research Symposium 2018, our faculty had the honour of welcoming Professor Albert Hofman, Chair of the Department of Clinical Epidemiology at the Harvard T.H. Chan School of Public Health and Clinical Epidemiology.

Hippocratic epidemiology uses diagnoses to paint bigger pictures, while etiology shines a light on the underlying causes of a disease. Together with prognosis and treatment, i.e. our intervention, these two aspects are the main questions covered by Hippocratic epidemiology, thereby bridging clinical and community medicine. After setting the scene as such, Dr. Hofman brought his audience back to 1662, when John Graunt, now considered as one of the first experts in epidemiology, published the "Bills of Mortality". Looking back at life expectancy (i.e. the *bigger picture*) throughout the years, Graunt lived in a world where the average life expectancy was approximately 35 years old. Around 1800, a linear increase can be observed up until recent years – today, in the record country, people reach the age of 90 years on average. However, at the beginning of the 20th century for example, the influenza epidemic was the cause of many deaths. This shows the "plasticity" of life expectancy and, if we detect a decline on time, our possibility to *intervene*. Going back to the four big questions in epidemiology, the *cause* of a disease is often difficult to pinpoint (e.g. why, at the age of 95, 50% are diagnosed with dementia?). Diseases are – contrary to popular belief - not caused by aging, but mostly genetics, environmental factors and/or the increasingly understood interaction between both give rise to a disease. Cohort studies and many new technologies can be applied to study this. Apart from family history, epidemiologic observation and molecular family studies, the added value of for example hypothesis-free genome wide association studies, cannot be underestimated (e.g. new insights in the role of genes and disease pathways in Alzheimer's disease). Non-genetic factors, including traumatic, endocrine, inflammatory and vascular factors, may also play a role. This brings the epidemiologist to the following question: what can *we* do about it? How can we *intervene* to prevent chronic disease? Epidemiology indeed holds enormous potential to positively influence public health and clinical medicine: over the years, clear strides have been made and while the effect occurs slowly, it is undeniably there.

8.10.3 Appendix X-C: Assessment of medication nonadherence – a UK perspective (Dr. Sangeeta Tanna)

Dr. Sangeeta Tanna was invited to our faculty to stress the importance of medication adherence and provide us with an interesting talk about different methods to assess this widely underestimated problem.

Medication nonadherence remains an incredibly frustrating problem for all health care workers. Once the administration of a medicine is in hands of the patient himself, unfortunately, nonadherence becomes a very pertinent and complex issue. The World Health Organisation even goes as far as stating more benefit would come from improving adherence than from developing new medicines. In sharp contrast with this, is the UK's National Health Service's (NHS) lack of funding for methods assessing medication adherence for disease areas other than bipolar disorders. However, the consequences of nonadherence are very serious, ranging from medicine wastage, increased healthcare cost and, ultimately, patients dying despite the availability of often very efficacious medicines. In the UK, for example, hospitals have their funding stopped for a certain period of time when patients are re-admitted within thirty days due to poor- or nonadherence. This example stresses the importance of both the pharmacist's and clinician's role in supporting the patient to be compliant, and the availability of methods assessing the patient's level of (non)adherence. Many indirect methods such as pill counts, pharmacy records, patient diaries and electronic monitoring are available and actively used in a wide range of countries across the globe. However, both the advantages and limitations of each of these methods should be kept in mind. Other, more objective methods approach the problem from a 'pharmacokinetics' point of view: medication adherence is assessed by directly monitoring drug levels in the blood or other biosamples. This is done through, for example, liquid blood samples taken from the patient in the clinic, or, more interestingly, via microsampling (e.g. dried blood spots). Analytical methods following such microsampling should then, of course, have both satisfactory sensitivity and specificity. Such instrumental analytical methods include immunoassays, chromatography-based methods and polymerase chain reactions (i.e. for diseases such as malaria and HIV/aids, where the assay measures the level of drug target instead of the drug itself). Such objective information about (non)adherence is invaluable in personalising pharmacotherapy and thus providing optimised care for each patient. In the UK, health care providers are burdened with the difficult task of implementing the described methods in an environment where such testing is not yet supported by the NHS, while having proved to be of utmost importance to make evidence-based clinical decisions.

Master dissertation submitted to the faculty of Pharmaceutical Sciences, performed in collaboration with the Laboratory of Toxicology.

Promoter: Prof. Dr. C. Stove

Commissioners: Dr. M. De Boevre and Dr. K. Van Uytfanghe

The information, conclusions and points of view in this master dissertation are those of the author and do not necessarily represent the opinion of the promoter or his/her research group.