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OPTIMIZACIJA STRUKTURNO ODVISNIH PARAMETROV PRI SKLOPLJENI MASNI SPEKTROMETRIJI ZA IZBRANE SINTEZNE KANABINOIDE TER NJIHOVA MODULACIJA IONSKIH KANALOV

OPTIMISATION OF COMPOUND-DEPENDENT PARAMETERS IN THE TANDEM MASS SPECTROMETRY FOR SELECTED SYNTHETIC CANNABINOIDS AND THEIR MODULATION OF ION CHANNELS

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All research for my master thesis was conducted at the research unit Toxicology and food chemistry at the Katholieke Universiteit Leuven in Belgium, headed by Prof. Dr. Apr. Jan Tytgat.

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Statement

I hereby declare that my master thesis was written independently by me under the supervision of Assoc. Prof. Dr. Lucija Peterlin Mašič and co-supervision of Prof. Dr. Apr. Jan Tytgat.

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I ABSTRACT

Synthetic cannabinoids are easily obtainable and legal, but untraceable with routine screening tests. After discovery they quickly became widely spread because of the effects very similar to marijuana - they act on the same pharmacological targets (cannabinoid receptors CB_1 and CB_2). Their mechanism of action could also be explained by their effect on cation channels in neurons. Synthetic cannabinoids therefore present an interesting direction for further research on novel analgesics and antispasmodics.

The aim of our work was to optimise compound-dependent parameters in tandem mass spectrometry for eight selected synthetic cannabinoids. In addition we tested whether these compounds modulate the voltage of cation channels.

Methanolic solutions of cannabinoids were introduced by direct infusion into the ion source of a triple quadrupole instrument. All parameters were successfully defined. For each compound we determined the four most abundant MRM transitions, which enables us the development of a targeted detection method for forensic samples.

The users of synthetic cannabinoid mixtures have reported unintended clinical effects on the cardiovascular (tachycardia, hypertension) and central nervous systems (panic attacks, psychosis, anxiety, nausea). In the second part of this work we tested the effect of these synthetic cannabinoids on the TRPV1 channel, known to be involved in nociception. Peripheral mechanisms of cannabinoids on nociceptive neurons appear to be independent from the modulation of cannabinoid receptors, because their level of expression in this region is very low. Therefore, we assumed that the mechanism of action could be associated with the modulation of TRPV1. Selectivity on a particular channel is crucial for the development of new therapeutics, so the compounds were additionally tested against voltage-gated sodium (subtypes Na_v 1.2, 1.3, 1.5) and potassium channels (subtypes K_v 1.1, 1.3, hERG) that show high expression in the brain and the heart. The most frequently reported adverse effects of synthetic cannabinoid mixtures are correlated particularly with these two body systems. cRNA of these channels was injected into stage V-VI oocytes isolated from *Xenopus laevis*. The electrophysiological behavior of voltage-gated ion channels and TRPV1 was monitored using the two-microelectrode voltage clamp technique. No effect was observed on the listed channel subtypes, including hERG; otherwise, the tested synthetic cannabinoids could potentially have fatal effects on the

cardiovascular system. The results of testing TRPV1 modulation are preliminary as the internship ended before the research was complete.

Keywords:

Synthetic cannabinoids, TRPV1, MS/MS, sodium channels, potassium channels

II POVZETEK

Sintezni kanabinoidi so lahko dostopni, legalni, vendar neizsledljivi z rutinskimi presejalnimi testi. Po odkritju so postali hitro razširjeni zaradi učinkov, zelo podobnih marihuani, saj prav tako izkazujejo delovanje na kanabinoidna receptorja CB_1 ter CB_2. Poleg tega pa bi lahko njihovo delovanje razložili z delovanjem na kationske kanalčke v nevronih, kar bi bila zanimiva iztočnica za nadaljnje raziskave novih skupin analgetikov ali spazmolitikov.

Namen našega dela je bila optimizacija strukturno odvisnih parametrov pri sklopljeni masni spektrometriji za rutinsko določanje osmih sinteznih kanabinoidov ter v nadaljevanju ugotoviti ali te snovi lahko modulirajo napetost kationskih kanalčkov.

Metanolne raztopine kanabinoidov smo direktno vbrizgali v ionizacijski izvor trojnega kvadrupol spektrofotometra. Vse parametre smo uspešno opredelili, pravtako pa smo za vsako spojino določiti štiri najbolj značilne masne fragmente, kar nam omogoča specifično detekcijo teh sinteznih kanabinoidov v forenzičnih vzorcih.

Uporabniki zeliščnih mešanic, ki vsebujejo sintezne kanabinoide, so poročali o številnih neželenih učinkih na kardiovaskularni (tahikardija, hipertenzija) in centralni živčni sistem (napadi panike, psihoze, anksioznost, slabost) kar bi lahko bilo povezano z delovanjem na kationske kanalčke. V drugem delu smo zato testirali aktivnost teh spojin na kanalu TRPV1, ki je vpleten v nocicepcijo. Periferni mehanizmi na nociceptivnih nevronih, v katere so vpleteni kanabinoidi, verjetno niso odvisni od delovanja na kanabinoidne receptorje, saj je ekspresija le-teh v tem predelu zelo nizka. Zaradi tega predpostavljamo, da je mehanizem lahko povezan z modulacijo TRPV1. Zaradi selektivnosti, ki je ključnega pomena pri razvoju novih zdravilnih učinkovin, smo spojine dodatno testirali na napetostno odvisnih natrijevih (podtipi Na_v 1.2, 1.3, 1.5) in kalijevih kanalih (podtipi K_v 1,1, 1,3, hERG), ki izkazujejo visoko ekspresijo v možganih in srcu. Najpogosteje poročani neželeni učinki po uporabi sinteznih kanabinoidov so namreč povezani prav s tema dvema telesnima sistemoma. cDNK navedenih kanalov smo injecirali v jajčne celice v fazi V-VI, izolirane iz *Xenopus laevis*. Elektrofiziološko obnašanje napetostno odvisnih ionskih kanalov in kanala TRPV1 smo spremljali z metodo vpete napetosti z dvema mikroelektrodama. Na nobenem od kanalov ni bilo dokazanega učinka. Noben testiran sintezni kanabinoid se ne veže na hERG kanale, kar bi lahko privedlo do usodnih učinkov

na srčno-žilni sistem. Rezultati testiranja modulacije kanala TRPV1 so preliminarni, saj se je moje raziskovalno delo zaključilo pred zaključkom raziskave.

Ključne besede:

Sintezni kanabinoidi, TRPV1, MS/MS, natrijevi kanali, kalijevi kanali

III LIST OF ABBREVIATIONS

AM-251 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide

AM-356 (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-[(1*R*)-2-Hydroxy-1-methylethyl]icosa-5,8,11,14-tetraenamide

AM-2201 1-[(5-Fluoropentyl)-1*H*-indol-3-yl]-(naphthalen-1-yl)methanone

CAD Collisionally activated dissociation

CAP Capsaicin

CB_1 Cannabinoid receptor I

CB_2 Cannabinoid receptor II

CE Collision energy

CEP Collision cell entrance potential

CP 55,940 2-[(1R,2R,5R)-5-Hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-5-(2-methyloctan-2-yl)phenol

CPZ Capsazepin

cRNA Complementary ribonucleic acid

CNS Central nervous system

CXP Collision cell exit potential

DP Declustering potential

DMSO Dimethyl sulfoxide

EP Entrance potential

ESI Electrospray ionisation

HEPES 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid

hERG Human ether-á-go-go related gene K^+ channel (K_v 11.1)

JWH-018 Naphthalen-1-yl-(1-pentylindol-3-yl)methanone

JWH-018*	Naphthalen-1-yl-(1-pentylindol-3-yl)methanone with additional methyl group on naphthyl ring
K_i	Inhibition constant
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NPS	Novel psychoactive substances
SC	Synthetic cannabinoid
TEVC	Two-microelectrode voltage-clamp technique
THC	Δ^9-Tetrahydrocannabinol
TRP(V)	Transient receptor potential (vanilloid) cation channel
TTX	Tetrodotoxin
VGIC	Voltage-gated ion channel
VGPC	Voltage-gated potassium channel
VGSC	Voltage-gated sodium channel
WIN 51,708	17-β-Hydroxy-17-α-ethynyl-5-α-androstano(3,2-b)pyrimido(1,2-a)benzimidazole
WIN 55,212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone; mesylate salt
WIN 62,577	17-β-Hydroxy-17-α-ethynyl-δ-4-androstano[3,2-b]pyrimido[1,2-a]benzimidazole

1 INTRODUCTION

1.1 NOVEL PSYCHOACTIVE SUBSTANCES

Novel psychoactive substances (NPS) range from natural plant-originated substances to semisynthetic and synthetic compounds, and possess psychoactive properties. They are also known as »legal highs«, »smarts«, »herbal highs« or »boosters«. The legal restrictions on their production, sale and possession are limited. The Internet with its online shops seems to be a global marketplace for selling NPS in many countries. The majority of the online shops sell »legal highs«, advertised as »air fresheners«, »herbal incenses«, »bath salts«, »plant fertilizers«, »collectibles« or »chemical reagents«. (1) Nowadays, the majority of early NPS have been legally restricted; however, entrepreneurs are synthesizing related substitutes on a daily basis. These substances are introduced as legal alternatives to popular but illicit recreational drugs and are designed to mimic their effects by slightly altering their chemical structure. (2) Usually no safety information or list of ingredients is provided.

NPS are classified into several groups: synthetic cannabinoids (SCs), tryptamines, phenethylamines, piperazines and cathinones. Between 2005 and 2011, 164 NPS were formally recognized through the Early Warning System established by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Synthetic cannabinoids and synthetic cathinones represent two thirds of the NPS discovered between 2005 and 2011. The group of synthetic cannabinoids was the largest out of the five different groups monitored. (2,3)

As most of these compounds have been developed/or discovered recently, there are only a limited number of reliable data on their pharmacology, toxicology and short-/long-term effects. Some synthetically produced cannabinoids have shown up to 100-times stronger binding affinity to cannabinoid receptors than natural THC, which may lead to unexpected hazardous or even lethal effects. (4) In this work, the focus will be on the group of synthetic cannabinoids (SCs).

1.1.1 Cannabinoids

1.1.1.1 History

Hemp or marijuana (*lat. Cannabis sativa, Cannabis indica and Cannabis ruderalis*) has been used by various societies for millennia for its euphoria-like effects. Besides recrea-

tional ends, it also has been used in (traditional) medicine. For example, its benefits against malaria and rheumatism were first mentioned by the Chinese emperor Sehn-Nung in 2737 BC. (5) Moreover, marihuana has been listed in the United States Pharmacopeia from 1850 until 1942. In general, it has been prescribed for clinical conditions like rheumatism, labor pains and nausea. (6) THC was first isolated in 1964, followed by the discovery of the CB_1 and CB_2 receptors in 1980s. (7,8) Today marijuana is one of the most frequently used illicit drugs.

Cannabinoids are a group of 66 compounds, chemically defined as C_{21} terpenophenols and originally extracted from hemp. In the 1990s, Dr. Huffman and his team isolated a large number of SCs with agonistic activity on the cannabinoid receptors, which have become known as the "JWH" compounds[1]. (9) Because of the cannabis-type euphoria, these compounds quickly became appreciated. Since 2004 they appeared for sale on the Internet and in smart shops as herbal blends/mixtures from exotic plant components, marketed under the name »Spice«. (10) By early 2009, »Spice« products have been reported in 21 European countries. (1)

Synthetic cannabinoids have also been studied for therapeutic purposes in patients with cancer, long-lasting chronic neuropathic pain, painful spasticity and related conditions because of their antiemetic, analgesic, appetite modulating and anti-glaucoma effects. (6,11)

1.1.1.2 Synthetic cannabinoids

SCs are lipid-soluble, non-polar and volatile. Most frequently, they are ingested orally, inhaled or smoked. (1) The contents of SC products vary in potency[2], purity and the number of other drugs and substances added, which increases the risk of accidental overdose. There have been several reported unintended clinical effects after the use of SC mixtures:

- central nervous system: agitation, panic attacks, loss of consciousness, anxiety, psychosis, paranoia, confusion, spasms, irritation, suicide attempts, changes in mood and perception (hallucinations, depression)
- cardiovascular system: tachycardia, hypertension, chest pain, cardiac ischemia
- metabolism: hyperglycemia, hypokalemia, acidosis

[1] The designation JWH refers to the initials of Dr. Huffman.

[2] The potency of HU-210 is 100 times the potency of THC. (12)

- gastrointestinal system: nausea, vomiting
- autonomic nervous system: fever, mydriasis (12,13)

SCs found in »Spice« and similar products (e.g. »Chill zone«, »Dream«, »K2«, »Fusion«, »Sensation«, »Magic Silver«, »Skunk«, »Scence« etc.) constitute a large family of chemically unrelated structures, that can be classified in 7 groups (see Figure 1):

(1) Naphthoylindoles (e.g. JWH-018, JWH-073, JWH-81, JWH-2201)

(2) Naphthylmethylindoles (JWH-185, JWH-199)

(3) Naphthoylpyrroles (JWH-369, JWH-370)

(4) Naphthylmethylindenes (JWH-176)

(5) Phenylacetylindoles (e.g. JWH-250, RCS-4)

(6) Cyclohexylphenols (CP 47,497 and its homologues) and

(7) Classical cannabinoids (e.g. HU-210 and nabilone). (14)

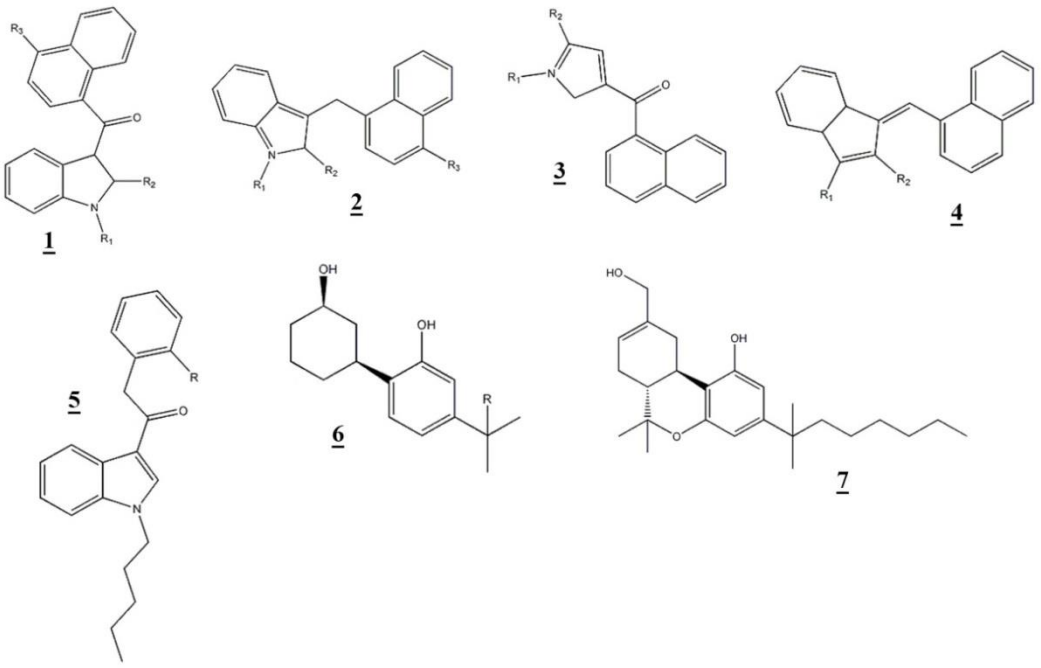

Figure 1: Chemical structures of synthetic cannabinoids used in »Spice«

1.1.1.3 Pharmacodynamics/Pharmacokinetics

There are several active constituents in cannabis, but the effects are primarily attributed to Δ^9-tetrahydrocannabinol (THC) and cannabidiol (see Figure 2), which are known agonists on the cannabinoid receptors 1 and 2 (CB_1 and CB_2)[3]. (6,11) Cannabinoid receptors are activated by a group of compounds called endocannabinoids (endogenous) and cannabinoids (exogenous), which produce a cascade of biochemical processes (e.g. inhibition of adenylate cyclase, enhancement of some types of potassium channels, inhibition of presynaptic N- and P/Q-type calcium channels etc.) that are involved in regulating mood, memory, appetite, anxiety, pain, cognition and emotion. (3,15)

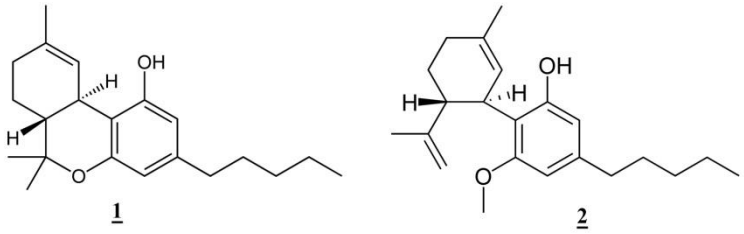

Figure 2: Chemical structures of THC (1) and cannabidiol (2)

Most of the SCs are functionally similar to THC and bind to the same CB receptors in the brain. However, the mechanism of action of cannabinoids is not limited only to CB receptors. Validation and characterisation of new pharmacological targets therefore remains a matter of research. For example, one of the most studied cannabinoids, cannabidiol, acts through many other biological pathways – it is a $5HT_{1A}$ receptor agonist, allosteric modulator of μ and δ receptors, G-protein coupled receptor 55 etc. (16–18) For SCs, which are a relatively new group of compounds, pharmacodynamic studies have barely started. Until date, only the affinities for CB receptors have been studied and are listed in Table I. The affinity of the ligand for the receptor, also called the inhibition constant (K_i), is expressed in molar units, usually in a nanomolar range. Some of the tested SCs (WIN 55,212-2, CP 55,940) were reported to modulate $5HT_{3A}$ receptors, TRPV1 and allegedly TRPV1-like

[3] Cannabidiol has low affinity on CB1 and CB2 receptors, but it is a high-potency antagonist of cannabinoid-receptor agonists.

receptors, non-CB and non-TRPV1 G-protein coupled receptors and neuronal receptors. (19–21)

Table I: Pharmacological properties and partition coefficient values of the investigated compounds, with THC for comparison

Name	Binding affinities	Partition coefficient (logP)[4]
THC	Agonist on CB_1(40.7±1.7 nM) and CB_2(36.4±10 nM) (22)	6.688
AM- 251	CB_1 inverse agonist (7,5 nM), direct agonist on mu opioid receptors, agonist on G-protein coupled receptor 55 (GPR55) (23)	5.622
AM-356	CB_1 agonist(20±1.6 nM) (24)	5.975
AM-2201	Full agonist at CB_1 (1.0 nM) and CB_2 (2.6 nM) (25)	6.114
CP 55,940	CB_1 (0.5 - 5.0 nM) and CB_2 (0.69 – 2.8 nM) agonist (21)	6.236
JWH-018	Full agonist at CB_1 (9±5.00 nM) and CB_2 (2.94±2.65 nM) (22)	6.713
WIN 51,708	Neurokinin 1 tachykinin receptor antagonist (26)	5.988
WIN 55,212-2	CB_1 (1.89 – 123 nM) and CB_2 (0.28 – 16.2 nM) agonist (21)	4.725
WIN 62,577	Neurokinin 1 tachykinin receptor antagonist (27)	5.923

[4] Partition coefficient values were calculated using interactive logP calculator from http://www.molinspiration.com/services/logp.html

Knowledge about absorption, metabolism, distribution and elimination of SCs is still limited. Some are subject to cytochrome P450-mediated oxidation with monohydroxylated and carboxylated derivatives as major metabolites, but this cannot be assumed for all compounds due to high structural diversity of SCs. (28)

1.2 OPTIMISATION OF COMPOUND-DEPENDENT MS/MS PARAMETERS FOR THE DETECTION OF SYNTHETIC CANNABINOIDS

1.2.1 Identification of synthetic cannabinoids in forensic samples

The main goal of forensic toxicology is to determine the intake of a particular drug, mainly by the analysis of biological matrices and seized drugs. These are usually blood and urine samples, however oral fluids have lately become an effective alternative since sample collection is easy, less invasive and therefore appropriate also for road-side screenings. The presence of psychotropic substances in samples is determined with screening tests, followed by confirmatory testing to identify specific drugs. LC-MS is applicable for both screening and confirmatory testing. (29) Using gas and liquid chromatography (GC/LC) combined with (tandem) mass spectrometry [MS(/MS)], methods have already been developed for the detection of certain synthetic cannabinoids (and THC) in human serum, while their metabolites were found in urine samples. (30,31)

1.2.2 Mass spectrometry

Mass spectrometers are best suited to three general applications: qualitative and quantitative analysis of small molecules (mass less than 1000 amu), and qualitative analysis of proteins and peptides. Qualitative analysis aims at the identification and structural characterisation of the compounds present in the investigated sample. (35)

A mass spectrometer consists of three essential parts: a) ion source; b) mass analyzer; and c) detector. The ion sources are subdivided into two main groups. The 1st group includes electron ionisation and chemical ionisation sources, where the sample is in gaseous state. The 2nd group consists of sources that are operating with sample solutions (mainly electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI)) or solid substrates (matrix-assisted laser desorption/ionisation (MALDI)). (37)

In our experiment we used ESI in positive ionisation mode. Three consequent processes take place in ESI: production of charged droplets (»Taylor cone«) at the capillary tip by applying a strong electrical field, followed by desolvatation of the charged droplets, leading to »Coulombic fissions« and production of gas phase ions from small/highly charged droplets. (37) These ions enter the mass analyzer.

In 3200 Q TRAP mass spectrometer the following scan techniques can be used: MS scan, MS/MS scan and MS/MS/MS scan. In MS scans, also referred to as single MS scans, ions are separated according to their mass-to-charge ratio. A single MS scan may be used to determine or confirm the molecular weight of a compound. Single MS scans can also be referred to as survey scans. MS scans do not provide any information as to the chemical make-up of the ions other than the molecular weight and therefore can not definitely determine the identity. To get more information about the ions and identify it, we needed to perform MS/MS. We know also the MS/MS/MS scan, which goes one step further and fragments the fragments produced in MS/MS.

The MS/MS scan we used can be thought of as a means by which to obtain the mass spectrum of a mass spectrum and is from a qualitative point of view most commonly used for identification of compounds relative to standards through pattern recognition, and mapping of fragmentation pathways. In this work, a triple quadrupole mass analyzer was used, a tandem-in-space MS/MS analyzer. A quadrupole filters ions according to their mass-to-charge ratio (*m/z*) as they pass along the central axis of the four parallel metal rods. Fragments of a molecular ion are created by CAD (collisionally activated dissociation), that takes place in the electromagnetic field. The latter is produced by a fixed direct current and altering radio frequency voltages applied to the two pairs of opposing rods. The magnitudes of these voltages, determine the *m/z* values that will reach the detector. Nonselected ions will not reach the detector because they follow an unstable trajectory and leave the quadrupole or collide against the rods. A triple quadrupole tandem MS/MS combines two quadrupole mass spectrometers in a series, with a radio frequency-only quadrupole in between, which acts as a collision cell. The instrument will be used in multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) mode. After ion generation with the selected ionisation source, ions enter the first mass analyser (Q1) where ions of interest (precursor ions) are selected. These ions are broken down into fragments by collisional excitation with neutral gas molecules in the pressurized collision cell (q2). Next, the frag-

mented products of the preselected ions are filtered in the last, third quadrupole (Q3). This mode is highly suitable for quantitative and sensitive analyses because of the monitoring of selective transitions from precursor ions to product ions. Ions passing through the mass analyzer enter the detector, which produces an electric signal. The signal represents the ion intensity for a particular *m/z* and the instrument displays this information as a mass spectrum. For the scheme of our mass analyzer see Figure 6.

1.3 MODULATION OF CATION CHANNELS

1.3.1 Molecular toxicology

Molecular toxicology combines the fundamental knowledge from biological and chemical sciences with a focus on the effects – adverse and beneficial – of naturally occurring herbs and food products, industrially produced environmental contaminants and designer drugs. Various molecular and computational methods are used to better understand how these agents interact with living organisms by affecting cellular and molecular mechanisms. The reported unintended clinical effects arising from the use of SCs are stated in section 1.1.1.2. Effects on the central nervous system (CNS) and cardiovascular system are the most common; the exact molecular mechanisms are not known yet. Endocannabinoids are known to modulate certain presynaptic Ca^{2+} and K^{+} channels, hence SCs could also exhibit activity on voltage-gated ion channels (VGIC) expressed in the brain and heart. (32) Secondly, the SCs are known to act on the same binding sites as other endocannabinoids, therefore they are interesting cue for research of novel drugs for clinical conditions such as neuropathic pain, spasticity etc. (11)

1.3.2 Voltage-gated ion channels

The application of molecular biology techniques and the development of new physiological methods have accelerated research on many different ion channels. (33) Ion channels are integral membrane proteins that are responsible for conducting across the membrane. (33) The difference in ion concentrations generates electrical signals required for generation and propagation of action potentials. Ion channels are very diverse and are classified into three major groups. The first group comprises voltage-dependent channels, activated by a sufficient change in membrane potential. (34) All channels tested in this thesis belong to this group. The next group consists of channels that are extracellularly-gated by ATP, glycine, acetylcholine or GABA. The intracellular ligand-gated channels, with ligands such

as ATP, Ca^{2+} ions, cAMP or cGMP, belong to the third group. (35) Voltage-gated cation channels include channels selective for Na^{+}, Ca^{2+}, K^{+} (namely, K_v, Na_v and Ca_v) and those that discriminate just cationic nature (TRP superfamily). They are engaged in diverse biological processes such as electric signaling in neurons, hormone regulation, cellular secretion, and contraction in excitable muscle cells. (36) Channelopathies are mutations in these channel genes, including hyperexcitability disorders and inheritable diseases such as neuromuscular diseases, cardiac long QT syndrome and epilepsy. (36) The highest expression, proposed functions and possible disease connections of tested channels are stated in Table II.

Table II: Overview of the function and expression profiles of the screened channels

Channel	Proposed function/possible disease connections	Highest expression
K_v 1.1	Controlling neuronal excitability / pain disorders, partial seizures, episodic ataxia, myokymia disorders, temporal lobe epilepsy (35,37)	Neurons, peripheral tissues in heart, the vasculature and the immune system (35,37)
K_v 1.3	Immune response / obesity, autoimmune diseases: multiple sclerosis, type-1 diabetes. Rheumatoid arthritis, psoriasis (35,37)	Peripheral tissues in heart, the vasculature and the immune system (35)
hERG	Cardiac repolarisation / cardiac disorders, long QT syndrome, torsade de pointes, ventricular fibrillation, schizophrenia, cancer (35)	Neurons, smooth muscle, cancer cells, neuroendocrine glands (38)
Na_v 1.2	Action potential initiation and conduction, repetitive firing / inherited febrile seizures, benign neonatal-infantile seizures, epilepsy (39,40)	Central neurons: unmyelinated and premyelinated axons, adult dorsal root ganglion(39,40)
Na_v 1.3	Action potential initiation and conduction, repetative firing / axatomy, nerve injuries, neuropathic pain (40,41)	Central neurons: primarily expressed in embryonic and early prenatal life; preferentially localized in cell bodies in adult rat brain, cardiac myocytes (40)
Na_v 1.5	Action potential initiation and conduction, repetitive firing / arrhythmia syndromes, long QT syn-	Cardiac myocites, immature and denervated skele-

	drome, idiopathic ventricular fibrillation (39,40)	tal muscle, certain brain neurons, dorsal root ganglion (40)
TRPV1	Detection of mild noxious heat, taste/ functional bowel disease, inflammatory bowel disease, ostheoarthritis, pancreatitis, impaired bladder contractions, impaired thermal hyperalgesia, asthma, cystitis, schizophrenia, gastroesophagal reflux (37)	TG, dorsal root ganglion neurons, urinary bladder, testis, brain, respiratory tract, gastrointestinal tract (37)

1.3.2.1 Voltage-gated sodium channels (VGSCs)

VGSCs family in mammals are multi-molecular protein complexes and consists of a nine large α-subunit isoforms (approx. 260 kDa; Na_v 1.1 – 1.9), encoded by ten genes (SCN1A-SCN11A), which are associated with four auxiliary β-subunit isoforms (33-36 kDa), encoded by four different genes (SNC1B-4B). (33,40) They are crucial for cardiac and nerve function. (40) The 10th isoform being identified is Na_x, which refers to a subfamily of sodium channel-like proteins. The pore-forming α-subunit is responsible for sodium channel functions – channel opening, ion selectivity and rapid inactivation; however, the β-subunit modifies the kinetics and voltage-dependence of the channels. (33) Every α-subunit contains four homologous domains (DI-DIV), each of which comprises six transmembrane α-helices (S1-S6) of 19 – 27 amino acid residues and an additional pore loop located between the S5 and S6 segments[5] (see Figure 3). VGSCs can exist in three different states: resting, activated and inactivated. At the resting membrane potential (negative), these positive charges are pulled inward. When the cell membrane of excitable cells is depolarized by a few millivolts, sodium channels activate (inward Na^+ current) and inactivate within few milliseconds (fast inactivation) or seconds to minutes (slow inactivation). (33) S1-S4 segment is a voltage sensor domain. In the S4 segment it contains positively charged amino acid residues spaced by two hydrophobic residues in an α-helix. These positively charged residues serve as gating charges and move across the membrane in order to initiate channel activation in response to depolarisation of the membrane. (33,40) Inactivation gate is located between DIII and DIV domain and isoleucine-phenylalanine-methionine (the IFM motif) fragment is crucial for both types of inactivation. (33) Activation of VGSCs can be modified by the following mechanisms: ion channel block → inhibition of ion transport

[5] Common structural motif for all VGICs.

(tetrodotoxin), persistent activation, slow inactivation (enhancement of persistent activation), transient repetitive activity and/or block (shift voltage-dependent activation to more negative potentials) or prolongation of action potential. (33,35) These mechanisms are acting on different binding sites and are triggered with a variety of structurally diverse modifiers: from biological poisons, including water-soluble heterocyclic guanidines (tetrodotoxin, saxitoxin), lipid-soluble polycyclic compounds (veratridine, aconitine, batrachotoxin) and low-molecular-weight polypeptide venoms isolated from sea anemone or scorpion venoms, to synthetic VGSC modifiers (DPI 201-106) and local anesthetics (lidocaine, procaine). (33)

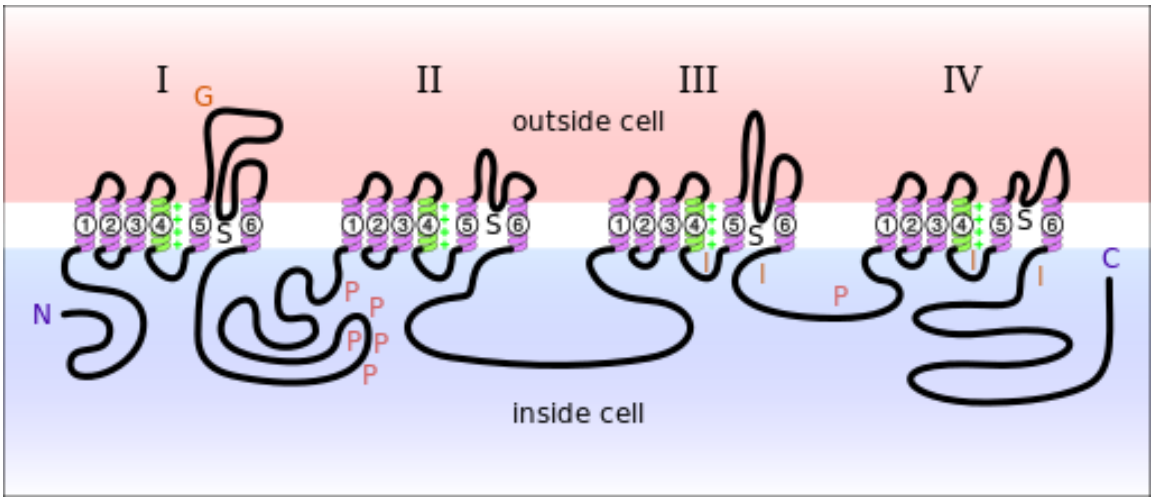

Figure 3: The voltage-gated ion channels (in sodium channels – α-subunit). Figure adapted from *www.wikipedia.org*

1.3.2.2 Voltage-gated potassium channels (VGPC)

Potassium channels are the most widely distributed of all ion channels and are found in virtually all living organisms. Their common feature is that they are highly selective for K^+ ions. There exist ligand-gated K^+ channels that open after the binding of an ion, small organic molecule or, in some cases, even a protein, and voltage-gated K^+ channels that are activated by the membrane potential. (34) 78 different types of potassium channels are present in the human body, of which 40 are voltage-gated. (35) Conduction of K^+ ions across the cell membrane is involved in a variety of cellular processes ranging from secretion, calcium signaling and cell volume regulation to repolarisation of cardiac and neuronal action potentials in both non-excitable and excitable cells. (35) K_v channels are involved in several diseases, so they are an important target in the development of new drugs for auto-

immune, neurological, metabolic and cardiovascular disorders, and cancer. Special attention should be paid to the member K_v 11.1 (hERG), which is relevant for cardiac repolarisation. Its blockage, either by the application of drugs or by rare mutations in some families, can cause potentially fatal long QT-syndrome and arrhythmias. (35) A lot of newly discovered drugs had a tendency to bind to hERG and are therefore inappropriate for treatment. The structure of VGPC is the same as that of VGSC (see Figure 3). Table II shows the correlation of diseases and tissues with the highest expression of tested channel subtypes.

1.3.3 Transient receptor potential superfamily and the vanilloid receptor 1

The transient receptor potential (TRP) multigene superfamily is one of the largest families of ligand-gated cation channels, found in many different species. So far there are 28 groups of identified TRPs found in mammalians, divided into 6 subfamilies[6], and two subfamilies of TRP channels found only in invertebrates and fish - TRPN and TRPY subfamily – TrpY1, found in yeast. They were first discovered in 1969 Cosens and Manning in *trp* mutant strain of fruit fly *Drosophila* photoreceptors and first cloned in 1997. (42,43) TRP channels are mainly non-selective cation channels, few of them are highly Ca^{2+} selective (transient receptor potential vanilloid cation channel (TRPV1)) and some are permeable for highly hydrated Mg^{2+} ions. They are expressed in many different tissues in the human body. Mutations in genes encoding TRP channels have been implicated in several inherited diseases (see Table II). TRP cation channels also play a central role in nociception under physiological and pathological conditions including inflammation and neuropathy. They are involved in variety of sensory processes such as osmosensation, thermosensation, olfaction, taste, mechanosensation and vision. Today they are one of the most ardently pursued targets for drug discovery. (42,44–46) Tetrameric structure of TRP proteins is common, as with VGIC; however, they show relatively little sequence homology (see Figure 3). Positive charges on the S4 segment and the P loop are poorly conserved in the TRP family. (37) Intracellular amino and carboxyl termini consist of a variety of domains and are variable in length, depending on the specific member of TRP family.

[6] TRPC (»Canonical«), TRPM (»Melastatin«), TRPV (»Vanilloid«), TRPA (»Ankyrin«), TRPP (»Polycystin«) and TRPML (»Mucolipin«)

TRPV1, also known as the capsaicin receptor or the vanilloid receptor 1, was the first isolated member of the TRP family. The name came from vanniloid moiety of capsaicin (CAP). CAP is the spicy ingredient of capsicum peppers and induces an intense burning sensation and pain. Paradoxically, after prolonged exposure to CAP, TRPV1 activity decreases (desensitisation), leading to reduced pain sensation. Ca^{2+} influx through the channel is required for this phenomenon, since it activates a Ca^{2+}-sensitive phospholipase C that causes hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2). The decreased PIP_2 concentration limits the channel activity. (42)

TRPV1 is expressed in small- to medium-sized sensory ganglia neurons and has been found to have a role in the management of chronic pain, migraine and gastrointestinal disorders; therefore it qualifies as a molecular target for pain treatment. (46) It is activated by noxious stimuli, heat (its threshold temperature is >43°C), protons, pH<5.9, and various natural and synthetic compounds. Since TRPV1 was not cloned until 1997, there is still a lot to unravel. TRPV1 agonists can affect the functioning of the channel directly or indirectly. Indirect agonists, called sensitizers, lower the thermal »physiological« activation threshold of TRPV1, while direct agonists bind directly to it. (46) Endogenous agonists include polyamines (spermine, spermidine) and various lipids from the fatty acid pool, divided into conjugates of biogenic amines and oxygenated eicosatetraenoic acids. The structure of endovanilloids resembles that of capsaicioids, with a polar head and a lipophilic moiety linked by an amide group. Endovanilloids are promiscuous agents, since *N*-arachidonylethanolamine and *N*-arachidonoyldopamine also bind to cannabinoid receptors. Exogenous agonists include capsaicinoids, capsinoids, piperine, eugenol, gingerol, plant toxins and animal toxins. (46) A structural characteristic of capsaicinoids and capsinoids is a lipophilic acyl group, which binds via an amide or ester linker to an oxygenated vanillyl aromatic moiety. Hydrogen bonds in vanillyl and the carbonyl linker are essential for activity, whereas lypophilic moiety interacts with the vanilloid binding site of TRPV1. (46) Piperine is an attractive lead compound in the synthesis of TRPV1 agonists. It exhibits greater efficacy than capsaicin for both activation and desensitisation; however, little is known about its structure-activity relationships. Vanillyl group has been shown to be critical for the activity of capsaicin and a more complex plant toxin, resiniferatoxin. (46) Animal TRPV1 activators are jellyfish and spider venoms, but the active principles and mechanism of action are still unknown. Surprising finding was the activation of TRPV1 with lidocaine – a common anesthetic and antiarrhythmic drug – by inducing a TRPV1-

dependent release of calcitonin gene-related peptides. Other activators are also pungent extracts from garlic and onion (allicin, alliin), mustard oil and membrane-permeable oxidizing agents like diamide, chloramine-T, and copper complexes. (46) Sensitizers influence TRPV1 channels indirectly, via G-protein coupled receptors (ATP, bradykinin, prostaglandins, trypsin or tryptase), receptors of intrinsic tyrosine kinase pathways (insulin, insulin-like growth factor), or via receptors coupled to the Janus tyrosine kinase/signal transducer (growth factor, glia-derived growth factor, neurotropin 3) and activator of transcription signaling pathway (stimulation of the metabotropic 5-hydroxytryptamine receptors $5HT_{2A}R$ and $5HT_7$). (46) TRPV1 antagonists are divided into two major groups: in classic antagonists, two moieties, usually with different polarity, are linked via carbonyl group of an ester-, amide-, urea-, or thiourea-. In non-classic antagonists, the carbonyl group is either unrecognizable or is present as a part of the heterocyclic ring. The first known competitive antagonist was capsazepine (CPZ), which inhibits capsaicin-mediated channel activation by competing for capsaicin-binding sites. The majority of the antagonists known so far are – unlike agonists – synthetically produced compounds, divided in the following groups: 1,3-dy(arylalkyl)thioureas (capsazepine), iodinatede vanillyl derivatives (5-iodo resiniferatoxin), di(arylalkyl)- and aryl(arylakyl)ureas (A-425619, ABT-102), cinnamides (SB-366791), carboxamides (SB-782443), imidazole derivatives (AMG517), noncompetitive antagonists (Ruthenium Red, AG 489). (46)

1.3.4 Two-microelectrode voltage-clamp technique

The electrophysiological behavior of ion channels was examined using the two-microelectrode voltage-clamp technique (TEVC), for scheme see Figure 4. During the experiment, two microelectrodes were inserted into the oocytes. The membrane potential is recorded by amplifier 1, which is connected to the first electrode, called the voltage-recording microelectrode (V1). Membrane potential is compared to the command potential, and the difference between those two signals amplified by the feedback amplifier 2 and compensated for by injecting a current through the current-passing microelectrode (V2). The membrane voltage is clamped at a constant value of command potential by injecting a current into the oocyte that is equal in amplitude but opposite in sign to the current flowing through the membrane of the oocyte. We indirectly measure the whole ion flow through channels expressed on the oocyte membrane by measuring the injected current. The number of open channels is proportional to the measured current. The two-electrode batch

clamp is actively controlling the bath potential via an analogous feedback system, so that the membrane potential is distinguishable from the bath potential.

Biological membranes are excellent capacitors; however, they have an enormous apparent surface area of ± 10^6 µm^2 that must be charged in order to clamp the cell. The current applied to the membrane first satisfies the requirement for charging the membrane capacitance and then the membrane voltage. The response time (τ) of a voltage clamp to step of voltage change is

$$\tau = \frac{(R_I * C_m)}{A},$$

where R_I is the resistance of the current-passing microelectrode (V2), C_m is the membrane capacitance and A is the gain of the command amplifier. In order to maintain the response time as low as possible, as it causes significant voltage errors when measuring channels with fast kinetics, we adapt the resistance and gain of the electrodes during the experiment (using the largest possible gain and the lowest possible resistance of the V2). The conditions and specific measuring protocols are described in section 3.

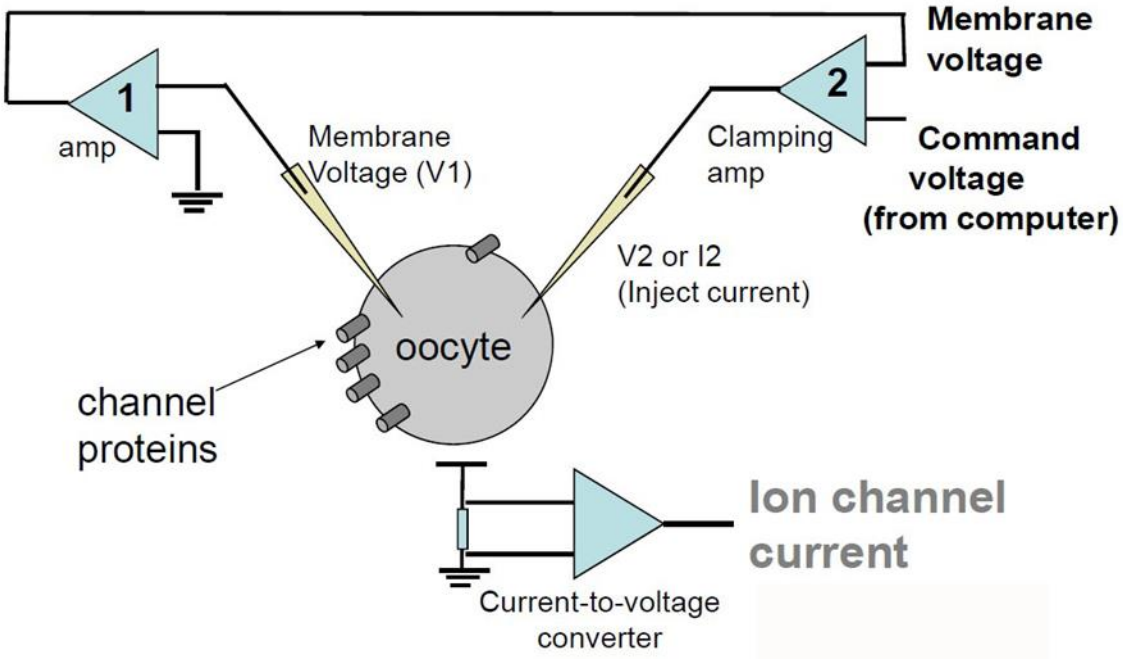

Figure 4: Scheme of the two-microelectrode voltage-clamp technique

2 AIM OF THE WORK

One of the objectives of forensic toxicology is the identification of compounds in biologic samples and seized drugs (pills, powders,...). Most of the newly emerged synthetic cannabinoids are still not systematically included in the screening panel in forensic labs. Therefore, the first aim of this study is to develop the MS/MS method in the MRM mode, with determination of the characteristic transitions (*m/z*) and optimal settings for the detection of eight SCs (see the chemical structures in Figure 5). The obtained results will be compared to the available literature on similar studies. Standards of SCs will be provided by the commercializing companies or from seized drug samples.

In the pharmacological part of the diploma thesis, the effect of eight SCs (Figure 5) will be directly tested on different ion channels in regard to potential inhibitory activity. The ligand-gated TRPV1, involved in a variety of diseases is a promising new target in ion channel research. Structures of our compounds were similar to already known inhibitors of the TRPV1 channel; therefore, we will test them to see if they exhibit any activity. Since the ion selectivity is important for the development of novel therapeutics, we will test these compounds on some subtypes of VGSC and VGPC as well. These channels are widely expressed throughout the cardiovascular and central nervous systems and could be correlated with unintended clinical effects arising from the use of synthetic cannabinoids mixtures. Some of these channel subtypes are involved in pain sensation and are therefore potential targets for our compounds, since cannabinoids are related with neuropathic and acute pain conditions (hyperalgesia, allodynia). We will inject complementary ribonucleic acid (cRNA) of channel subtypes in oocytes isolated from *Xenopus laevis,* which are a popular model for the expression of heterologous proteins. We will use the two-microelectrode voltage-clamp technique for electrophysiological measurements of current traces.

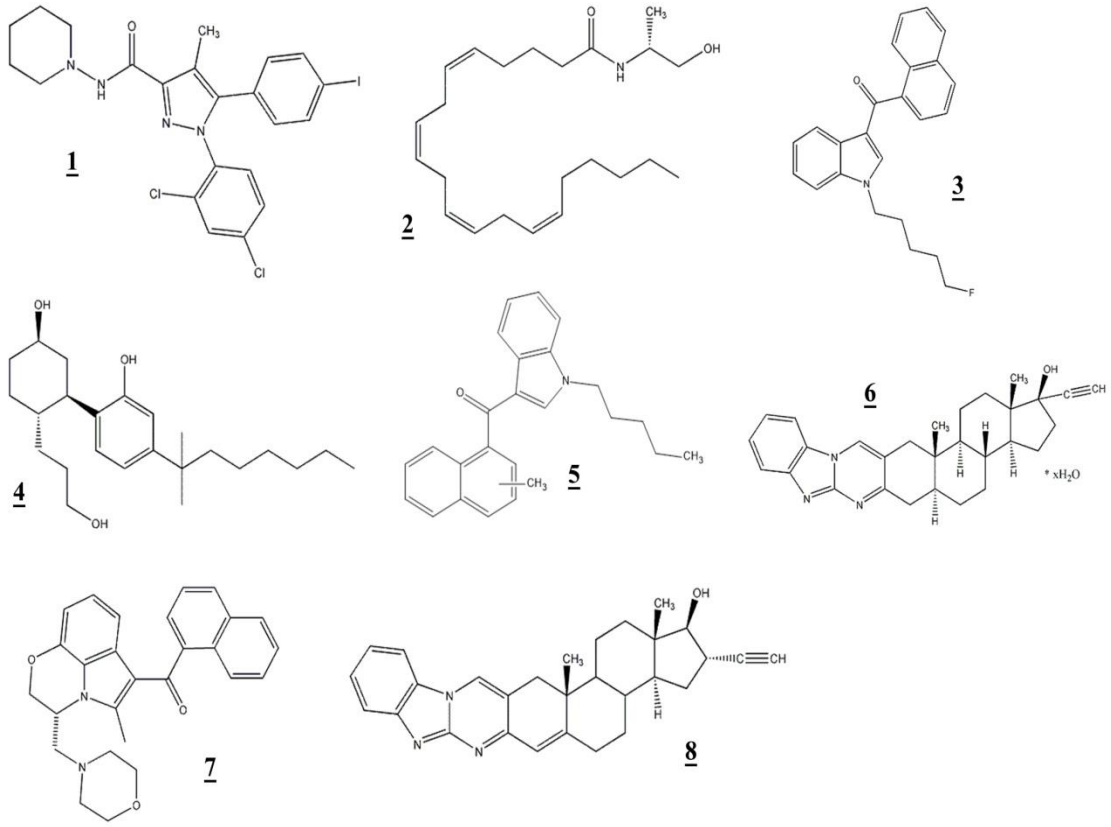

Figure 5: Chemical structures of the investigated compounds

3 MATERIALS AND METHODS

3.1 OPTIMISATION OF COMPOUND-DEPENDENT MS/MS PARAMETERS FOR THE DETECTION OF SYNTHETIC CANNABINOIDS

3.1.1 Chemicals and standards

Methanol (LC-MS grade) was purchased from Biosolve LTD (Valkenswaard, The Netherlands), and ethanol from Merck (Darmstadt, Germany). Water was prepared using a Milli Q Water Purification System (Millipore, Brussels, Belgium). 1.5 mL screw cap vials were purchased from Agilent (Diegem, Belgium). All analytical standards of SCs used in experiments, except JWH-018 with added methyl group on naphthyl ring (hereinafter referred to as JWH-018*) and AM-2201, were purchased from Sigma-Aldrich (Bornem, Belgium). JWH-018* and AM-2201 were obtained from seized drug samples, available due to the unique position of the host laboratory in the field of forensic toxicology. The identity of the forensic samples JWH-018* and AM2201 was determined using gas chromatography-MS, but no purity analysis or supplementary structural analysis has been performed. My thesis was within the PhD of Anne-Marie Gruyters.

Stock solutions of each analyte (AM-251, AM-356, AM-2201, CP 55,940, JWH-018*, WIN 51,708, WIN 55,212-2 and WIN 62,577) were prepared at a concentration of 1 mg/mL in methanol (MeOH). Working solutions were prepared using MeOH by independent dilution of each stock solution at the following concentrations: 1 µg/mL (1/10 from 10 µg/mL), 10 µg/mL (1/100 from 1 mg/mL) and 100 ng/mL (1/100 from 10 µg/mL). According to the signal intensity, the concentrations shown in Table III were used. All solutions were stored at -20°C until 30 minutes before analysis.

3.1.2 MS/MS analyses

In my thesis I determined the optimal parameters for accurate determination and distinction of the following synthetic cannabinoids in forensic samples with MS/MS: **1** 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide (AM-251), **2** (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-[(1*R*)-2-hydroxy-1-methylethyl]icosa-5,8,11,14-tetraenamide (AM-356), **3** 1-[(5-fluoropentyl)-1*H*-indol-3-yl]-(naphthalen-1-yl)methanone (AM-2201), **4** 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP-55,940), **5** naphthalen-1-yl-(1-pentylindol-3-

yl)methanone with additional methyl group on naphthyl ring (JWH-018*), **6** 17-β-Hydroxy-17-α-ethynyl-5-α-androstano(3,2-b)pyrimido(1,2-a)benzimidazole (WIN 51,708 hydrate), **7** (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone mesylate salt (R-(+)-WIN 55,212-2) and **8** 17-β-hydroxy-17-α-ethynyl-δ-4-androstano[3,2-b]pyrimido[1,2-a]benzimidazole (WIN 62,577) (see Figure 5 for chemical structures[7]). Compound-dependent MS/MS optimisation was carried out using a 3200 Q TRAP (ABSciex, Halle, Belgium), a hybrid triple quadrupole / linear ion trap mass spectrometer equipped with a Turbo V™ ion source (see Figure 6), using electrospray ionisation in positive mode and an electron multiplier detector. The ESI source was installed and was used in positive mode. Analyst® software (version 1.5) was used for system control and data processing.

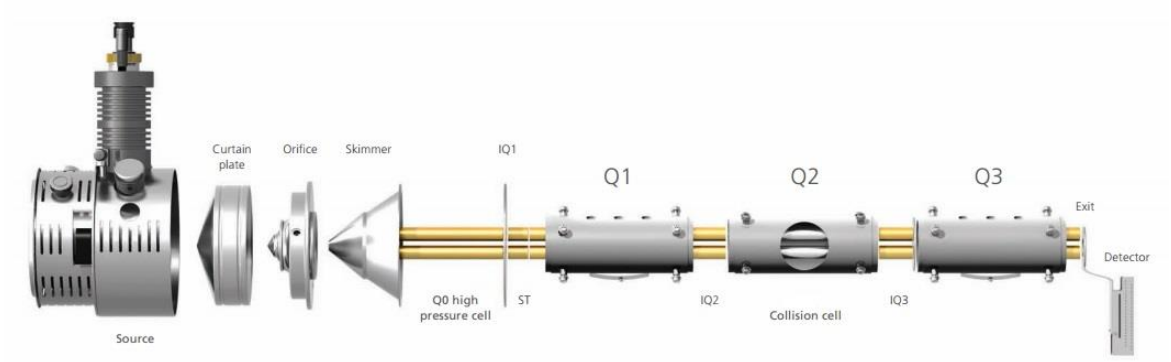

Figure 6: 3200 Q TRAP mass spectrometer. Figure adapted from (47).

Automatic optimisation was performed five times for each compound, introduced by direct infusion. Flow rates were adjusted according to the signal intensity (see Table III). The ESI settings were as follows: gas 1 and 2: nitrogen, 50.0 psi; ion-spray voltage: 5000 V; ion-source temperature: 550°C; curtain gas: nitrogen, 10.0 psi. The mass spectrometer was operated in MRM mode. The detector voltage was set to 1.72 kV. All other settings were analyte-specific and were auto-optimized by flow injection of the analyte solution.

[7] All structural formulas were drawn with ChemDraw Ultra 12.0 software (PerkinElmer Inc., Waltham, Massachusetts, USA)

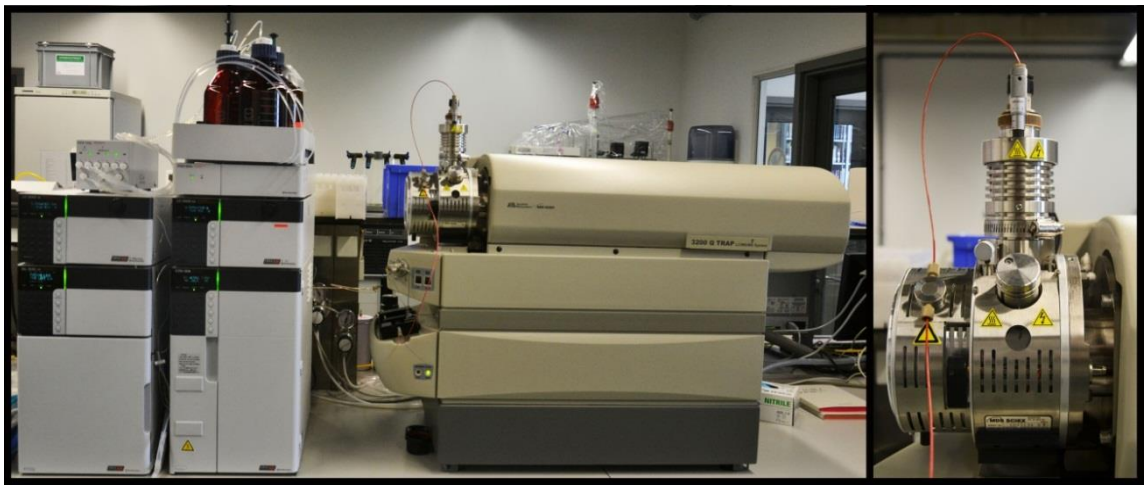

Figure 7: Left - LC/MS-MS; Right - ionisation chamber

Table III: Experimental conditions

Compound	Used concentration (in MeOH)	FR (µL/min)
AM-251	100 ng/mL	10
AM-2201	100 ng/mL	10
CP 55,940	1 µg/mL	10
JWH-018*	100 ng/mL	10
R(+)-methanandamide	1 µg/mL 10 µg/mL	10 10; 5
WIN 51,708	100 ng/mL	10
WIN 55,212-2	100 ng/mL	10
WIN 62,577	100 ng/mL	10

3.2 MODULATION OF CATION CHANNELS

3.2.1 Chemicals and standards

Origins of the compounds used are stated in section 3.1.1. AM-356 was purchased dissolved in ethanol, which was removed under a stream of N_2 prior to reformulation in dimethylsulfoxide (DMSO). 100% DMSO, capsaicin, capsazepin and tetrodotoxin (TTX) were obtained from Sigma Aldrich (Bornem, Belgium). Collagenase type I 370 μ/mg CLS1 was purchased from Worthington Biochemical Corporation (Lakewood, New Jersey, US).

Perfusion solution ND-96 was composed of 2 mM KCl, 96 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and neutralized to pH 7.5 with NaOH. For defolliculation of oocytes, collagenase was dissolved in Ca^{2+} free ND-96.

3.2.2 Sample preparation

The solubility data was obtained from manufacturers[8]. Accordingly, powders of SCs were dissolved in DMSO and were stored at -20°C. Stock solutions of AM-251, AM-2201 and WIN 55, 212-2 were 40 mM, of CP 55,940 was 30 mM, of JWH-018*, R(+)-methanandamide and WIN 51,708 were 20 mM, and of WIN 62,577 was 10 mM.

For electrophysiological measurements on VGIC, stock solutions were diluted with ND-96 to a concentration of 200 μM. When measuring TRPV1, diluted stock solutions of SCs in ND-96 and capsazepin (used as an antagonist) were 10 μM and capsaicin (used as an agonist) was 2 μM. pH of the solutions was 7,4 and the total DMSO concentration in testing solutions was at most 0.5%. As a perfusion solution was used ND-96.

3.2.3 Isolation and injection of cRNA

Oocytes were harvested from adult female *Xenopus laevis* frog anaesthetized with tricaine (Sigma Aldrich, Bornem, Belgium) in ice water (1 g/L) by partial ovariectomy through an abdominal incision. The frogs were kept on ice during operation (see Figure 8). Lobes of oocytes were manually teased apart and defolliculated using 2.5 mg/mL collagenase in Ca^{2+} free ND-96 for 2 – 2.5 hours with gentle agitation. Between 1 and 24 hours after defolliculation, stage V-VI immature oocytes were selected for microinjection of cRNA. In-

[8] At www.caymanchem.com and www.sigmaaldrich.com.

dividual oocytes were injected with 10 to 60 nL of cRNA solutions using a microinjector Nanoinjector (WPI, Sarasota, Florida, USA). Oocytes were incubated at 16°C in ND-96 solution supplemented with gentamicin (50 mg/L) for one to five days, until sufficient expression of the channels was achieved. Incubation solution was refreshed daily.

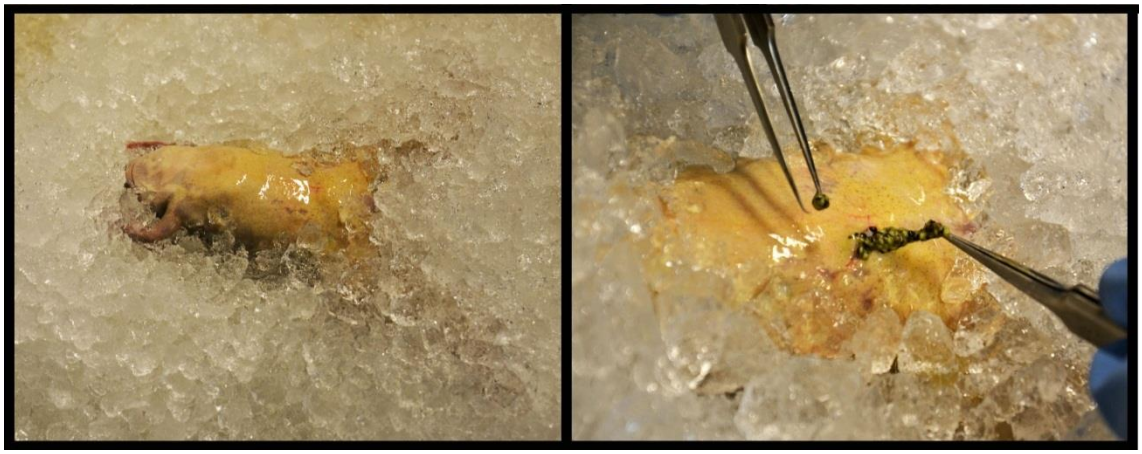

Figure 8: An anesthetized *Xenopus laevis* on ice

cRNA used were: rat K_v 1.1, human K_v 1.3, rat α-subunit Na_v 1.2, rat α-subunit Na_v 1.3, human α-subunit Na_v 1.5, rat β1-subunit, human ether-á-go-go related gene (hERG), and rat TRPV1. The cRNA of sodium channel α- and β-subunits were mixed prior to injection, using a 1:1 ratio. The cRNAs were obtained by transforming the cDNA encoding for these channel subunits into *E. coli* XL10-Gold Ultracompetent cells (Agilent Technologies, Santa Clara, California, US) using the heat shock method. After isolation, purification and linearisation of the plasmids containing the cDNA, the cDNA was transcribed into the desired cRNA by using the mMESSAGE-mMACHINE transcription kit (Ambion, Austin, Texas, USA). All cRNAs were provided by a colleague, Bea Mille.

3.2.4 Electrophysiological recordings

After sufficient expression, the electrophysiological behaviors of VGICs and TRPV1 channel were examined using TEVC, described in section 1.3.4 (see Figure 10, where Top left represents the selection of the cells for microinjection; top right is the instrument for injection of RNA consisting: binocular dissecting microscope with light source, microinjector, control switch for microinjector, microscope stage with oocyte injection chamber ; bottom left - injection of RNA and bottom right is the two-microelectrode voltage-clamp technique). Oocytes were impaled on two microelectrodes filled with 3 M KCl solution, pulled from 1.0 mm o.d. borosilicate glass tubing using PUL-1 micropipette puller

(WPI, Sarasota, Florida, USA). The resistances of both electrodes were maintained as low as possible (between 0.5 and 1.50 MΩ). The elicited currents were sampled at 20 kHz and filtered at 1 kHz (TRPV1) and 2 kHz (VIGC), using a four-pole, low-pass Bessel filter. We controlled the bath potential by a two-electrode bath-clamp in order to eliminate the voltage drop across the bath grounding electrode. The cells selected for the experiments were required to have leakage lower than 500 nA. For all measurements, digital leak subtraction was performed using the –P/4 protocol. Each experiment was repeated on three to five different oocytes.

Currents were recorded at room temperature (18-22°C), using a GeneClamp 500B voltage and patch clamp amplifier (Molecular Devices, Sunnyvale, California, USA) controlled by a DIGIDATA 1322A 16-bit data acquisition system (Molecular Devices, Sunnyvale, California, USA). Pulse protocols were executed using CLAMPEX module of pCLAMP v. 9.2.1.9 software (Molecular Devices, Sunnyvale, California, USA).

When recording, VGIC oocytes were placed in a bath containing ND-96 solution (200 µL). All current traces of sodium channels were evoked by a 100 ms depolarisation to the voltage corresponding to the maximal activation of VGSCs (i.e. 0 mV), starting from a holding potential of -90 mV. Following depolarisation, membrane voltage was repolarized to -90 mV during 100 ms. K_v 1.1 and K_v 1.3 channels were evoked by depolarisation from a holding potential of -90 to a test potential of 0 mV, followed by a test potential of -50 mV. hERG currents were induced by depolarisation to +40 mV, holding potential was -90 mV, then clamped back to -120 Mv (see Figure 9). 200 µM solutions of SCs were applied into the bath with clamped cell using a pipette after approximately 20 stable (overlapping) sweeps.

TRPV1 channel currents were measured in ND-96 solution using a series of 400 ms step pulses from -90 to +90 mV. The recording chamber was perfused at a rate of 2 mL/min with a ND-96 solution. As previously described, all solutions of SCs and capsazepine were 10 µM and capsaicin was 2 µM.

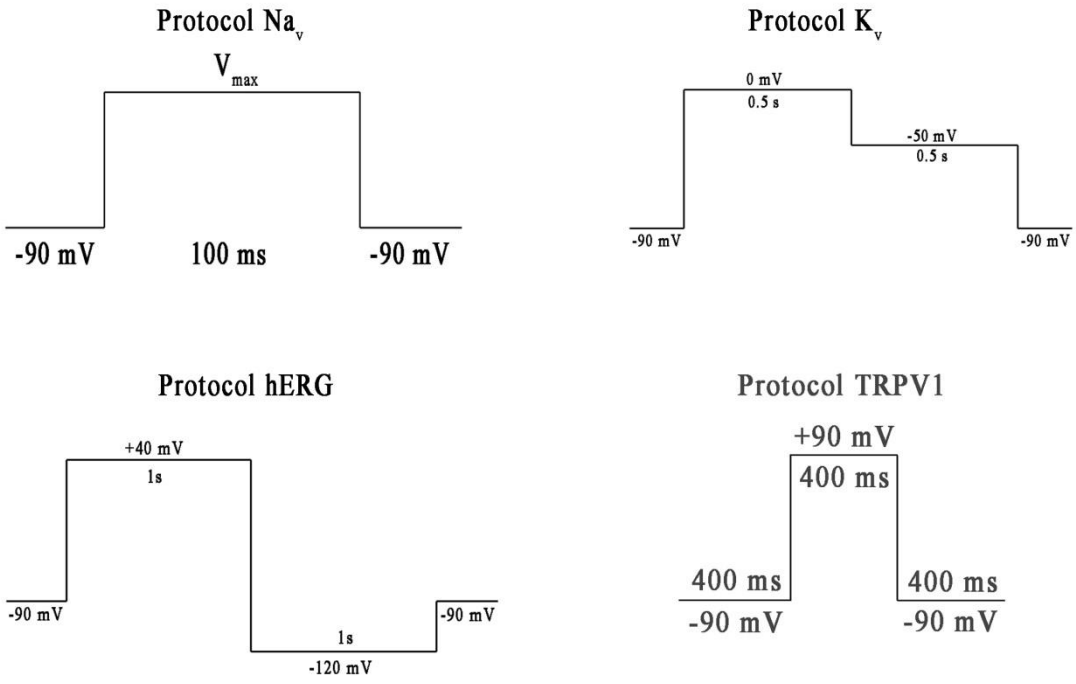

Figure 9: Used measurements protocols

Obtained current traces were transferred to Clamfit v. 10.0.0.61 software (Molecular Devices Corporation) and the final graphs were drawn with Origin v. 7.5 SR4 software (OriginLab Corporation).

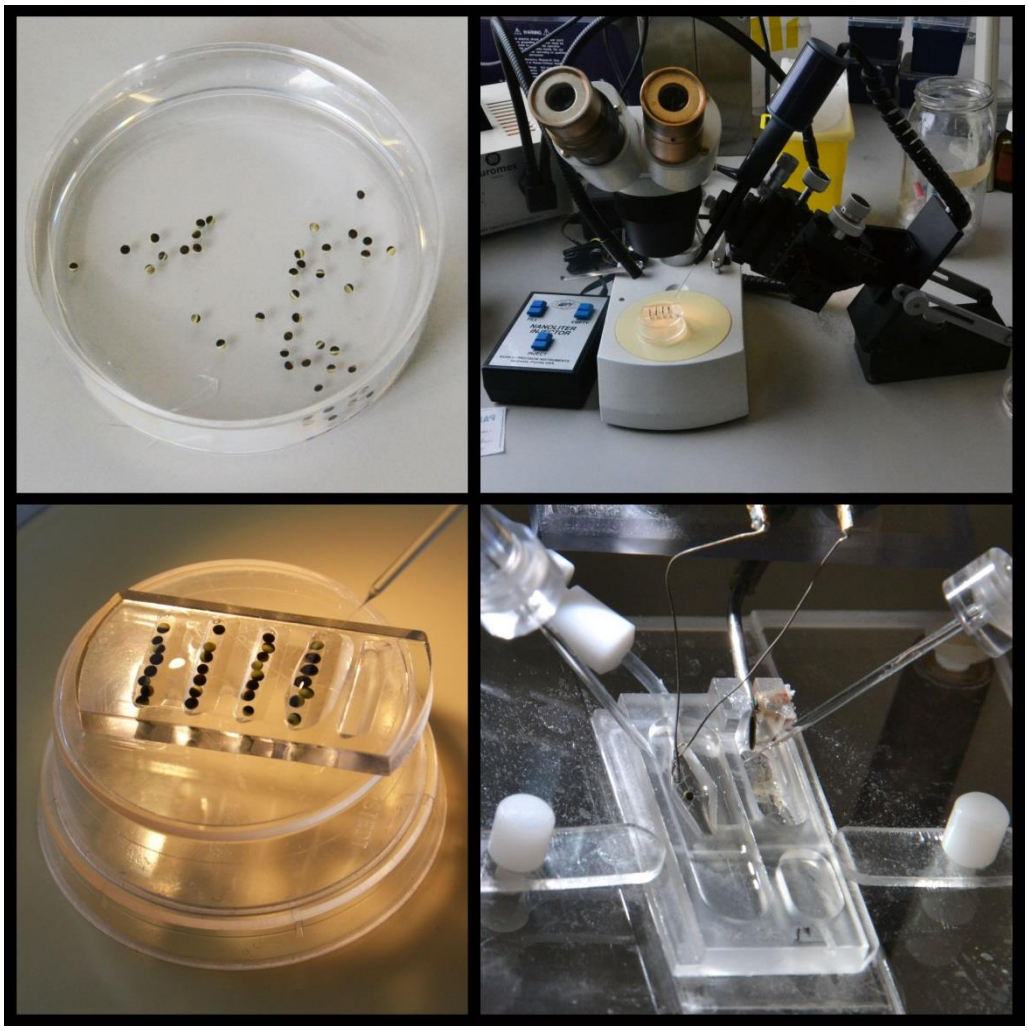

Figure 10: Selection and injection of the cells and TEVC technique

4 RESULTS AND DISCUSSION

In this diploma thesis we investigated SCs, that are emerging in herbal mixtures and are available in a growing number of websites as Spice products. Most of them are already illicit by law, therefore the detection in forensic samples is an important issue. Identification of these compounds is a constant issue in forensic laboratories, because of the frequent emergence of new SC substances. Currently there are more than 100 different SC available on the market. (48)

SCs themselves are also an intriguing group of compounds for which a lot of research remains to be done. Previous studies used variable and not always channel-specific methods. Characterizing non-CB targets and discovering its ligands, which are able to selectively activate or block them with reasonable potency is still an intriguing task. Pharmacodynamic profile of SCs is poorly studied, therefore we tested our compounds on variety of cation channels.

4.1 OPTIMISATION OF COMPOUND-DEPENDENT MS/MS PARAMETERS FOR THE DETECTION OF SYNTHETIC CANNABINOIDS

We optimized compound-dependent MS/MS parameters in MRM mode for the detection of AM-251, AM-356, AM-2201, CP 55,940, JWH-018*, WIN 51,708, WIN 55,212-2 and WIN 62,577. The parameters were: collision energy (CE), declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP) and collision cell exit potential (CXP) (see the meaning of each under the Table IV). The compounds of interest were introduced by direct infusion of the sample solution. This is a continuous flow introduction of sample at low flow rates into the ionisation source by a syringe pump. MeOH was selected as a solvent as most of the SCs have a low solubility in water. (15) Based on the extensive literature search and the coupling of the MS/MS instrument to an LC separation, ESI was chosen as ionisation source. ESI is also a softer ionisation method than any other and a recommended starting point for method development. (29) It is suitable for the ionisation of a broad range of compounds – from small molecules (polar analytes with in the *m/z* 100 -1500 range) to large protein and peptide characterization. It is used for sample concentrations as low as 10^{-6} M and can be combined with all types of mass analyzers. In combination with ESI, the use of tandem mass spectrometry is beneficial, because ESI is a soft ionisation technique. ESI allows efficient ionisation, but there are some drawbacks. At

concentrations higher than 10^{-3}M, saturation effects can appear and further increases in concentration have little effect on the signal. (18; 20) For mass analysis we used a triple quadrupole mass analyzer, because its straightforward operation, ultratrace-level quantification and greater discrimination against chemical background than single MS. The pressure in the collision cell can be controlled according to the number of collisions during the CAD process. By selecting representative ion transitions (MRM scan) the triple quadrupole MS shows better sensitivity than tandem-in-time mass spectrometry. (50) For the correct identification of SCs, five MRM transitions were selected for each analyte. We developed the final method using the 4 most abundant product ions for each compound (Q1 – Q4 in Table IV), and proved that the MRM method is suitable for detecting all of the eight tested SCs. MRM transitions of AM-356 (*m/z* 362.2; 76.0 and 91.1), AM-2201 (m/z 360.2; 155.1 and 127.1) and WIN 55,212-2 (*m/z* 427.3; 155.2, 127.2 and 100.1) were as well used in previous studies. (30,32,51) To our knowledge, no previous study did report the compound-dependent data for the remaining five compounds. Unfortunately we did not register the full scan mass spectra of these compounds, so we can not provide it in this paper.

Flow rates and concentrations of analytes were adjusted according to the intensity of the obtained response, as described in section 3.1.2. All compounds are listed in Table IV with their respective MRM transitions and optimal compound-dependent parameters.

Detection and identification of synthetic cannabinoids is becoming increasingly important, as they are used as a substitute for cannabis due to their legal status, and the fact that most are still untraceable using routine drug screening techniques. A high prevalence of SC-positive samples was found in forensic psychiatric and rehab centres. (52) We developed our method for the parent compounds, not including the metabolites. Hence it is unlikely to be used for the analysis of urine samples[9]. (53) In further research we could improve it by including metabolites, which could be predicted by computational method. However, our method can potentially be used to analyze blood samples, seized drugs and oral fluid samples. Oral fluid was proven to be adequate as a biological matrix with application in road-side drug screening, in cases where typical symptoms of cannabis intoxication are observed without detecting THC or its metabolites. (52) Oral fluid samples can be collected using non-invasive methods and without privacy intrusion. Problems with

[9] SCs in urine samples are mostly already in metabolised form.

substitution and adulteration, often associated with urine collection, are also reduced. (52) We did not test the suitability of our method for analyzing and quantifying the presence of SCs in oral fluids. Therefore we cannot confirm whether it complies with standards for sensitivity and reproducibility required by forensic analysis.

Table IV: Multiple reaction monitoring (MRM) transitions and the optimal compound-dependent parameters for each of the eight tested SCs.

	AM-251	AM-356	AM-2201	CP 55,940	JWH-018*	WIN 51,708	WIN 55,212-2	WIN 62,577
DP (V)	76	41	56	36	71	111	61	96
EP (V)	9	5	7	9.5	8	6	8	10
CEP (V)	24	14	16	16	14	20	18	18
Q1 (amu)	557.0	362.3	360.2	377.4	356.2	440.3	427.3	438.3
$Q3_1$ (amu)	129.2	76.0	77.1	71.1	115.2	220.2	77.1	232.2
$Q3_2$ (amu)	229.2	91.1	126.6	77.1	141.2	234.2	100.1	233.2
$Q3_3$ (amu)	330.1	79.1	127.1	121.2	169.2	236.2	127.2	234.3
$Q3_4$ (amu)	457.0	105.1	155.1	233.3	214.2	344.3	155.2	246.3
CXP_1 (V)	4	4	4	4	4	4	4	4
CXP_2 (V)	4	4	4	4	4	4	4	4
CXP_3 (V)	4	4	4	4	4	4	4	4
CXP_4 (V)	6	4	4	4	4	6	4	4
CE_1 (V)	81	29	116	31	91	103	129	107
CE_2 (V)	99	63	108	105	55	89	56	71
CE_3 (V)	61	60	66	26	91	71	79	67
CE_4 (V)	41	43	33	17	32	59	33	65

DP, EP and CEP are pre-collision cell voltages. Optimisation of these values involves gradually changing the voltage range while monitoring the signal intensity of the compound. The potential applied to the orifice plate has the greatest effect on the amount of declustering in the orifice region of the interface.

The DP controls the potential difference between ground (usually the skimmer) and the orifice plate. It is used to minimize solvent cluster ions, which may attach to the sample. The higher the voltage, the greater the amount of fragmentation, or declustering. If the declustering potential is too high, the sample ion itself may fragment. The optimal DP is usually between 20 and 200 in positive mode and between -200 and -20 in negative mode. Recommended step size 5 or 10 V.

The EP parameter controls the potential difference between the voltage on Q0 and ground. The entrance potential guides and focuses the ions through the high-pressure Q0 region. Entrance potential affects the value of all the other ion path voltages. For nearly all applications, a value of EP equal to +10 V (positive ion mode) or equal -10 V (negative ion mode) is acceptable and should not be changed. In special cases involving very fragile analytes you may find a lower EP to be beneficial.

The CEP parameter controls the collision cell entrance potential, which is the potential difference between Q0 and IQ2. It focuses ions into Q2 (collision cell). The optimal CEP gives the greatest intensity for the ions of interest. For MS-type scans, the default value is appropriate. For MS/MS-type scans, optimize CEP for the precursor ion. The optimal CEP value is usually between 10 and 70 V in positive mode, -70 and -10 in negative mode. Recommended step size: 1V

The CAD gas parameter controls the pressure of collision gas in the collision cell during MS/MS-type scans. The collision gas acts as a target to fragment the precursor ions. When the parent ions collide with the collision gas, they can dissociate to fragment ions. For LIT scans, the collision gas also helps to focus and trap ions in the LIT. Although this parameter is displayed on the Source/Gas tab of the Tune Method Editor, this parameter is compound-dependent and not dependent on the sample flow. Typically in quadrupole mode MS/MS type scans, a setting of medium is recommended. For LIT mode scans, a setting of high is recommended. We therefore used a setting of medium.

The CE parameter controls the collision energy, which is the potential difference between

Q0 and Q2 for MS/MS-type scans. This is the amount of energy that the precursor ions receive as they are accelerated into the Q2 collision cell, where they collide with gas molecules and fragment. The optimal CE value gives the desired fragmentation of the precursor ion. For quantitative analysis, the optimal CE produces the greatest intensity of the fragment ions of interest. For qualitative analysis, the optimal CE gives the desired distribution of fragment ions. Recommended step size: 5 V

The CXP parameter controls the collision cell exit potential, which is used to focus and accelerate the ions out of the collision cell (Q2). The optimal CXP gives you the greatest intensity for the ions of interest. For Q3 scans, the default value is appropriate. For MS/MS-type scans, optimize for the fragment ions of interest. The optimal CXP value is usually between 0 and 20 in positive mode, and -20 and 0 in negative mode. Recommended step size: 0.5 V.

4.2 MODULATION OF CATION CHANNELS

Research interest in neuropathic treatment increased in recent years. Over a hundred new chemical entities were investigated, but none of them yet made it to the market. (54) Preclinical data showed that TRPV1 antagonists might be useful for treatment of neuropathic pain, pancreatitis, cough, incontinence and migraine; hence TRPV1 is an intriguing novel target in the research of next-generation drugs, focusing analgesics. (46)

The objectives of this research were to find new ligands for TRPV1 channels and use them as pharmacological tools in further elucidation of the working mechanism of TRPV1. We compared known modulators of the TRPV1 channel (see chapter 1.3.3) to our synthetic cannabinoids. According to known structure-activity relationships, AM-251 and AM-356 fit the structural profile of TRPV1 antagonists. (46) Even though 15 years have passed, since the first cloned TRPV1 channel, the knowledge on ligand-binding regions on TRPV1 is still limited. The exact structure-activity relationship studies for the rational design of novel therapeutics is dubious and the prediction of modulators is not fully reliable. (46)

Selectivity of the interacting channel is important when binding to the TRPV1 channel to prevent unwanted effects. Therefore we also tested selected SCs on VGSC and VGPC subtypes that our compounds were most likely to modulate, according to the tissues with highest levels of expression and reported adverse effects after the use of SCs mixtures (see section 1.1.1.2 and Table II).

The methods of introducing recombinant cRNA into *Xenopus laevis* egg precursors (oocytes) and transfected mammalian cell cultures have enabled a breakthrough in studies of ion-channel function, structure-function relationships and modulation. For expressing desired channel subtypes in this paper we used *X. laevis* oocytes. Their most important advantages are high efficiency expression, very few endogenous channels, simplicity of electrophysiological recording and the ability to inject varying ratios of subunits. (55) However, we also encountered some of the drawbacks of this technique during our experiments. Oocytes are an animal model so the quality varies from batch to batch. The biggest issue were unstable (oocytes expression system exhibits poor voltage control) and poor quality cells, over which we had no control. During incubation we used daily-refreshed gentamicin containing solution medium to prolong lifespan and prevent infection, and after injecting cRNA we placed each cell in its own compartment. Incubation time depended on channel subtype. This presented a particular difficulty with TRPV1 channel, where sufficient expression was only achieved after approximately 5 days. Nevertheless, even then TRPV1 in most cases did not exhibit any expression at all, or the cells were unresponsive to CAP and CPZ. We did not have enough time to test all of the selected SCs on TRPV1, so the results in this paper are preliminary. We successfully completed tests on VGPC, VGSC and hERG, where expression was sufficient and with stable current traces much sooner (normally 1-2 days). All the electrophysiological measurements on the different cloned rat or human ion-channel subtypes expressed in *X. laevis* oocytes were carried out using the TEVC technique. Solutions in methanol of the following compounds were applied: AM-251, AM-356, AM-2201, CP 55,940, JWH-018*, WIN 51,708, WIN 55,212-2 and WIN 62,577.

4.2.1 Activities against voltage-gated ion channels

All of the compounds were tested on the following channel subtypes of VGIC: K_v 1.1, K_v 1.3, hERG, Na_v 1.2, Na_v 1.3 and Na_v 1.5. Representative current traces (see Figure 11, where * marks the trace recorded in the presence of SCs, none of the SCs show any visible effect on tested channel subtypes; ° marks the trace recorded in the presence of 1 μM tetrodotoxin, a potent blocker of TTX-sensitive channel subtypes) showed no visible effect of 2 μM SC solutions on any of the channel subtypes tested. A higher concentration of 5 μM tested on Na_v 1.3, Na_v 1.5 and hERG, and also showed no effect. Positive control was

carried out on the sodium channel subtype Na_v 1.3 using a potent blocker tetrodotoxin[10] (1 μM); 100% inhibition was achieved (see Figure 11). Lack of activity on the hERG channel is beneficial, since approximately 60% of the chemical entities developed as potential therapeutic targets are withdrawn from subsequent studies due to the QT prolongation and hepatotoxicity, caused by activity on hERG. (38) Tested SCs can still be used as agents if further studies demonstrate their therapeutic value on some other biological target. Previously conducted studies on VGSC in mouse brain synaptoneurosomes report that anandamide, WIN 55,212-2 and CP-55,940 are operating as concentration-dependent allosteric inhibitors of [3H]batrachotoxinin A 20-α-benzoate[11] binding and that the WIN 55,212-2 also inhibits veratridine-dependent[12] depolarisation of synaptoneurosomes. (56) This findings suggest a novel mechanism for cannabinoid depression of transmitter release. Anandamide, an endogenous cannabinoid neurotransmitter, showed strongly analgesic effect in CB1 knockout mice, which supports the fact that cannabinoid receptors are not the only physiological target. (56) Further research is required to determine the exact mechanisms and consequences of blocking sodium channels. This is promising for the development of novel, more effective drugs to treat anaesthesia, sleep disorders, epilepsy, anxiety, cardiac arrhythmias and coma. (56) However, in our experiments we were using the TEVC technique, where only one subtype of ion channel was overexpressed at once. Indirect effects can be possibly related to multiple ion channels or an indirect pathway could not be detected with our technique.

[10] Neurotoxin produced by organisms belonging to the *Tetradontidae* order (puffer fish, ocean sunfish and porcupine fish)

[11] Batrachotoxins are extremely potent cardiotoxic and neurotoxic steroidal alkaloids found in some species of animals (poison dart frog, melyrid beetles etc.)

[12] Neurotoxin derived from plants in the *Liliaceae* family

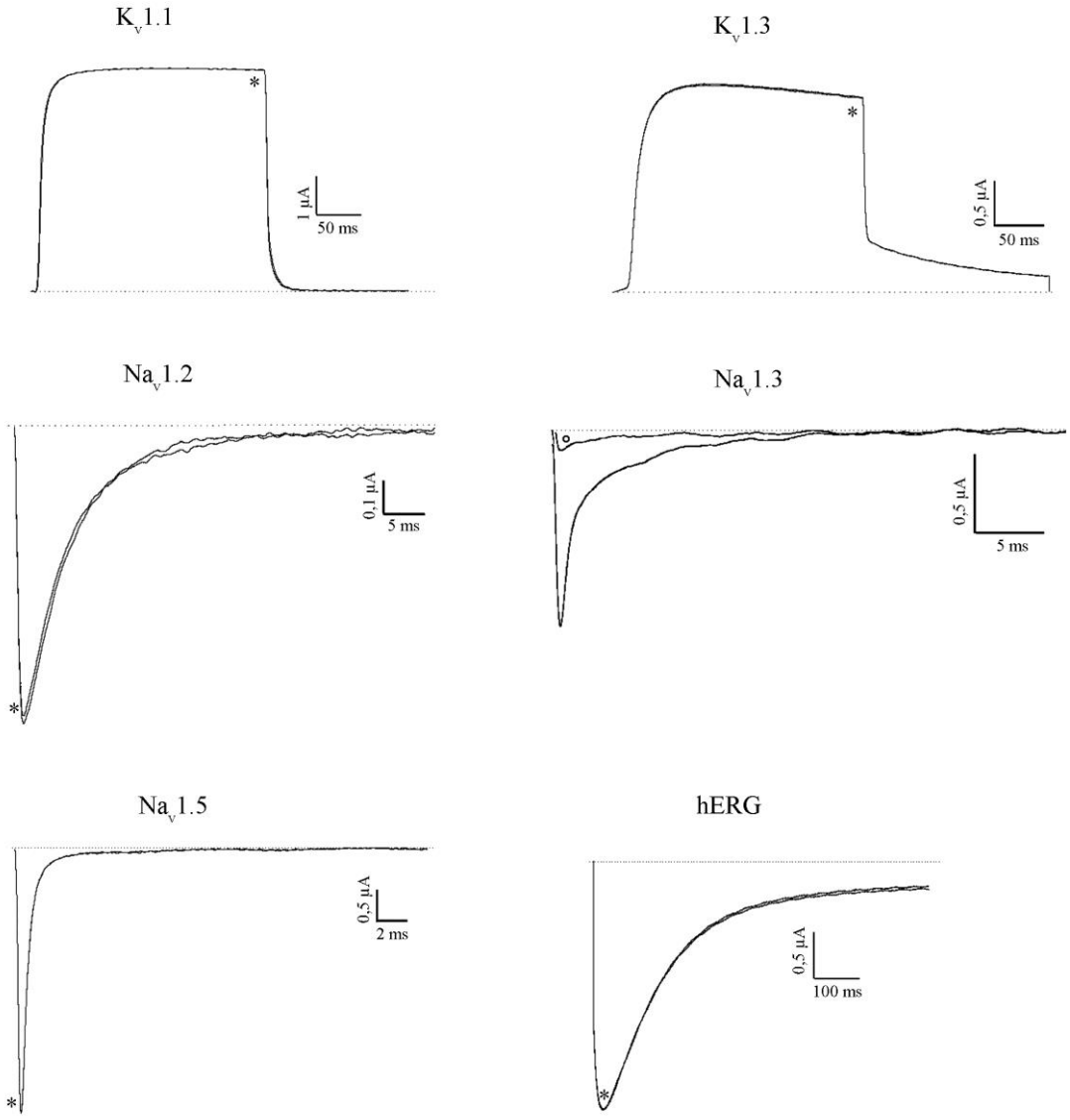

Figure 11: Representative VGIC current traces

4.2.2 Activities against TRPV1 channel

TRPV1 is located on the primary sensory neurons and represents a potential target for novel analgesics. The reduction of pain through TRPV1 can be achieved either by agonists, who cause desensitisation, or antagonists, which pharmacologically block the TRPV1 receptor. As previously described in section 3.2.1, capsaicin (2 µM) was used as an agonist and capsazepine (10 µM) as an antagonist of the TRPV1. Administration of 10 µM solutions of SCs alone showed no visible effect on TRPV1 (see the representative current trace

in Figure 12). When AM-2201, CP 55,940, WIN 62,577 were applied together with capsaicin, a small down-shift was detected (see Figure 12, where we first activated the channel with CAP and closed it with CAP+CPZ). Application of 10 μM SCs alone showed no visible effect, so we activated the channel for the second time with CAP (the channel is desensitised) and test the solution with CAP+SC combined. Plausible allosteric effect was observed in case of the three aforementioned SCs (just one graph is shown, as the effect was the same for all three). As the down-shift was very faint we can not confirm or refute this effect; it could have been the result of a change in the flow when we switched between solutions of CAP alone and CAP+SC combined. In order to confirm or refute the allosteric effect, the experiment should be repeated at least three times, but due to lack of time, we could not conduct all the measurements with higher concentrations as we had planned (20 μM). Even if the allosteric effect were present, this probably would not represent a significant result in the field of TRPV1 agonists, since the potency would be too low (drugs appropriate for additional research act as a potent inhibitors already at the 1 μM concentration; see Figure 12). The main obstacle for to conducting all required experiments was the *X. laevis* heterologous expression system, since the quality (viability and expression levels) and quantity of isolated oocytes are dependent on the seasonal influences and could not be predicted. (57)

There are many opportunities for further study. While acid solutions are known to evoke ion currents in TRPV1 channel, another approach of testing would be to lower the pH of ND-96 perfusion solution to ~5.4. (46) Surprisingly, gadolinium (III) ions are also known to potentiate TRPV1 activation by capsaicin, therefore they are an interesting approach that could be also added to the perfusion ND-96 solution. (46) Since synthetic cannabinoids and the TRP family of ion channels are relatively new discoveries, few studies have been conducted to date. However, the results of *Patwardhan* study show that pre-treating isolated male rat neurons with WIN 55,212-2 directly and reversibly inhibited capsaicin-induced calcium influx by dephosphorylating and desensitizing TRPV1 in nociceptors via a calcium calcineurin-dependent mechanism. (20) The inhibition depends on the presence of Ca^{2+} influx that activates protein phosphatase 2B (calcineurin), which is involved in regulation of TRPV1 responses. (20) We therefore also used Ca^{2+} ions in our perfusion solution ND-96 (for the composition of the ND-96 see section 3.2.1), but as mentioned before, we de-

tected no direct effect on TRPV1. Some studies also hinted that cannabidiol[13], which reverses thermal or mechanical hyperalgesia, might be mediated via TRPV1 and that the action of WIN 55,212-2 on CB1 receptor suppresses TRPV1-induced inflammatory responses to corneal injury. (7; 45) SCs therefore have a potential to be used as novel therapeutics, not only abused as drugs as they have been so far.

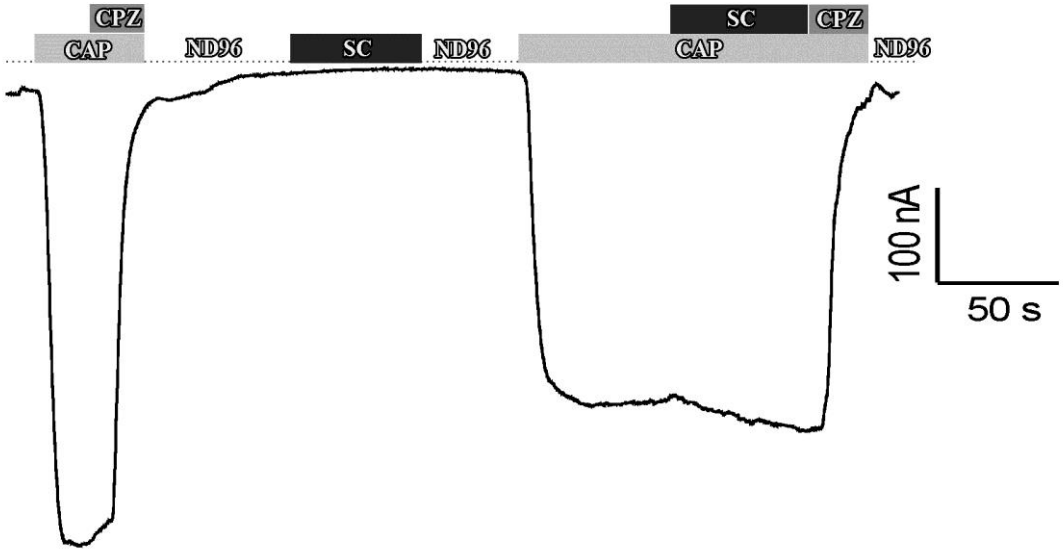

Figure 12: Electrophysiological tests on TRPV1

[13] One of the cannabinoids found in marijuana – represents up to 40% in its extracts.

5 CONCLUSIONS

Identification of novel psychoactive substances, including synthetic cannabinoids, represents a recent challenge for clinical, forensic and workplace drug-testing laboratories. Over a hundred SCs were synthesized so far, with new ones appearing regularly. These new substances cannot be detected using routing screenings for THC or its metabolites. Therefore, suitable analytical methods for these new substances must be developed.

We successfully optimized the compound-dependent parameters to detect eight different synthetic cannabinoids by tandem mass spectrometry using a 3200 Q TRAP LC/MS/MS system. We determined the optimal parameters to detect these compounds with the highest intensities (see Table IV) and the four most abundant MRM transitions for each compound. These were 129.2, 229.2, 330.1 and 457.0 for AM-251; 76.0, 91.1, 79.1 and 105.1 for AM-356; 77.1, 126.6, 127.1 and 155.1 for AM-2201; 71.1, 77.1, 121.2 and 233.3 for CP 55,940; 115.2, 141.2, 169.2 and 214.2 for JWH-018*; 220.2, 234.2, 236.2 and 344.3 for WIN 51,708; 77.1, 100.1, 127.2 and 155.2 for WIN 55,212-2; and 232.2, 233.2, 234.3 and 246.3 for WIN 62,577. The MRM transitions were compared to findings from previous studies.

When testing the pharmacological effects, the main purpose was to find out if any of the selected SCs modulate the tested channel subtypes and are consequently related to intoxication symptoms after the use of SC mixtures. Our results showed that none of the tested SCs modulate TRPV1 channels. The results for the three SCs tested on TRPV1 are preliminary; additional experiments will be necessary to confirm or refute the small down-shift after the application of combined solution of SC with capsaicin. We also tested SCs on other types of voltage-gated ion channels to confirm the selectivity. However, again none of SCs showed any inhibition of VGIC. The lack of visible effect on hERG channel is beneficial, since its modulators cause serious side effects and can not be used as pharmacological agents. While no channel was directly modulated, additional research is necessary to discover the physiological targets and the correlation with reported unwanted effects.

In further research the metabolic transformations of SCs should be tested, since they are frequently related to side effects. Based on the effects of cannabinoids, such as euphoria and stress reduction, I would test these compounds on the group of serotonin receptors.

Some studies already suggested that 5-HT_3 and 5-HT_{1A} may also be stimulated by cannabinoids. (11) The VGPC subtype K_v 1.2 should also be tested, since it is an important player in multiple sclerosis spasticity, where cannabinoids have already shown some therapeutic potential. (11)

Synthetic cannabinoids and voltage-gated ion channels remain intriguing targets for further studies and have a big potential in discovery of new therapeutics. The research has only started a few years ago, so there is still a lot to unravel.

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