Instituto Superior de Ciências da Saúde Egas Moniz

MSc Erasmus Mundus in Forensic Science

A Comprehensive Study of Herbal Blends from Portuguese Smart Shops: Isolation, Analysis and Toxicological Impact.

Inês Costa Lopes

Julho 2014

Instituto Superior de Ciências da Saúde Egas Moniz

MSc Erasmus Mundus in Forensic Science

A Comprehensive Study of Herbal Blends from Portuguese Smart Shops: Isolation, Analysis and Toxicological Impact.

Supervisor: Alexandre Quintas

Co-supervisor: Rita Soeiro

Inês Costa Lopes

Julho 2014

Certificate of Originality

This is to certify that I am responsible for the work submitted in this thesis and that the work is original and not copied or plagiarised from any other source, except as specified in the acknowledgements and in references. Neither the thesis nor the original work contained therein has been previously submitted to any institution for a degree.

Signature:

Name: Inês Costa Lopes

Date: 15th July 2014

Acknowledgements

To all members of my family for all the patience, availability and encouragement throughout the Masters.

To my colleagues and friends.

To Professor Alexandre Quintas for all the encouragement and support.

To Professor Rita Soeiro for all the support, patience and help.

To Joana Couceiro who perfomed the biological assays.

To Tiago Outeiro from Cell and Molecular Neuroscience unit (Instituto Medicina Molecular) who kindly provided the Human Neuroblastoma-Glioma (SH-SY5Y cell line).

To Dr. João Ruivo and Dr. Patrícia Ruivo from the LAD (Laboratório de Análises de Dopagem) who kindly performed the LC-MS analysis.

To Dr. Mário João Dias from INML (Instituto Nacional de Medicina Legal – delegação do Sul) who kindly performed the GC- MS analysis.

To my teachers at University of Cordoba, Spain and University of Lincoln, UK for their support and teaching which laid the foundation for this research project.

To Executive Agency Education, Audiovisual and Culture (EACEA).

To Erasmus Mundus Programme.

Abstract

In the last few years, several samples were found to contain different combinations of multiple synthetic cannabinoids, fact which may end up in a serious health threat since these substances are not fully studied and the toxic as well as the pharmacological effects are not known. The isolation and quantification of psychoactive substances from the herbal blends, known as 'Spice', was performed by HPLC-UV and the identification by LC-MS/MS. The HPLC-UV method was validated for the quantification and has proven to be fit for purpose. The sample 1 has shown the coexistence of JWH-122 (29,07mg/g) and JWH-210 (175,9mg/g) while the sample 2 was found to contain one single synthetic cannabinoid, the JWH-018 (149,7mg/g). The sample 3 presented the same multiple psychoactive substances as sample 1, JWH-122 (7,3mg/g) and JWH-210 (129,9mg/g). Since these drugs are mainly smoked, a smoking machine was created* in order to characterize the chemical composition of the pyrolysis. The GC-MS analysis performed to the pyrolysis solutions has shown no degradation of the JWH-018, drug used for the assay. The toxicity impact of the characterized substances was assessed on human neuroblastoma SH-SY5Y cells, applying a combination of multiple synthetic cannabinoids (JWH-122 and JWH-210) and single one (JWH-018). The preliminary results indicate that these psychoactive substances are not toxic to the cells in the concentrations ranging from 0µM to 50µM.

*Designed by Dr. Alexandre Quintas and is under a patenting process

Keywords: synthetic cannabinoids, JWH-018, JWH-122, JWH-210, isolation, quantification, HPLC-UV, LC-MS/MS, GC-MS, smoking machine, toxicity

Contents

Figure Content

Abbreviations

CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EU	European Union
EWS	Early Warning System
GC-MS	Gas Chromatography - Mass Spectrometry
HPLC	H
LC-MS	Liquid Chromatography - Mass Spectrometry
MTT	MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution
NMR	Nuclear Magnetic Resonance)
NPS	New Psychoactive Substances
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
PBS	Phosphate buffered saline
SH-SY5Y	human neuroblastoma SH-SY5Y
THC	Δ^9-tetra-hidrocanabinol
UNODC	United Nations Office on Drugs and Crime

1. Introduction

Novel Psychoactive Substances (NPS) are substances which are not controlled by the convention from 1961 'Single Convention on Narcotic Drugs' or either from 1971 'Convention on Psychotropic Substances' that may result in a health threat. (UNODC, 2013b) These are defined as 'compounds that are formally (not synthetically) derived from the structure of a well-known compound' (EMCDDA, 2012) exhibiting slight chemical modifications, however still similar to the basic structures of the studied compounds.

On the global market over the period 2009-2013, the number of NPS more than doubled - by December 2013, the number of such substances reported to UNODC reached 348. These synthetic drugs can range from synthetic cannabinoids (known as incense or spice) to synthetic amphetamine-like drugs (UNODC, 2013b). The present research study is focused on synthetic cannabinoids, substances which have been highly reported in the last two years. (Figure 1).

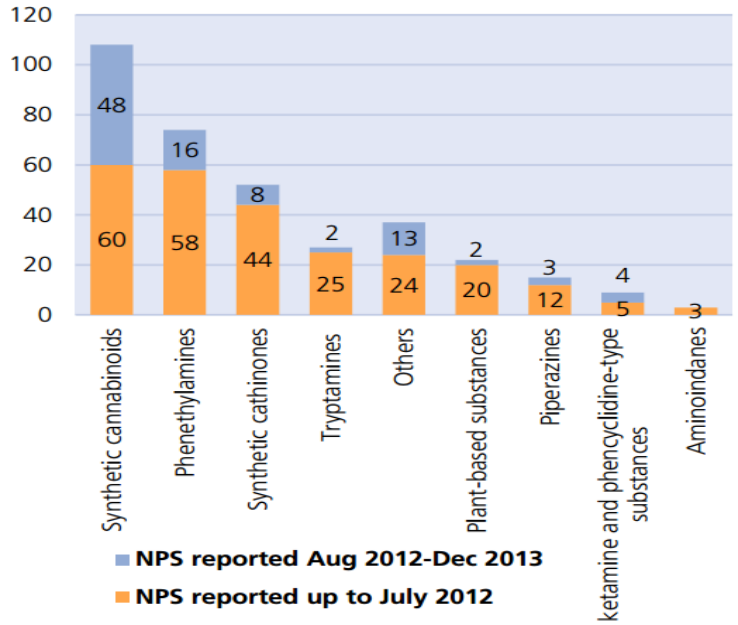

Figure 1- New psychoactive substances reported to UNODC by December 2013 (UNODC, 2014)

1.1.Synthetic Cannabinoids

Back in 2008, the Europol joint with the European Monitor Centre for Drugs and Drug Addiction (EMCDDA) identified in herbal mixtures (so called 'Spice') synthetic cannabinoids which are defined as pharmacologically active ingredients that affect both cannabinoid 1/cannabinoid 2 receptors (CB_1/CB_2), being functionally similar to Δ-9-deltahydrocannabinol (THC), principal molecule of cannabis (EMCDDA, 2008). The CB_1 receptor is present mainly in the brain and spinal cord and is responsible for psychotropic (mood and behavior alterations due changes in chemical levels in the brain) and physiological effects of cannabis. The CB_2 is mainly located in the cells of the immune system and in the spleen being able to mediate immune-modulatory effects. (Porter & Felder, 2001)

Sold from 0,5g to 3g per packet and known as well as incense or herbal blends, the first generation of these 'Spice' products had names as 'Spice gold', 'Smoke', 'Yucatan Fire' or 'Spice silver' (Figure 2). The highly attractive packets describe the ingredients as plant material (wide range of various herbs) without mentioning the addition of chemical additives. Some of these products state on the label that the herbs are able to produce similar effects to cannabis to make believe that the pharmacological effects are due to the natural constituents. With the increase of popularity, these products which started being commercialized mainly online started being available in physical shops, so called as 'smartshops' or 'headshops'. (UNODC,2011)

Figure 2 - 'Spice' packet

1.2.Mode of administration

The main route of the administration of synthetic cannabinoids is usually through inhalation (smoking) (UNODC, 2011), being the main reason the quick absorption and consequent fast onset of the desired effects. These substances are present in the market as pills however the oral administration leads to a delayed onset of the effects due to the metabolism that takes place before the substance reaches the blood. The use of synthetic cannabinoids as tea (infusions) is not so common due to the low solubility in water. The administration through parenteral routes has not been described so far.

1.3.Chemical classification

Cannabis is widely considered as the illicit drug most used in developed societies. This all started as a consequence of THC isolation in the 1960s, when a wide range of different synthetic exogenous cannabinoid receptor agonists were synthesized. At that time, J.W.Huffman focused his research on the synthesis of THC analogues and metabolites initially with the purpose of studying the interaction of drugs in the brain, in order to develop pharmaceutical products. Later on, these substances started to be commercialized with a second purpose. The JWH-018, which is by far the most known synthetic cannabinoid and is considered to be up to three times more potent than THC, belongs to the aminoalkylindoles group (as most of the JWH compounds) This group is divided into seven considered structural groups: a) Naphthylmethylindoles (e.g. JWH-018, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210), b) Phenylacetylindoles (e.g.JWH-250, JWH-251), c) Benzoylindoles, d) Naphthylmethylindoles, e) Cyclopropoylindoles, f) Adamantoylindoles and g) Indole carboxamides. (UNODC, 2013b)

In addition to these substances, there are other synthetic cannabinoids, which include the cannabinoid produced by Raphael Mechoulam (1060s) at Hebrew University named as 'HU-210' and considered the first synthetic analogue of THC. Due to its chemical similarity with THC, it is regarded as 'Classical Cannabinoid' (Figure 3). The 'Non-Classical Cannabinoids' were developed by Pfizer in the 1970s as potential analgesics and are named as 'CP' (cyclohexylphenol). Alexandros Makriyannis synthesized the 'AM' compounds which are other indole-derived cannabinoids detected as well in many herbal products. These are considered as 'Hybrid Cannabinoids' since they combines features from both 'classical' and 'non-classical' cannabinoids. (Seely, 2012) The 'Eicosanoids' group are endocannabinoids (e.g. anandamide – AEA) and their synthetic analogues. (UNODC, 2013a)

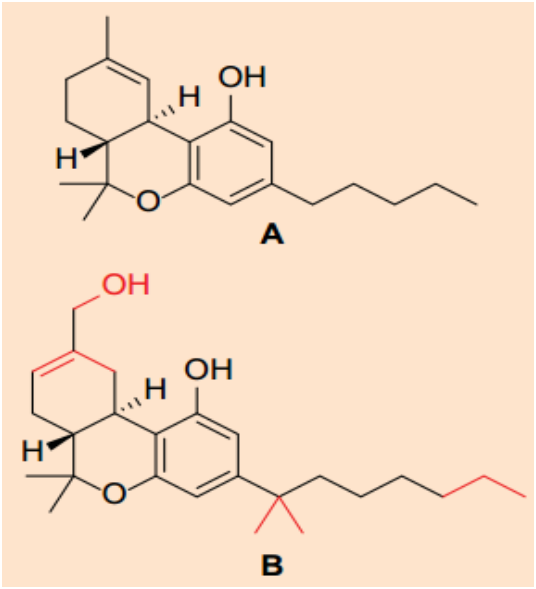

Figure 3 - Chemical structure of (A) Delta-9-THC; (B) HU-210 (UNODC, 2013b)

1.4.Effects

The packages frequently state that the content is 'not for human consumption', however the belief that synthetic cannabinoids are safe, licit and non-toxic is still persistent. Actually, some of these compounds, considered as legal alternatives to the illegal cannabis, might be more powerful than the basic THC (Δ^9-tetrahydrocannabinol) however, any safety information (doses and side effects) as well as chemical information (list of active pharmacological agents) is not provided due to lack of regulation (Seely *et al.*, 2012). Although the cannabis adverse effects are known, the information available concerning the short and long-term of synthetic cannabinoid effects is limited. Although unreliable, nowadays the most abundant source with some of the side effects of these new substances is within the internet, namely in forums where the consumers exchange knowledge about the body reaction after its consumption. These range from disorientation, psychosis, sedation, delusions, agitation to tachycardia. (Lapoint *et al.*, 2011; Simmons *et al.*, 2011; Zimmermann *et al.*, 2009)

Due to the ever-changing cocktail blend of chemicals that characterize these substances, the effects in the users are not the same. Studies to assess how similar these packages were, found that the chemical composition (namely the presence of synthetic cannabinoids) was the principal variable to account for sample differentiation. Moreover, it was found that correlation of substances found among products takes into account the flavor of the herbal preparation however not the name from which is known. (Zuba *et al*., 2011) This may be a potential danger since the consumers rely on the fact that the products with the same name end up containing the same substances. Since the user has no information related with the active substances present in the herbal product, this phenomenon should be considered as a threat since it may culminate in a series of toxic effects not expected by the consumer.

1.5.Most relevant studies

Along the past few years, several studies have focused on the identification and quantification of synthetic cannabinoids and respective derivatives in herbal products as adulterants (Nakajima *et al*., 2010; Uchiyama *et al*., 2010; Dunham *et al*., 2012) or even more recently, the study of their metabolites (Moran *et al*., 2011; Rajasekaran *et al*., 2013; Scheidweiler & Huestis, 2014), however the secondary and toxic effects hidden behind

these products still remain uncertain. Actually, Zuba *et al*. (2011) identified a range of active compounds present in herbal products and compared the chemical composition found in each one of them. Although many of these substances have been submitted to several studies (Nakajima *et al*, 2010; Durham *et al*., 2012; Ernst *et al*., 2012) and these are already considered illegal, there are no experiments that assess the toxicity of these in combination with other derived substances.

Aggravating the situation, several studies revealed that several samples contained different combinations of multiple synthetic cannabinoids, which may end up in a serious health issue (UNODC, 2013b) since these substances are not fully studied and the toxicity as well as the pharmacological effects are not known (Camilleri *et al*., 2010). Moreover, the presence of multiple synthetic cannabinoids in herbal mixtures has been recurrent and the assessment of the toxicity inherent to the co-existence of more than one compound has not been studied so far. This phenomenon characterized by the multiple presence of different synthetic substances in herbal products has become common that countless studies (Uchiyama *et al*., 2010, 2011; Zuba *et al*., 2011; Choi *et al*., 2013) are reporting findings of different combinations of substances. Subsequent to this fact, Atwood *et al.* (2011) have shown that most of these new substances that nowadays are part of herbal blends composition, have similar or higher affinities and potencies to CB_1 and CB_2 receptor when compared with the JWH-018. Although the psychoactive effects produced by these substances are due to this pharmacodynamic occurrence meant to mimic Δ9-THC substance, similar affinities and potencies detected may result in higher toxicity than are known for pure cannabis.

In 2011, Tomiyama & Funada (2011) studied the cytotoxicity of some synthetic cannabinoids (CP-55,940, CP-47,497 and CP-47,497-C8) upon NG 108-15 cells and it was found that these were cytotoxic to the cells (on a concentration dependent manner - ≥75–100 μM), inducing cell apoptosis. However, this cytotoxicity was mediated mainly by the CB_1 receptor, but not by the CB_2. Later on in 2014, along with the synthetic cannabinoids that had been tested previously, HU-210, JWH-018, JWH-210, AM-2201, MAM-2201 were also added to the study, this time using mouse brain neuronal cells (Tomiyama & Funada, 2014). These results allowed the same conclusion to be made: the cytotoxicity induced (cell apoptosis) by synthetic cannabinoids was mediated by CB_1 but not CB_2 receptor, depending as well on the concentration applied. Summary, the recurrent use of synthetic cannabinoids may end up in neuronal cell damage.

1.6.Emergence of synthetic cannabinoids

New variants of these substances are synthesized every day by clandestine laboratories in order to circumvent the current legislation, these being defined as 'compounds that are formally (not synthetically) derived from the structure of a well-known compound', exhibiting slight chemical modifications however still similar to the basic structures of the studied compounds (EMCDDA, 2012). This occurrence has been followed and discussed by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) as well by the United Nations Office on Drugs and Crime (UNODC). Based on the latest figures obtained from last year's report (2013), in 2012 the European Union (EU) seized 73 new types of psychoactive substances from which 30 were synthetic cannabinoids. It also indicates that 84 synthetic cannabinoids in total are being monitored by EU Early Warning System (EWS). Europe is by far where synthetic cannabinoids are seized (Figure 4). The emergence of these substances in Europe arose in a large scale in 2008, reaching its peak in 2010 when ten countries have reported these compounds (Belgium, Bulgaria, Croatia, Lithuania, Luxembourg, Malta, Netherlands, Slovakia, Spain and Turkey) (UNODC, 2013b). As dominant compounds, the 'JWH' series headed the list of compounds detected in the 'Spice' products. Up to 2012, JWH-018, JWH-073, JWH-250 and JWH-081 were the most widespread synthetic cannabinoids in the market (UNODC, 2013b).

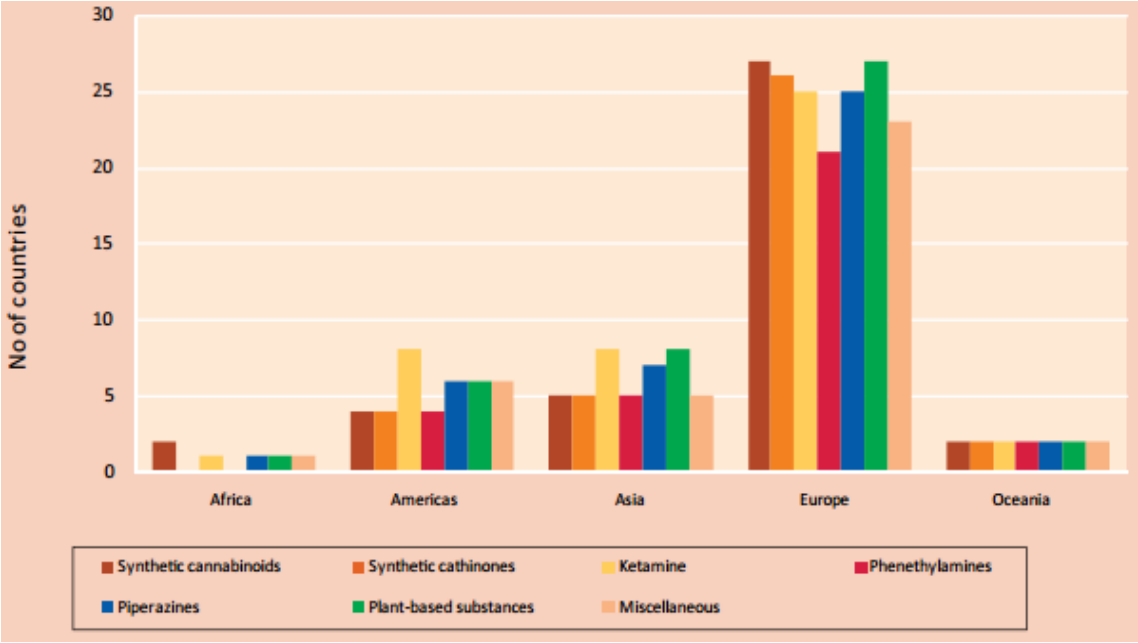

Figure 4 - NPS seizures by region, 2009 – 2012. (UNODC, 2013b)

1.7.Legislation

Synthetic cannabinoids became popular due to the relative chemical dissimilarity to the original cannabis, what led it to being considered 'legal' by the users. Over the past few years, drugs created in laboratories as derivatives and analogues of a parent drug (cannabis in this case) have shown a startling rise in popularity and suddenly became one of the most common misused substances (NPS). However, the prohibition did not stop the trade of these products and it is known that the demand and use of these drugs after being banned by local authorities did not slow (Vandrey *et al.*, 2012). While cannabis and THC are substances which are internationally controlled under international conventions (1961 and 1971 conventions) none of the synthetic cannabinoids are. The legal status of these substances are defined by each country (UNODC, 2011) and in Portugal, these substances were banned in 2013, later than in the other European Countries. Efforts have been made in the last few years by governments around the world, which are fighting against the growing threat of synthetic drugs, banning their import, sale, and use. However, the problem lies on the chemistry behind this drugs, which seems to be capable to circumvent the laws by changing a single molecule and an illegal synthetic drug can suddenly become legal again.

1.8.*Methods of analysis*

The analysis of herbal blends which are suspected to contain synthetic cannabinoids requires an extraction technique able to extract the lipophilic compounds from the herbal matrix, using an organic solvent. Liquid chromatography–mass spectrometry (LC-MS) and gas chromatography –mass spectrometry (GC-MS) are the most used techniques to identify the main active compounds present in the sample. Whenever new substances are found (no correspondence with the spectra library), there is the need to isolate and characterize these compounds. The isolation of substance is commonly performed by high performance liquid chromatography (HPLC) (collecting the fraction that corresponds to the unknown peak) and the structure elucidation achieved by ^{1}H- and ^{13}C-NMR (Nuclear Magnetic Resonance). This methodology is recommended by UNODC (2013a), however it has been used in several studies and confirmed the efficiency of the processes (Uchiyama *et al.*, 2010; Nakajima *et al.*, 2010; Zuba *et al.*, 2011; Dunham *et al.*, 2012; Ernst *et al.*, 2012; Moosmann *et al.*, 2013).

1.9.Challenges: Lack of Standards

The current available literature about synthetic cannabinoids leads to the creation of new and more complex derivatives of these already studied substances by illegal laboratories. These slight changes performed in the chemical structure of the compound are made in order to circumvent the law but keep the cannabinoids-type effects. Over the last few years, it has been found more frequently the presence of new compounds in herbal products, which were not identified beforehand. Since illegal laboratories are the producers of these substances, they are always one step ahead from chemical companies due to the latter not having the capacity to produce synthetic cannabinoids standards as fast as new derivatives are produced. The use of reference material is required in any analysis in order to obtain reliable qualitative and quantitative results. The lack of standards of these substances might turn the identification process of these compounds into complex and high cost procedure.

Moosmann *et al.* (2013) described a method to isolate new synthetic cannabinoids from herbal products with such high purity that can be used as reference material (standards). The substances present in the herbal blends were identified by GC-MS and isolated by a new flash chromatographic method, which allows the isolation of a great percentage of the compound. In addition, a Nuclear Magnetic Resonance (NMR) technique was used as complementary technique, in order to elucidate the chemical structure of the new substances identified previously. In a previous study, Moosmann *et al.* (2012) reported the identification of two new synthetic cannabinoids in herbal blends, with a parallel methodology resorting also to the isolation of the compounds to be used as own standards.

Since it is expected that new substances will often be found and the respective standards are still not being commercialized, there is the need to isolate those substances and obtain the respective reference material. Exceeding a purity of 99%, this approach has proven to be fit for purpose and the isolated compounds were able to be used as a reference material.

1.10. Cytotoxicity

The SH-SY5Y human neuroblastoma cell line has widely been used since these contain important functional and biochemical properties of neurons. The cannabinoid receptors present in these cells allows the investigation of synthetic cannabinoid-induced cytotoxicity. (Hong-rong *et al.*, 2010) The simplicity of the MTT method as well as short time that is needed to obtain results, turns this assay into one of the most used assays to assess cytotoxicity and cell viability. It consists of a colorimetric test which measures the intrinsic ability of the mitochondrial dehydrogenases to reduce the MTT (yellowish color) to formazan (purple color). The formazan product can be detected colorimetrically and estimated by spectrophotometry. (Stockert *et al.*, 2012)

1.11. Pyrolysis

The characterization of the chemical composition of some of the smoke constituents has been performed by Daw *et al*. (2014). A Borgwaldt KC smoking machine was used to test JWH-018, JWH-250 and AM-2201. It was found that the pyrolysis process leads to the production of several active pyrolysis products, which can include other synthetic cannabinoids -JWH-018 and JWH-122 have been formed. These reaction products, apart from being active, have a certain toxicity associated which at present is unidentified. Moreover, these products might have dissimilar affinities to CB_1 and CB_2 receptor and thus, extend or even aggravate the adverse effects. This is a single study in the pyrolysis content since this process has not been a search target.

1.12. Aims

In 2013, Zuba *et al*. (2013) presented results of a long-term study (between 2008 and 2011) on 'legal highs' seized samples from head shops which analysis culminated in inconsistency of qualitative and quantitative compositions when it came to the analysis of identical labelled products. In this sense, the present study introduces a new component that might be crucial to understanding and exploring the toxicity that might be associated with the presence of multiple synthetic cannabinoids in the same sample. The main aim of this research is to study the toxicity induced on cells (human neuroblastoma SH-SY5Y), when a combination of different synthetic cannabinoids is applied. In order to

explore the possibility that fact that the coexistence of two or more synthetic cannabinoids in the herbal products might produce higher toxicity on the cell, rather than exposed to just one compound. In order to achieve this, a complete process of isolation and identification of the synthetic cannabinoids present in the herbal blend is needed. On the other hand, as a second perspective of the toxicity, a smoking system was created in order to mimic the consumption of these compounds and characterize the chemical composition of the products.

2. Methods and Materials

2.1.Chemicals and reagents

The methanol used in the extraction of the substances from the herbal matrix was bought from VWR. The formic acid and acetonitrile (Carlo Erba) used as analytical reagents grade for the HPLC gradient were purchased from SigmaAldrich®. The MiliQ water used in the dilutions was obtained through Millipore UV Simplicity equipment (Merck Millipore).

2.2.Samples for analysis

A total of 3 herbal packages sold as herbal blends (Figure 5) were purchased in smartshops localized in Lisbon, in March of 2013, before the ban of these substances in Portugal. It were named as sample 1, sample 2 and sample 3.

Figure 5 - (A) Sample 1; (B) Sample 2; (C) Sample 3

2.3.Extraction

The extraction procedure was based on the 'Recommended methods for the Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials' (UNODC, 2013) The procedure performed up to the dryness as well as the amount of solvent was similar in order to compare results. 1 mg of each herbal mixture was precisely weighed and then extracted using an extraction solvent under sonication bath for 10 min. Different extraction solvents (methanol and hexane) were tested. The extraction process described above was performed using methanol and hexane as extraction solvents and the fraction concentration quantified by HPLC. The solution was passed through a 0.45 µm filter (Interlab) to obtain the final extract solution. In order to increase the extraction, the process was performed twice using a total volume of 2ml.

2.4.Isolation and quantification of substances by HPLC

The UNODC (2013) recommends the use of LC in order to obtain the fraction containing the compound of interest. The isolation of the compounds was performed by HPLC (HITACHI LaChrom from Merck) with an UV detector (L-7400) at 315nm of wavelength, using LiChrospher® 100 RP-18 (5 µm) LiChroCART® 125-4 column bought from Merckmillipore. The analysis was carried out with a binary mobile phase, consisting of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). An elution program (1) with a linear gradient was used for the analysis of the substances, defined by a gradient starting at 70% A (2 min hold) to 0% A (3 min hold) at a flow rate of 1 ml/min (total of 30 min run). A second elution program (2) with a linear gradient was used for the isolation of the substances, defined by a gradient starting at 30% A (2 min hold) to 0% A (3 min hold), at a flow rate of 1 ml/min (total of 15 min run). Using the second elution program, 20 µl were injected for quantification of the substances (same amount used for the calibration curve) and 100 µl for isolation. Later on, the procedure was carried on using a semi-preparative VDSpher 100 C18-M (5 µm) 250x10mm column bought from VDS Optilab in order to collect larger amounts of the analyte. The scale-up optimization from the analytical method to the preparative method was performed using standard formulas (Huber & Majors, 2007) to define the flow (Figure 6) and the injection volume (Figure 7). The elution program (2) with a linear gradient was used (gradient starting at 30% A (2 min hold) to 0% A (3 min hold)), however at a flow rate of 5 ml/min (total of 15 min run). The integration of the peak areas was performed used the software Merck-Hitachi Model D-700 Chromatography Data Station Software from Merck. In order to use the extracts on cells, the solutions obtained were evaporated to dryness and re-suspended in pure dimethyl sulfoxide (DMSO)(Sigma Aldrich, USA).

$$\frac{V_1}{V_2} = \frac{{r_1}^2}{{r_2}^2}$$

Figure 6 - formula to calculate the flow where: V_1 - Flow column 1; V_2 - Flow column 2; r_1^2- radius column 1; r_2^2- radius column 2

$$\frac{x_1}{\pi \text{ x } {r_1}^2} = \frac{x_2}{\pi \text{ x } {r_2}^2} \text{ x } \frac{1}{C_L}$$

Figure 7 - formula to calculate the volume injected where: X_1 – max. volume column 1; X_2 – max. volume column 2; r_1^2- radius column 1; r_2^2- radius column 2; C_L – ratio lengths of columns

2.5.Evaporation to dryness

A sample was chosen for this optimization procedure. The extraction described above was performed and the total volume obtained divided in three equal aliquots. One of the aliquots was chosen to go under the lyophilization, other through rotary evaporator and another through nitrogen stream. The lyphilization required the samples to be frozen at -80ºc before the process, which was carried using a Modulyo Freeze Dryer from Thermo Electron Corporation at an average of -47ºC and 200 mbar. No pre-treatment of the sample was needed for the nitrogen stream as well as for the rotary evaporator used was a Heidolph Laborota 400ºC at an average temperature of 60ºC and.

2.6.Calibration curve

Two calibration curves were performed using a JHW-018 standard. The calibrations solutions were prepared with acetonitrile, containing 0, 5, 10, 15, 20, 25, 30, 35 and 40 µl/ml. Each calibrator was injected in triplicate.

2.7.Method validation

The accuracy (bias), precision (intra- and inter-assay precision), linearity, limit of detection (LOD) and limit of quantification (LOQ) were evaluated in order to validate the analytical method. The two calibration curves were performed on two successive days in order to access the accuracy as well as the precision (intra- and inter-assay precision). The accuracy and precision were compared with the acceptance criteria (<20 % coefficient of variation for the precision and <±20 % for bias). Nine different calibrators were used in each calibration curve (being five the minimum recommended amount of calibrators), being the linearity assed from this data set. (STGTOX, 2013)

2.8.Identification of substances by LC-MS

The identification of the compounds was performed by liquid chromatography–mass spectrometry (LC/MS/MS) (LC - Waters 2795 MS - Waters Quattro micro) using an XTerra MS C18, 5 µm 2.1 x 150 mm column brought from Waters Quattro micro. The solution that was injected was made of 10µl of the sample and 50µl of internal standard

(deuterated standard JWH-018-d3 1 µg/mL), reconstituted in 200 µL of mobile phase (initial composition). The analysis was carried out with a binary mobile phase, consisting of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). A linear gradient was used for the analysis of the substances, defined by a gradient starting at 25% A (1 min hold) to 80% A (13,5 min hold) and then decreasing to 25% A (3min hold) at a flow rate of 0,3 ml/min (total of 30 min run). The tandem mass spectrometry (MS/MS) analysis was performed in full scan from 0 to *500m/z* in ESI mode.

2.9.Pyrolysis

Taking into account that smoking is the main route of administration of synthetic cannabinoids, a smoking system was created (Figure 8) in order to characterize the chemical composition from the pyrolysis products. With the purpose of mimicking the lungs, which are mainly constituted by water, an aqueous solution was used (Milipore water) as well as a methanolic solution to retain the lipophilic compounds.

For this experiment, it was used purified JWH-018 from sample 2, having methanol as solvent. The identification of compounds present in both solutions was performed on a GC-MS Agilent 6890A coupled with a MS detector Agilent 5973N using two spectra libraries, PMW_TOX2.L and SWGDRUG.L. The separation was done using an HP-5MS 5% phenyl methyl siloxane. The initial column temperature was set at 80ºC and was increased at a rate of 10ºC/min to 290ºC and help during 15min, with a total run time of 37min. 2µl of sample were injected using the automatic injector Agilent 7683 in the split mode (6:1) with the transfer line temperature set at 280ºC. The MS was operated under EI in full scan (50 to 550 *m/z*). For the GC-MS analysis the water solution was evaporated to dryness using the rotary evaporator and re-suspended in methanol.

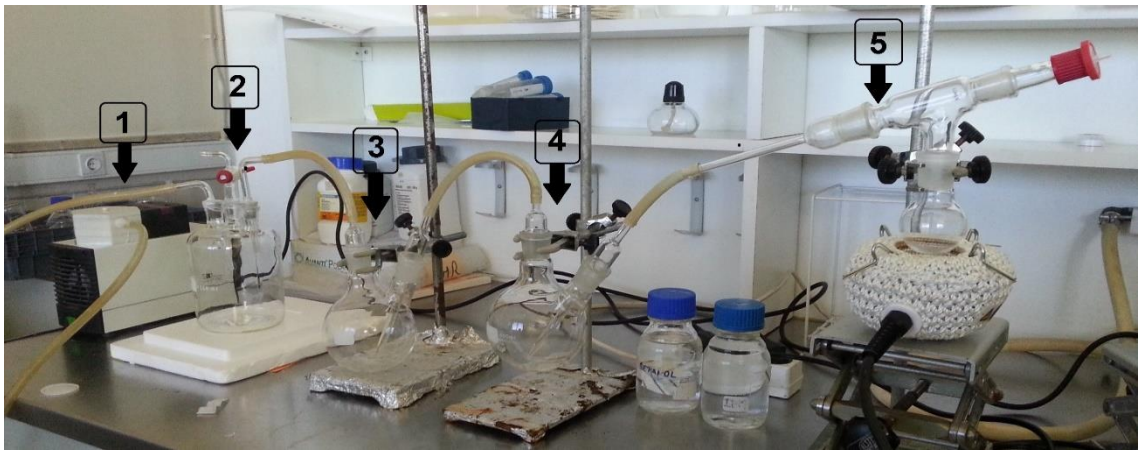

Figure 8 - Smoking system (non-steril environment). (1) Pump, (2) Security valve, (3) Methanol, (4) Water, (5) Heating blanket + volumetric flask with herbal blend

2.10. Cell Culture

The Human Neuroblastoma-Glioma (SH-SY5Y cell line), kindly provided by Dr. Tiago Outeiro, Cell and Molecular Neuroscience unit, Instituto Medicina Molecular, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, USA) supplemented with 10% Fetal Bovine Serum (PAA Laboratories GmbH, Austria), L-glutamine 2mM, Penicillin 100U/ml, Streptomycin 0.1mg/ml (all from Sigma Aldrich, USA). The PBS (phosphate buffered saline) (Fisher Scientific) was diluted to the physiological concentration using double distilled, filtered through 0.2µm (Orange Scientific Filters) water in sterile environment.

2.11. MTT Assay

After 24h of cell grown, the cells were exposed to 10, 25 and 50mM of the drugs being studied, in a 0,2%DMSO (VWR). After 24h incubation in optimum conditions (37ºC; humidified atmosphere, 5% CO_2), the medium is removed from each well of the microplate and each well is washed with DMEM to remove all traces of the test compounds. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution (MTT) was prepared at a concentration of 0,5mg/ml in culture medium and 200ul of this MTT solution was added to each well and each plate was incubated for 2,5 hours under the same standard conditions. After incubation, the MTT solution was removed and each well washed with 50 µl of PBS solution 1x. After washing with PBS, 200 µl of DMSO

(Dimethyl sulfoxide) solution was added to each well to dissolve the formazan crystals formed. Negative and positive controls were performed, being part of the negative control the cells with medium (without the drug) and an equivalent amount of solvent (0,2%DMSO) while the positive control was 0,1%Triton X-100 (Sigma Aldrich, USA). The plate was analyzed at 595nm under a microplate reader. The % of reduced MTT can be calculated dividing the absorbance mean of the sample by the negative control, then multiplying by 100 times.

3. Results and Discussion

3.1.Samples for analysis

The samples bought were suspected to contain synthetic cannabinoids in a random smartshop. Bought as herbal incenses, the packages advertised that was only for domestic use. All three stated the supposed main herbal compounds (e.g. coconut leaf, damiana, camomile) that they were produced in European Union (EU), however no further details were given.

3.2.Extraction

From the solvents tested, methanol was the solvent chosen as the extraction solvent. Apart from being a hazardous solvent, hexane did not achieve as good recovery percentages in the extraction procedure as methanol (50% less).

The extraction procedure was done right before the analysis in order to proceed with an accurate quantification of the compounds, taking in to account the possibility instability of the solutions (e.g. degradation of the compounds along the time). Multiple extractions performed to the same original sample tend to give better yields. Taking into account the ratio herbal matrix:solvent used (100mg:1ml) for the first extraction, the solvent may saturate and only a fraction of the total sample is recovered. This may explain the extraction of still a third of the total amount while performing the second extraction. For the same reason, a third extraction might increase the final yield, allowing a total recovery from the original sample. On the other hand the time of exposure to the vortex or/and the sonic bath might another variable that should be taken into account.

On the other hand the time of exposure to the vortex or/and the sonic bath might be another variable that should be taken into account. A longer exposure to these may promote the extraction of compounds from the herbal matrix and increase the overall recovery percentage.

3.3.Isolation and quantification of substances by HPLC

During the isolation process, it was found that both sample 1 and sample 3 presented two close peaks while sample 2 presented one single peak (Figure 18).Chromatograms from different aliquots obtained along the time, from the same sample, shown differences in the intensity of peaks. The chromatograms (A) refer to the first extraction performed and the chromatograms (B) refer to an extraction performed around one month later. In sample 1 (Figure 9), the ratio between peaks (I) is 0,60 and (II) from sample 1 (A) and (B) is 0,61 while the ratio among sample 2 (A) and (B) is 1,83. The main cause of these area differences might be related with the sampling process since the extraction process was the same (analyte, extraction solvent, time of exposure to vortex and sonication bath). The heterogeneity of the herbal blends turns the sampling process a crucial step to achieve some sort of consistency in results. Turn the herbal blend into powder could allow a better homogeneity of the sample, however, the conservation of this material during long periods might need to care since it can be exposed to humidity and some of the compounds may deteriorate (oxidize).

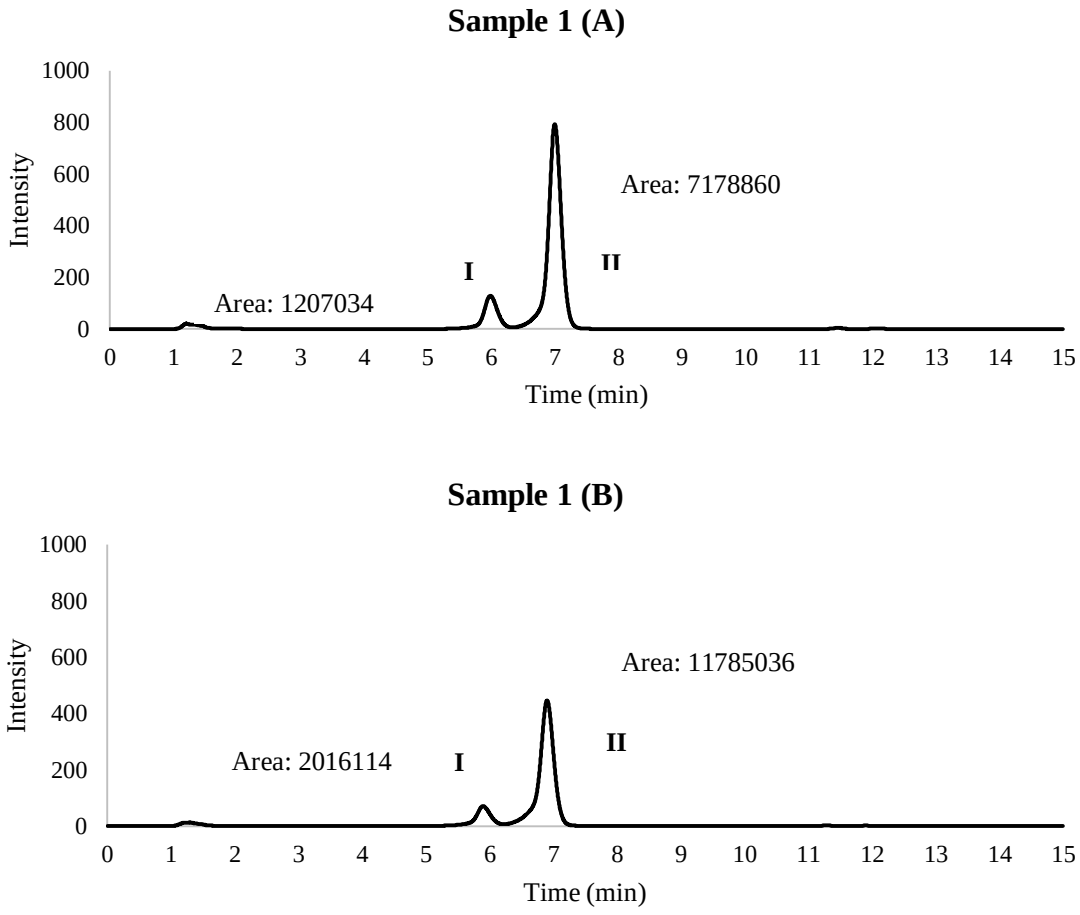

Figure 9 - HPLC chromatograms of Sample 1 (A) Primary data; (B) Secondary data

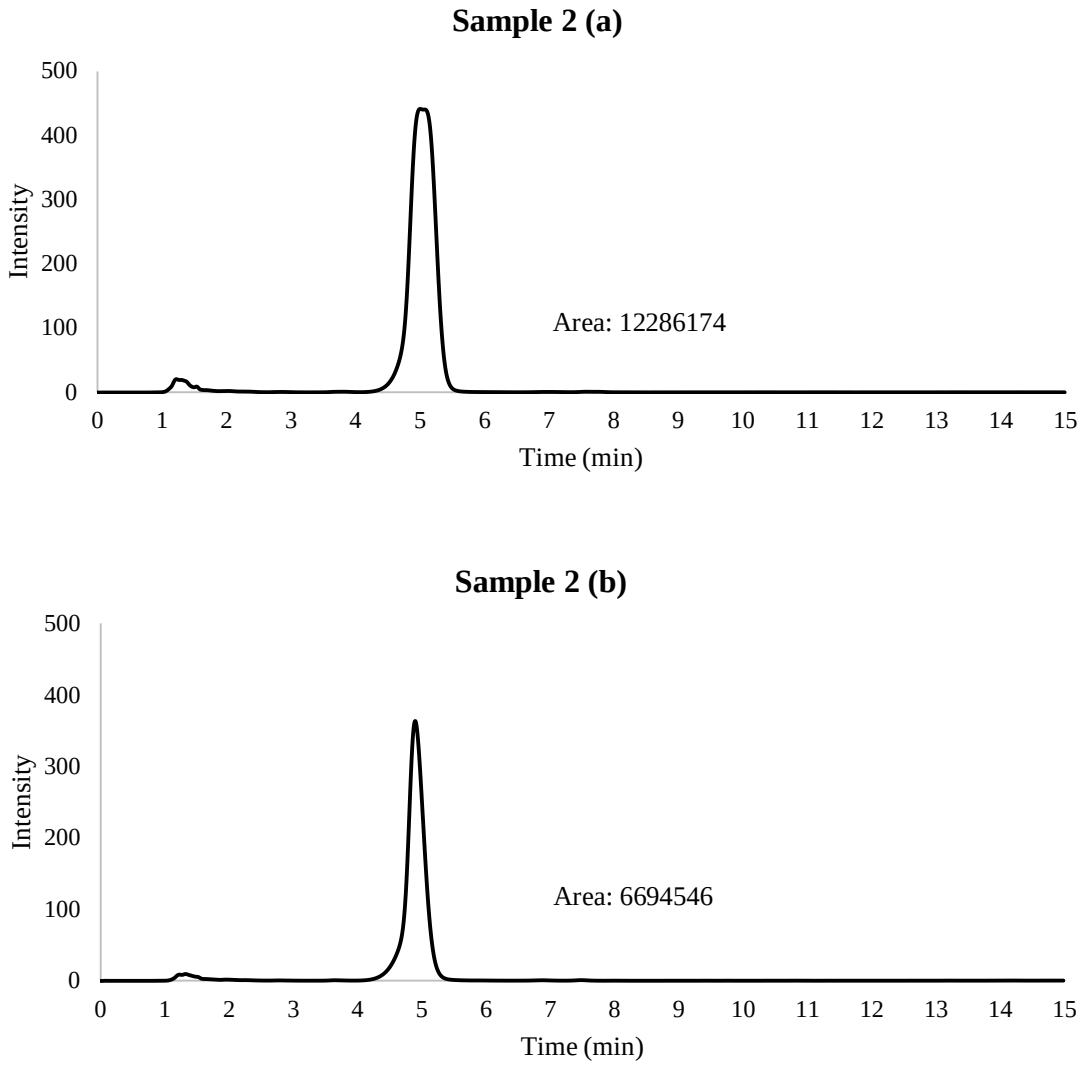

Figure 10 - HPLC chromatograms of Sample 1 (A) Primary data; (B) Secondary data

On the other hand, factors related with the HPLC equipment may affect the resolution of the separation. The resolution of the column tends to decrease with the use which can end up in a poorer separation of the compounds. Moreover, the collection of the purified fractions was made manually, which can lead either to a loss of analyte collected or the collection of a co-eluent peak. The low recovery percentages obtained might be strongly related with this fact, the manual collection of the fraction can lead to a great loss of the analyte to be collected. The best recovery percentage was achieved from sample 1 (20,51%), followed by sample 2 (14,97%) and sample 3 (13,73%). Not only the isolation process of a substance takes into account the loss of analyte in order to get this purified and avoid adjacent compounds, the extraction process might introduce a relative loss of compound.

Not least, it has to be taken into account that the HPLC reads in a single wavelength, being only possible to detect compounds which are read at the wavelength used. An UV spectra of the JWH-018 standard (Figure 11) was performed using a Jasco V-530 UV/VIS spectrophotometer in order to get the wavelength at which it reaches the maximum absorbance. This was found to be 315nm which means that all the other compounds which do not have the ability to absorb at this wavelength, are not detected. Subsequently to this, additional compounds may be present in the run without being detected and the isolation might compromised. Although the fractions collected were injected to confirm the presence of just one peak in the chromatogram), the purity of the analyte could not be determined.

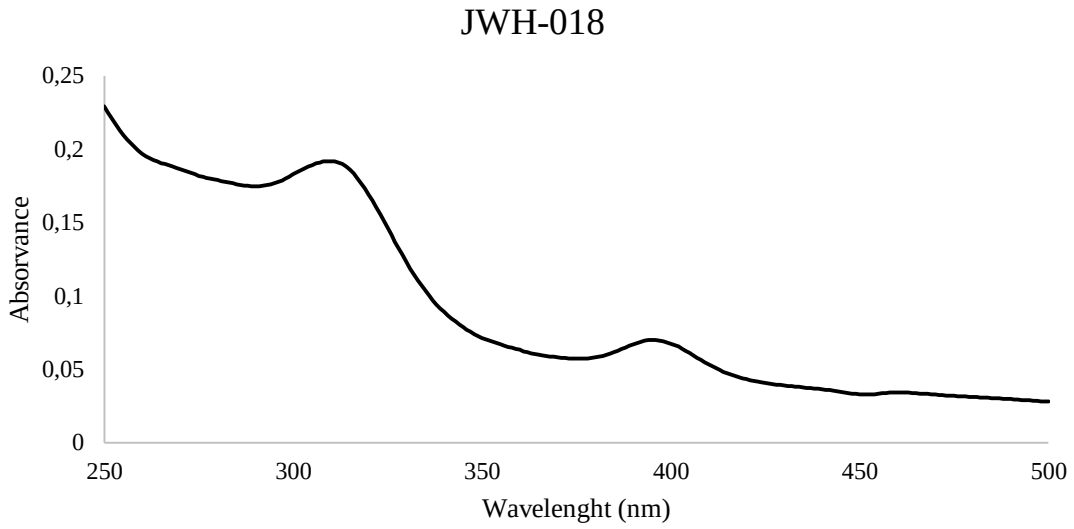

Figure 11 - UV spectra of JWH-018

The quantification was performed using the calibration curve with higher correlation value ($R^2 = 0,9982$) (Figure 12). In order to minimize possible negative effects from peak broadening, the peak area was used to determine the concentration of the substance being analyzed. To avoid extrapolation, the solutions were diluted in order to fit on the calibration curve. The content of synthetic cannabinoids has shown to be characterized by a wide range of concentrations, from 7,3mg/g to 176,6mg/g taking into account that the packages usually contain 1g of herbal mixture. The sample 1 has shown to contain 29,07mg/g of JWH-122 and 175,9mg/g of JWH-210, sample 2 contained 149,7mg/g of JWH-018 while sample 3 contained 7,3mg/g of JWH-122 and 129,9mg/g of JWH-210.

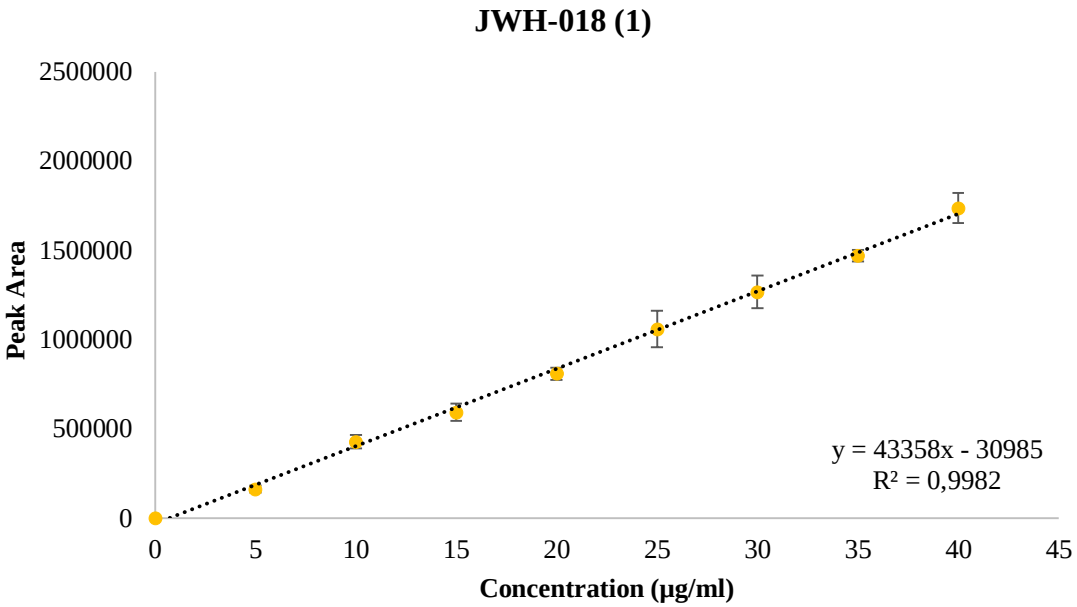

Figure 12 - Calibration curve (1) using JWH-018 standard

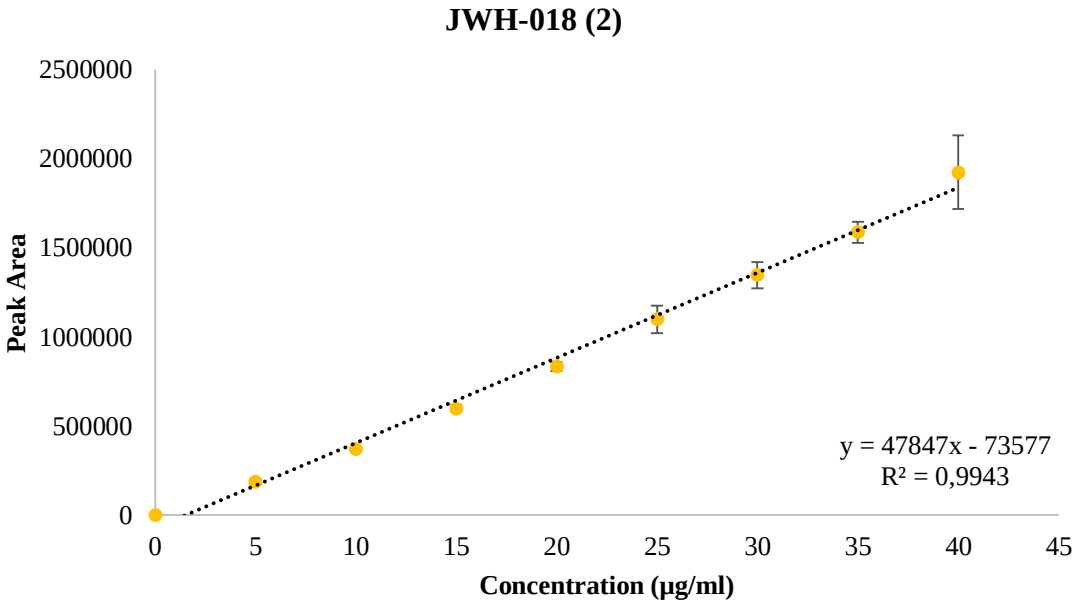

Figure 13 - Calibration curve (2) using JWH-018 standard

These values are an alert to the wide range of concentration of synthetic cannabinoids that might be present in the herbal products which comply with several studies that quantified synthetic cannabinoids in 'Spice' products, ranging from 1,1mg/g up to 303mg/g (Uchiyama *et al.*, 2010; Nakajima *et al.*, 2011; Dunham *et al.*, 2012; Ernst *et al.*, 2012; Valoti *et al.*, 2012; Langer *et al.*, 2013).

The concentrations values obtained are an approximation due to several factors. Even though the software performs the integration of the peaks to obtain the area, this step frequently has to be adjusted manually, which inserts a random error to the measurement. On the other hand, an internal standard calibration (e.g. JWH-018 d3) would have been relevant taking into account the complex matrix that is the herbal mixture, however the respective standard was not available. Lastly, the calibration curve used to calculate the concentration of all the compounds was performed using the JWH-018 standard, since no other standards were available. Due to this fact, the quantification of substances as JWH-122 and JWH-210 might not be accurate.

These results warn to the problem of multiple presence of synthetic cannabinoids in the same sample. The lack of toxicological studies and the possible mixtures are might be done may be potential harmful to the consumer, not only just because of the wide concentration range that these synthetic cannabinoids might present in the packages as well as the wide variety of substances that might present simultaneously.

3.4.Evaporation to dryness - optimization

The lyophilization technique (56% recovery) has shown to be time consuming and the recovery percentage was not profitable. On the other hand, the nitrogen stream (52% recovery) revealed to be time consuming as well expensive taking into account the amount of gas needed for a unique evaporation. Finally, the rotary evaporator (68%) using methanol as solvent was found to be the best procedure to concentrate the purified compound, achieving a higher recovery percentage in a relative short time procedure.

3.5.Method Validation

The quantification method of JWH-018 in herbal blends by HPLC-UV was validated. The parameters of the validation were calculated using Microsoft Excel 2010 (Version 14.0.7106.5003). From the two calibration curves performed, it was used the data set from the calibration curve with better correlation value (R^2 = 0,9982), being the method linear over the whole range of calibrators considered (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 µg/mL). Using Grubb's test to detect potential outliers, it was found that the null hypothesis for Grubbs' outlier test is that there are no outliers in the data set. The limit of detection (LOD) was determined as 1,56µg/mL while the limit of quantification (LOQ) was 15,57µg/mL. The repeatability (within-run precision) was calculated from the calibration curve with better correlation value (R^2 = 0,9982). The %RSD obtained was 2,24%, 4,16% and 12,6% respectively for low (5µg/ml), medium (20µg/m) and high (35µg/m) concentration, values which are in compliance with the criteria (<20% RSD). The intermediate precision (between-run) was calculated from both calibration curves performed since they were performed in different days. The %RSD obtained was 3,71%, 2,8% and 7,05% respectively for low (5µg/ml), medium (20µg/m) and high (35µg/m) concentration, values which are in compliance with the criteria (<20% RSD). Beyond the experimental error associated with any laboratory experiment due to the systematic errors (namely from micropipettes), the lack of more accurate results might also be due to human errors (random errors), since the injections were made manually.

3.6.Identification of substances by LC-MS

The qualitative analysis was carried out at the Laboratório de Análises de Dopagem (LAD) in Lisbon. Usually the LC identification is performed by comparison of the retention time of the standard with the retention time of the analyte, however no standards were available. The identification of the substances was made by comparison of the mass transitions (molecular ion → product ion 1; product ion 2; product ion 3; etc;) with literature references (Table 1).

Table 1 – Major ions for selected synthetic cannabinoids. (Kneisel, S. and Auwärter, V., 2012).

Analyte	Protonated molecular ion (m/z)	Fragment ion (m/z)
JWH-018	342.2	155.1
		127.1
JWH-122	356.2	169.1
		141.1
JWH-210	370.2	183.1
		155.1

In the sample 1 (Figure 19) and 3 (Figure 24) the ions at *m/z* 356.27 and 370.31 were identified as the protonated molecular ions $[M+H]^+$ of JWH-122 and JWH-210, respectively (Table 1). The fragmentation ions obtained through MS/MS at *m/z* 356.27 (*m/z* 141.08 and 169.12) were compatible with JWH 122 (Figure 20 and Figure 25). The fragment ion at *m/z* 141 is due to 2-methylnaphthalene fragment while a fragment at *m/z* 169 corresponds to 6-methylnaphthalene-2-carbaldehyde. The present at *m/z* 370.31 (*m/z* 183.11 and 155.1) were compatible with JWH 210 (Figure 21 and Figure 26), from which the base peak at *m/z* 183 corresponds to $[M-186]^+$ due to the loss of 1-phenyl-1H-indole fragment ($-C_{13}H_{17}N$, -186 Da). The fragment ion at *m/z* 155 is due to 2-ethylnaphthalene fragment.

The molecular ion 342.29 was identified in the sample 2 (Figure 22), which fragmentation *m/z* 342.29 (*m/z* 155.08 and 127.19) corresponds to JWH 018 (Figure 23). In this case, the fragment ion at *m/z* 155 is due to naphthalene-2-carbaldehyde and the m/z 127 to the single naphthalene fragment. Although the fragment ion m/z 155 is common to the JWH-018 and JWH-210, the molecules correspondents are not the same. Although this ion is a specific ion from the structural point of view, cannot be used as specific taking into

account just the *m/z* value. Due to this, the identification is done based on the fragment ion *m/z* 127 and on the molecular ion *m/z* 342.

The fragment at m/z 214 is common to all these three compounds due to the presence of 1-phenyl-1H-indole-2carbaldeheyde ($C_{14}H_{17}NO$), reason why is not considered as a specific ion.

All the substances found in the herbal products are known to mimic the pharmacological activity of the cannabinoids and taking into account the co-presence of multiple active compounds in the same products, it may culminate in a health problem. Actually, these synthetic cannabinoids found in the herbal blends were already studied by J. W. Huffman, namely the receptor affinities with the receptors CB_1 and CB_2. JWH-122 and JWH-210 are both analogues of JWH-018. It was stated that JWH-018 has a K_i (nM) of 9 ± 5 (CB_1) and 2.9 ± 2.6 (CB_2) while JWH-122 and JWH-210 have as K_i (nM) values of 0.69 ± 0.5 (CB_1) and 1.2 ± 1.2 (CB_2) and 0.46 ± 0.03 (CB_1) and 0.69 ± 0.01 (CB_2) respectively - data reported as mean values ± SEM (standard error of the mean) (UNODC, 2011). Considering the Δ^9- THC as a reference, all these synthetic substances have higher binding affinities with both cannabinoid receptors that the original molecule. This increased potency is might be related with an increased likelihood of adverse effects or even cause overdose incidents.

3.7.Pyrolysis

A smoking system was used to characterize the products from the pyrolysis of JWH-018, retained in methanol and water solutions. The JWH-018 was burnt as having methanol as solvent. The qualitative analysis as well as the identification of the compounds was done by GC-MS, carried out at the Instituto Nacional de Medicina Legal (INML) in Lisbon.

The methanolic solution (Figure 14) has shown to retain more compounds than the aqueous solution (Figure 15). From the innumerous pyrolysis products detected, the search against the library identified caffeine and the synthetic cannabinoid JWH-018 in both methanolic and aqueous solutions. From these results it is also possible to conclude that caffeine is an ingredient present in the sample 2. These compounds should not be taken into consideration as products of pyrolysis but as not degraded compounds. Actually, caffeine was already reported as an additive substance to be added with the synthetic cannabinoids (Dresen *et al.*, 2010, Grigoryev *et al.*, 2011)

It would be expected to get degradation products of JWH-018 since the heat induced should lead to the cleavage of the molecule bonds. The occurrence of not degraded compounds might be due to an incomplete pyrolysis which can occur from not achieving the optimum temperature for the pyrolysis. It is stated that the heating blanket may reach 400ºC, however there is no panel indicating the temperature that the device is operating.

Beyond the caffeine and the JWH-018 substance, phthalates were also identified. These may have origin in plastic hoses (used to link the devices in the smoking system) or in the solvent used, namely the methanol which is from analytical grade and actually sold in plastic bottles. Although there are various compounds which were not identified (no correspondence with the library), it is not expected to reach the same results as Daw *et al* (2014) who obtained other synthetic cannabinoids as result of pyrolysis products.

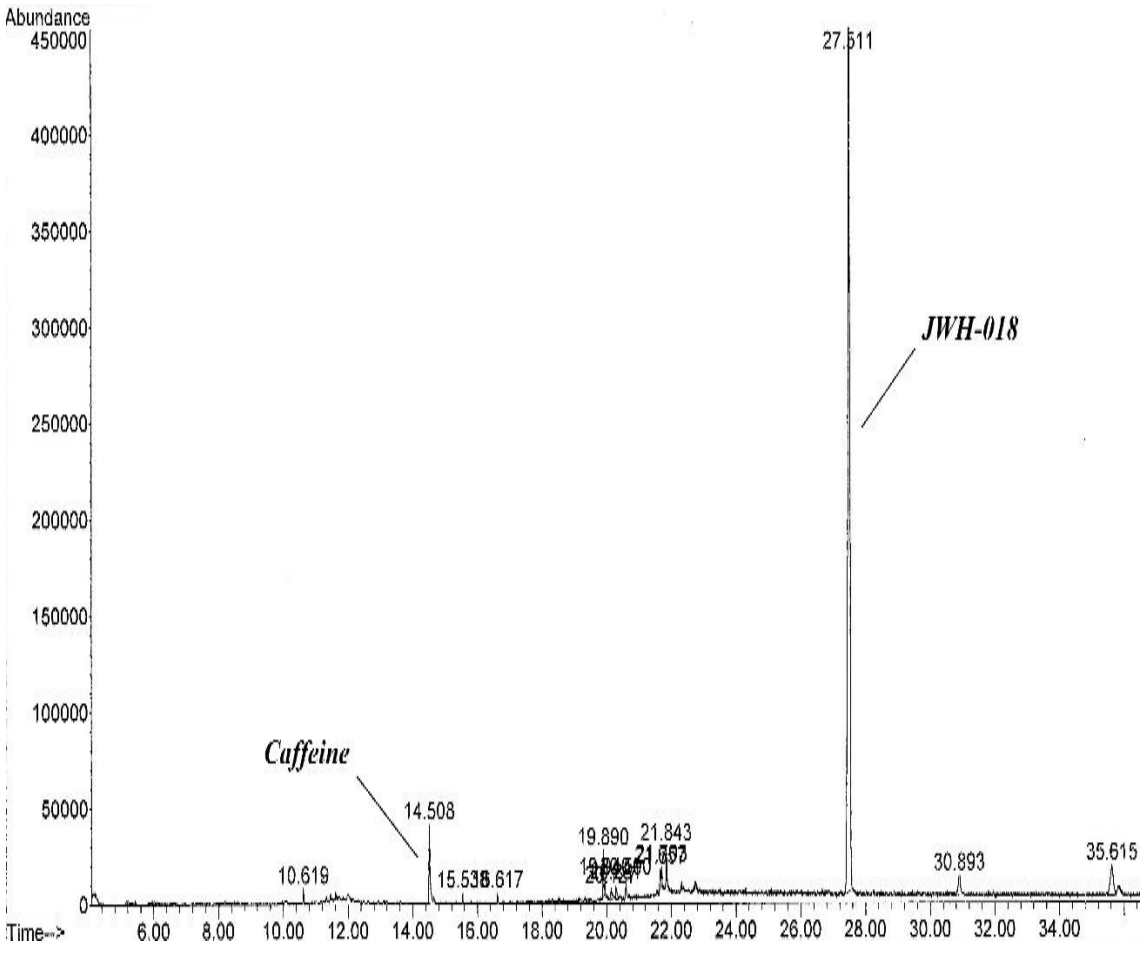

Figure 14 - Chromatogram from the aqueous solution

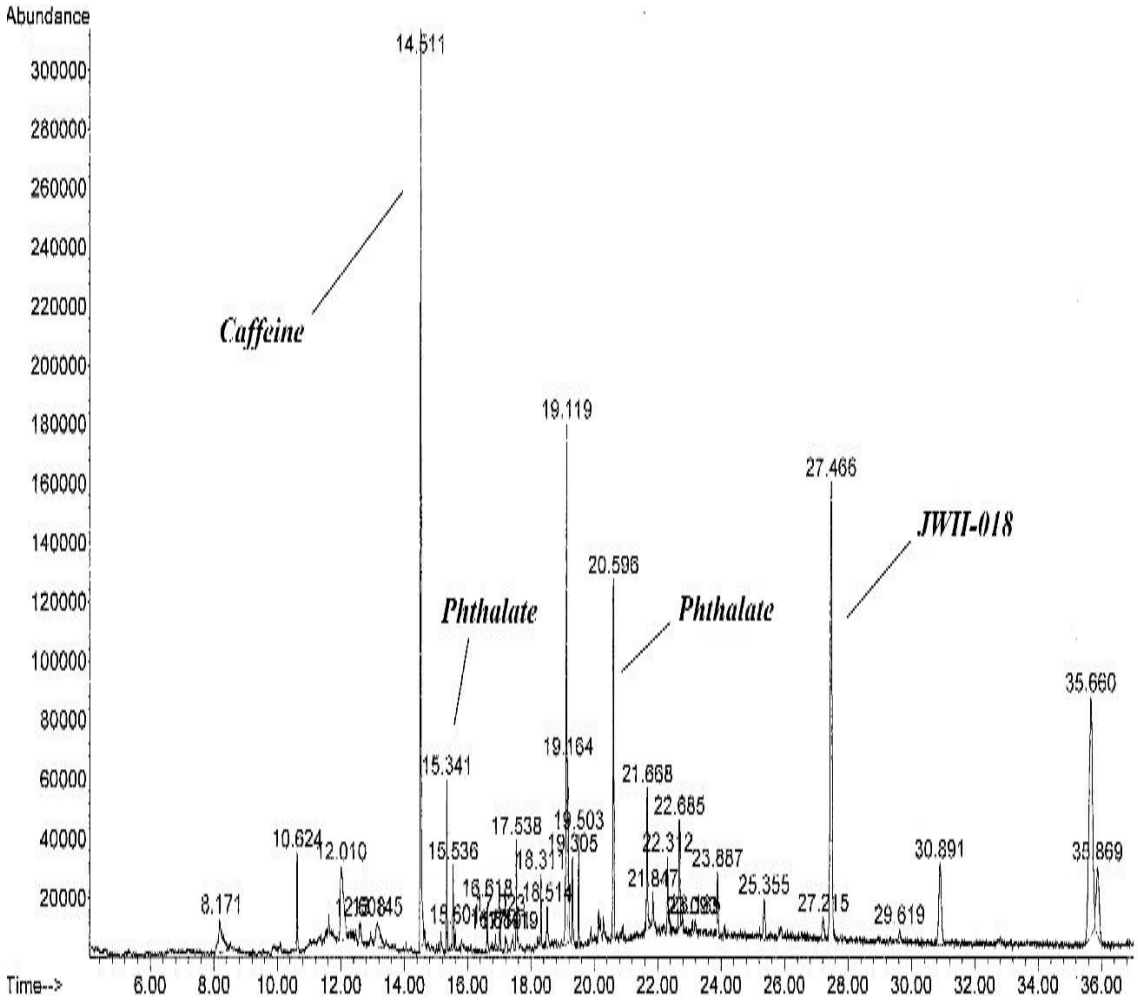

Figure 15 - Chromatogram from the methanolic solution

3.8.MTT assay – toxicological impact

The cytotoxic test was performed using the drugs found in the herbal blends analyzed. Purified JWH-018, JWH-122 and JWH-210 were applied to the cells, being the latters applied simultaneously and in the same proportions as it was found in the sample 1.

Taking into account that the total amount of formazan produced is proportional to the number viable of cells, it is shown that the number of viable cells did not decrease in contact with the drug within the concentration range studied (FIGURES). Actually, both assays have shown the tendency the number of viable cells has shown general tendency. In both toxicological assays, the results indicate that these psychoactive substance are not toxic to the cell in the concentrations used.

The results obtained from the MTT assay were not conclusive since this is just a preliminary analysis. Further assays should be performed in order to get a supportive result and reliable conclusions.

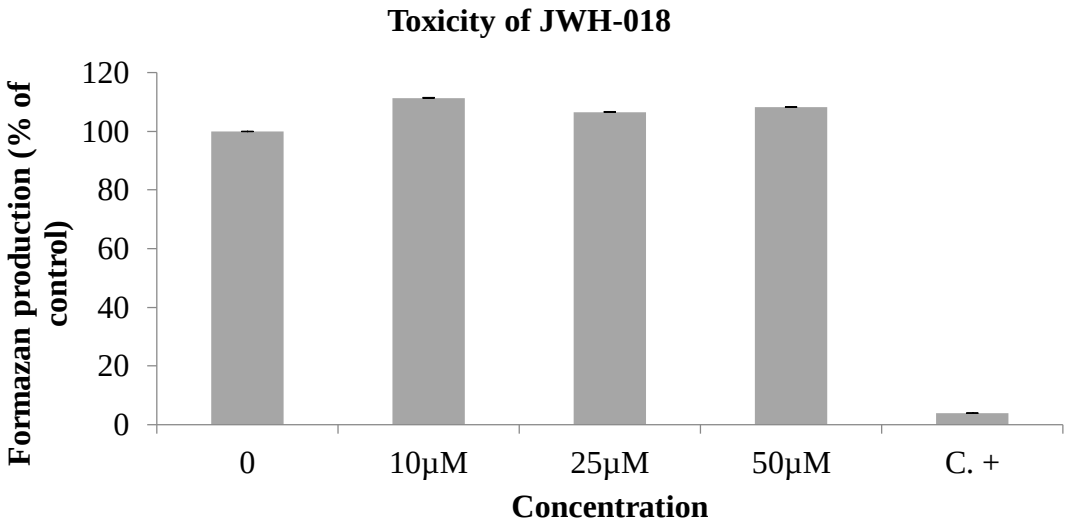

Figure 16 - Cytotoxic evaluation of JWH-018 on SH-SY5Y cells.

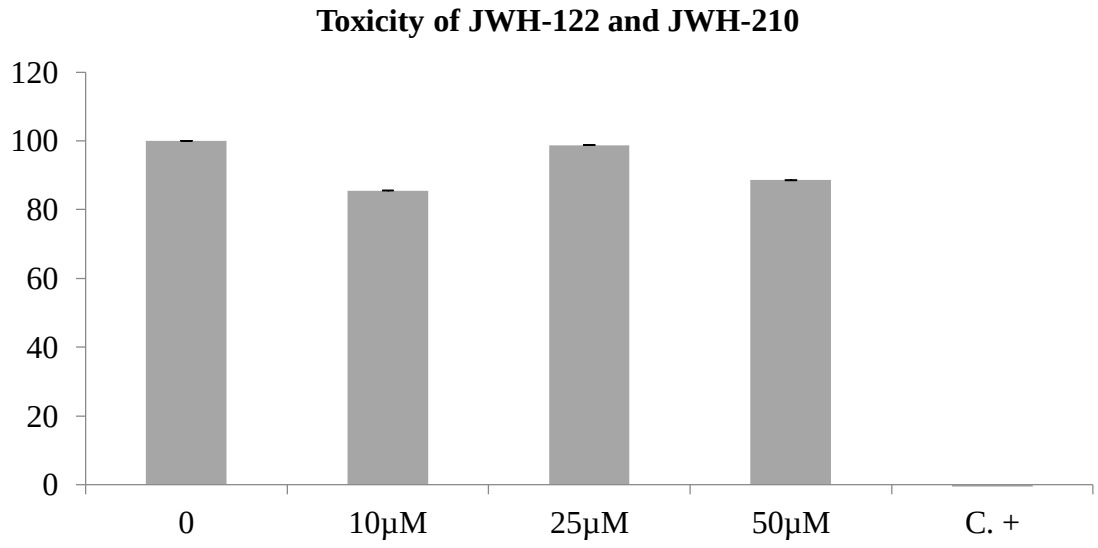

Figure 17 - Cytotoxic evaluation of JWH-122 and JWH-210 on SH-SY5Y cells.

4. Conclusions

In terms of optimization, the methanol has shown to be more efficient as extraction solvent when compared with hexane and the rotary evaporator the most effective method to evaporate to dryness taking into account factors as time, cost and recovery percentage when compared with the lyophilization and the nitrogen stream. The data acquired for the present HPLC-UV method to detect and quantify synthetic cannabinoids proved to be within the acceptable limits (criteria) and the method has proven to be fit for purpose. The method was validated over a concentration range of 0-45µg/ml and the samples quantified. The isolation and consequent quantitative analysis by HPLC allied with the identification of the compounds by LC-MS/MS. The sample 1 it has shown the coexistence of JWH-122 (29,07mg/g) and JWH-210 (175,9mg/g) while the sample 2 was found to contain the synthetic cannabinoid JWH-018 (149,7mg/g). The sample 3 presented the same multiple psychoactive substances as sample 1, JWH-122 (7,3mg/g) and JWH-210 (129,9mg/g). Since these drugs are mainly smoked, a smoking machine was created in order to characterize the chemical composition of the pyrolysis. From the GC-MS analysis performed to the pyrolysis solutions has shown no degradation of the JWH-018 or degradation in the same fragments that appears in the mass spectra. Phthalates compounds were found, which may be derived from the plastic hoses or from an impure solvent that was used. The preliminary results obtained from the two toxicity assays performed on human neuroblastoma SH-SY5Y cells, one applying a combination of multiple synthetic cannabinoids (JWH-122 and JWH-210) and a second one testing the toxicicty of JWH-018 itself) indicate that these psychoactive substance are not toxic to the cells in the concentrations ranging from 0µM to 50µM.

5. Recommendation for further work

It is known that NPS are a threat for the public heath since these are not known or fully described. This approach aims to have a comprehensive characterization of the unknown substances present in the herbal blends, from the isolation up to the toxicity impact that they might have. The present methodology, which includes isolation and identification of the synthetic cannabinoids and assessment of toxicological impact should be carried by the competent authorities in order to control and characterize the psychoactive substances present in Portugal.

The approach presented should be developed and validated in order to get better and more accurate results, either in the identification of a wider range of substances or in a better accurate and realistic toxicological impact. In future studies when it comes to quantifying synthetic cannabinoids, it is recommended further optimization of the extraction process in order to increase the overall yield. Cytotoxic studies should be carried on, adding variables to the study the time of exposure of the drug to the cell, the concentration range used or even the number of synthetic cannabinoids applied simultaneously. Perform these studies taking into account the different classes of synthetic cannabinoids would be interesting in order to evaluate if there is some association between the drug classes and toxicities.

Allying epidemiological with toxicological research would allow to assessing the dimension of this phenomenon. Further studies taking into account metabolites of these substances should be taken into account since the metabolism of these compounds is unknown. Apart from the toxicity that might be detected along the metabolism of these compounds, a deeper knowledge about specific metabolites can lead to a faster and easier screening of the consumption of these drugs.

6. References

Atwood, B.K., Lee, D., Straiker, A., Widlanski, T.S., Mackie, K. (2011). The CP47,497-C8 and JWH073, commonly found in 'Spice' herbal blends, are potent and efficacious CB_1 cannabinoid receptor agonists. *European Journal of Pharmacology*, 259(2-3), 139-145. doi:10.1016/j.ejphar.2011.01.066.

Camilleri, A., Johnston, M., Brennan, M., Davis, S., Caldicott, D. (2010). Chemical analysis of four capsules containing the controlled substance analogues 4-methylmethcathinone, 2-fluoromethamphetamine, a-phthalimidopropiophenone and N-ethylcathinone. *Forensic Science International*, 197(1-3), 59-66. doi:10.1016/j.forsciint.2009.12.048.

Choi, H., Heo, S., Choe, S., Yang, W., Park, Y., Kim, E. (2013). Simultaneous analysis of synthetic cannabinoids in the materials seized during drug trafficking using GC-MS. *Analytical and Bioanalytical Chemistry*, 405 (12), 3937–3944. doi:10.1007/s00216-012-6560-z.

Daw, R.C., Grabenauer, M., Pande, P.G., Cox, A.O., Kovach, A.L., Davis, K.H., Wiley, J.L., Stout, P.R., Thomas, B.F. (2014). Pyrolysis studies of synthetic cannabinoids in herbal products. *Drug and Alcohol Dependence*, 140, e44. doi: 10.1016/j.drugalcdep.2014.02.142.

Dresen, S., Ferreirós, N., Pütz, M., Westphal, F., Zimmermann, R., Auwärter, V. (2010). Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. *Journal of Mass Spectrometry*, 45(10), 1186-1194. doi: 10.1002/jms.1811.

Dunham, S.J.B., Hooker, P.D., Hyde, R.M. (2012). Identification, extraction and quantification of the synthetic cannabinoid JWH-018 from commercially available herbal marijuana alternatives. *Forensic Science International*, 223(1-3), 241-244. doi:10.1016/j.forsciint.2012.09.010.

Ernst, L., Krüger, K., Lindigkeit, R., Schiebel, H.M., Beuerle, T. (2012). Synthetic cannabinoids in ''spice-like'' herbal blends: First appearance of JWH-307 and recurrence of JWH-018 on the German market. *Forensic Science International*, 222(1-3), 216–222. doi:10.1016/j.forsciint.2012.05.027.

European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2008) Annual Report on the implementation of Council Decision. 2005/387/JHA. Lisbon: European Monitoring Centre for Drugs and Drug Addiction. Available from: http://www.emcdda.europa.eu/html.cfm/index132901EN.html [Accessed on 5th February 2014]

European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2012) Annual Report on the implementation of Council Decision. 2005/387/JHA. Lisbon: European Monitoring Centre for Drugs and Drug Addiction. Available from: http://www.emcdda.europa.eu/publications/implementation-reports/2012 [Accessed on 5th February 2014]

Grigoryev, A., Savchuk, S., Melnik, A., Moskaleva, N., Dzhurko, J., Ershov, M., Nosyrev, A., Vedenin, A., Izotov, B., Zabirova, I., rozhanets, V. (2011). Chromatography–mass spectrometry studies on the metabolism of synthetic cannabinoids JWH-018 and JWH-073, psychoactive components of smoking mixtures. *Journal of Chromatography B*, 879(15-16), 1126-1136. doi: 10.1016/j.jchromb.2011.03.034.

Hong-rong, X., Lin-sen, H., Guo-yi, L. (2010). SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. *Chinese Medicine*, 123(8), 1086-1092. doi:10.3760/cma.j.issn.0366-6999.2010.08.021.

Huber, U., & Majors, R. (2007). Principles in preparative HPLC. Germany: Agilent Technologies. Source: https://www.chem.agilent.com/Library/primers/Public/5989-6639EN.pdhonKneisel, S. & Auwärter, V. (2012). Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. *Journal of Mass Spectrometry*, 47(7), 825-835.

Lapoint, J., James, L.P, Moran, C.L., Nelson, L.S., Hoffman, R.S., Moran, J.H. (2011). Severe toxicity following synthetic cannabinoid ingestion. *Clinical Toxicology*, 49(8), 760-764. doi:10.3109/15563650.2011.609822.

Langer, N., Lindigkeit, R., Schiebel, Hans-Martin, Ernst, L., Beuerle, T. (2013) Identification and quantification of synthetic cannabinoids in 'spice-like' herbal mixtures: A snapshot of the German situation in the autumn of 2012. *Drug Testing and Analysis*, 6(1-2), 59-71. doi: 10.1002/dta.1499.

Moosmann, B., Kneisel, S., Girreser, U., Brecht, V., Westphal, F., Auwärter, V. (2012). Separation and structural characterization of the synthetic cannabinoids JWH-412 and 1-[(5-fluoropentyl)-1H-indol-3yl]-(4-methylnaphthalen-1-yl)methanone using GC-MS, NMR analysis and a flash chromatography system. *Forensic science international*, 220(1-3), e17-22. doi: 10.1016/j.forsciint.2011.12.010.

Moosmann, B., Kneisel, S., Wohlfarth., Brecht, V., Auwärter, V. (2013). A fast and inexpensive procedure for the isolation of synthetic cannabinoids from 'Spice' products using a flash chromatography system. *Analytical and Bioanalytical Chemistry*, 405(12), 3929-3935. doi:10.1007/s00216-012-6462-0.

Moran, C., Le, V.H., Chimalakonda, K.C., Smedley, A.L, Lackey, F.D., Owen, S.N., Kennedy, P.D., Endres, G.W., Ciske, F., Kramer, J., Kornilov, A., Bratton, L.D., Dobrowolski, P.J., Wessinger, W.D., Fantegrossi, W.E., Prather, P.L., James, L.P, Moran, J.H. (2011). Quantitative Measurement of JWH-018 and JWH-073 Metabolites Excreted in Human Urine. *Analytical Chemistry*, 83(11), 4228–4236. doi:10.1021/ac2005636.

Nakajima, J., Takahashi, M., Seto, T., Suzuki, J. (2010). Identification and quantitation of cannabimimetic compound JWH-250 as an adulterant in products obtained via the Internet. *Forensic Toxicology*, 29(1), 51-55. doi:10.1007/s11419-010-0101-2.

Porter, A.C. & Felder. C.C. (2001) The endocannabinoid nervous system: Unique opportunities for therapeutic intervention. *Pharmacology & Therapeutics*, 90(1), 45-60. doi:10.1016/S0163-7258(01)00130-9.

Rajasekaran, M., Brents, L., Franks, L.N., Moran, J.H., Prather, P.L. (2013). Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors. *Toxicology and Applied Pharmacology*, 269(2), 100–108. doi:10.1016/j.taap.2013.03.012.

Scheidweiler, K. & Huestis, M. (2014). Simultaneous quantification of 20 synthetic cannabinoids and 21metabolites, and semi-quantification of 12 alkyl hydroxy metabolitesin human urine by liquid chromatography–tandem massspectrometry. *Journal of Chromatography A*, 1327, 105– 117. doi:10.1016/j.chroma.2013.12.067.

Seely, K., Lapoint, J., Moran, J.H., Fattore, L. (2012). Spice drugs are more than harmless herbal blends: a review of the pharmacology and toxicology of synthetic cannabinoids.

Progress in neuro-psychopharmacology & biological psychiatry, 39(2), 234-243. doi:10.1016/j.pnpbp.2012.04.017.

Simmons, J., Cookman, L., Kangm C., Skinner, C. (2011). Three cases of "spice" exposure. *Clinical Toxicology*, 49(5), 431-433. doi:10.3109/15563650.2011.584316.

Stockert, J. C., Blázquez-Castro, A., Cañete, M., Horobin, R.W., Villanueva, A. (2012) MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta histochemica*, 114(8), 785-796. doi: 10.1016/j.acthis.2012.01.006.

Scientific Working Group for Forensic Toxicology (SWGTOX). (2013). Standard Practices for Method Validation in Forensic Toxicology. Available from: http://www.swgtox.org/documents/Validation3.pdf. [Accessed on 16th June 2014]

Tomiyama K. & Funada M. (2011). Cytotoxicity of synthetic cannabinoids found in "Spice" products: The role of cannabinoid receptors and the caspase cascade in the NG 108-15 cell line. Toxicology Letters, *Toxicology Letters* 207(1), 12–17. doi:10.1016/j.toxlet.2011.08.021.

Tomiyama K. & Funada M. (2014). Cytotoxicity of synthetic cannabinoids on primary neuronal cells of the forebrain: the involvement of cannabinoid CB_1 receptors and apoptotic cell death. *Toxicology and Applied Pharmacology*, 274(1), 17–23. doi:10.1016/j.taap.2013.10.028.

Uchiyama, N., Kikura-Hanajiri, R., Ogata, J., Goda, Y. (2010). Chemical analysis of synthetic cannabinoids as designer drugs in herbal products. *Forensic Science International*, 198(1-3), 31–38. doi: 10.1016/j.forsciint.2010.01.004.

Uchiyama, N., Kikura-Hanajiri, R., Y., Goda, Y. (2011). Identification of a Novel Cannabimimetic phenylacetylindole, cannabipiperidiethanone, as a designer drug in a herbal product and its affinity for cannabinoid CB_1 and CB_2 receptors. *Chemical & pharmaceutical bulletin*, 59(9), 1203-1205.

United Nations Office on Drugs and Crime (UNODC). (2011). *Synthetic cannabinoids: in herbal products.* New York: United Nations. Available from: https://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf. [Accessed on 16th March 2014]

United Nations Office on Drugs and Crime (UNODC). (2013a). *Recommended methods for the Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials.* New York: United Nations. Available from: http://www.unodc.org/documents/scientific/STNAR48_Synthetic_Cannabinoids_ENG.pdf. [Accessed on 5th May 2014]

United Nations Office on Drugs and Crime (UNODC). (2013b). *The challenge of new psychoactive substances.* New York: United Nations. Available from: http://www.unodc.org/documents/scientific/NPS_2013_SMART.pdf. [Acessed on the 20th of June 2014]

United Nations Office on Drugs and Crime (UNODC). (2014). *World Drug Report 2014.* New York: United Nations. Available from: http://www.unodc.org/documents/wdr2014/World_Drug_Report_2014_web.pdf. [Acessed on the 20th of June 2014]

Vandrey, R., Dunn, K.E., Fry, J., Girling, E.R. (2012). A survey study to characterize use of Spice products (synthetic cannabinoids). *Drug and alcohol dependence*, 120(1-3), 238-241. doi:10.1016/j.drugalcdep.2011.07.011.

Valoti, E., Casagni, E., Dell'Acqua, L., Pallavicini, M., Roda, G., Rusconi, C., Straniero, V., Gambaro, V. (2012) Identification of 1-butyl-3-(1-(4-methyl)naphtoyl)indole detected for the first time in ''herbal high'' products on the Italian market. *Forensic Science International*, 223(1-3), e42-e46. doi: 10.1016/j.forsciint.2012.08.009.

Zimmermann, U.S., Winkelmann, P.R., Pilhatsch, M., Nees, J., Spanagel, R., Schulz, K. (2009). Withdrawal phenomena and dependence syndrome after the consumption of "spice gold". *Deutsches Ärzteblatt international*, 106(27), 464-470. doi:10.3238/arztebl.2009.0464.

Zuba, D., Byrska, B., Maciow, M. (2011). Comparison of "herbal highs" composition. *Analytical and Bioanalytical Chemistry*, 400(1), 119-126. doi: 10.1007/s00216-011-4743-7.

Zuba, D., & Byrska, B. (2013). Prevalence and co-existence of active components of 'legal highs'. *Drug Testing and Analysis*, 5(6), 420-439. doi:10.1002/dta.1365

7. Appendices

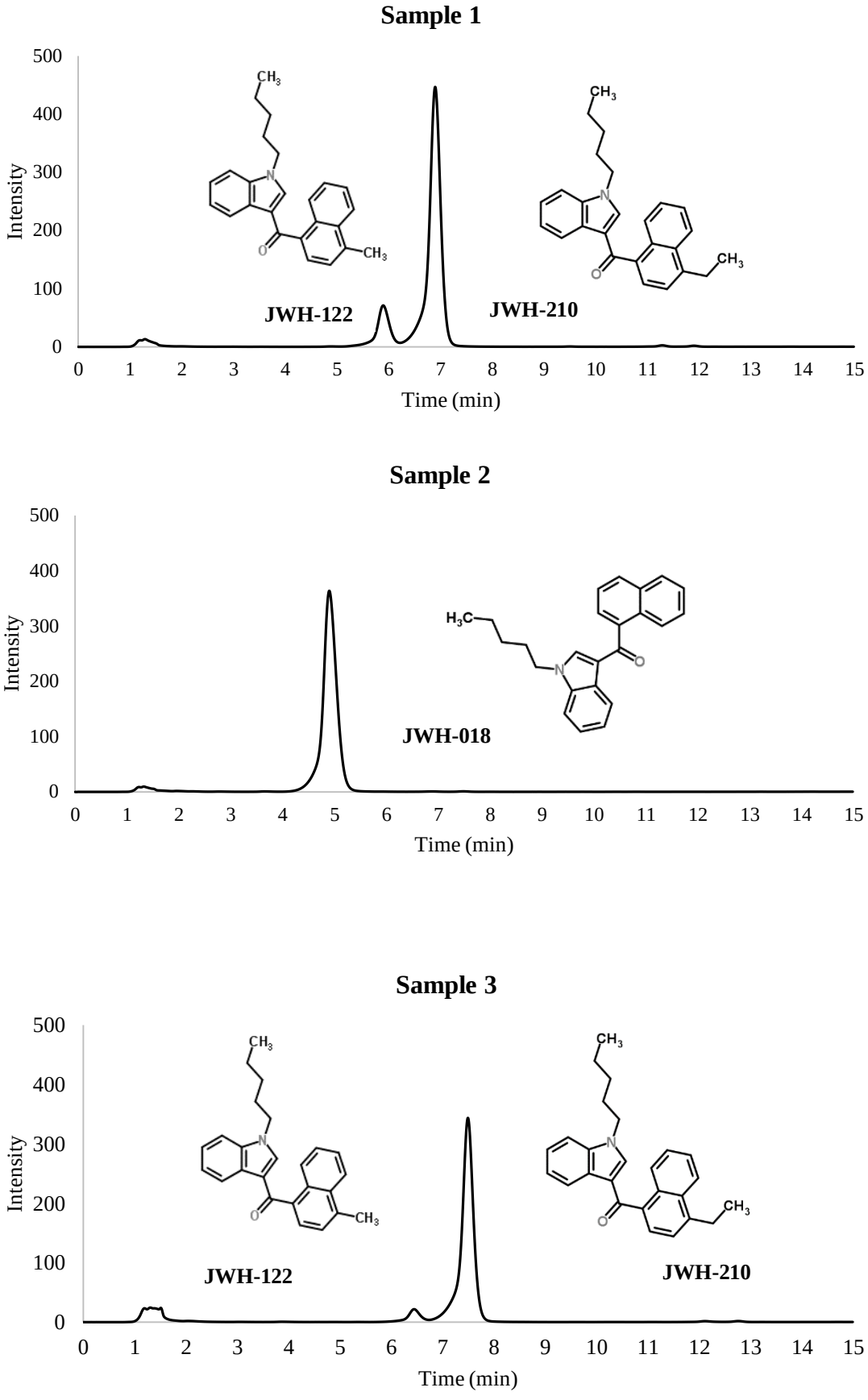

Figure 18 - HPLC-UV chromatograms of the three different samples

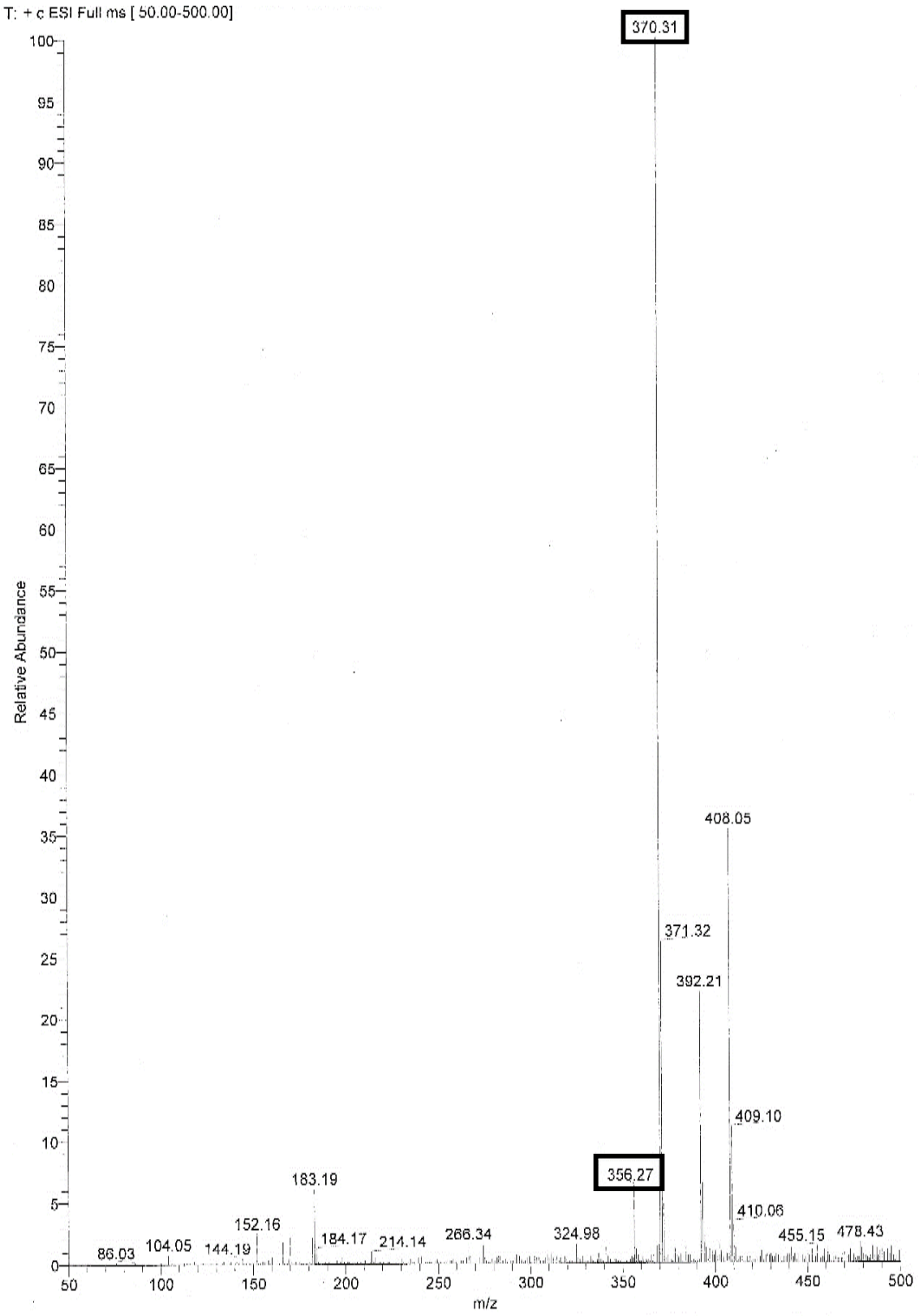

Figure 19 - Full MS spectra from the sample 1, containing the molecular ion at m/z 356.2 (JWH-122) and m/z 370.3 (JWH-210)

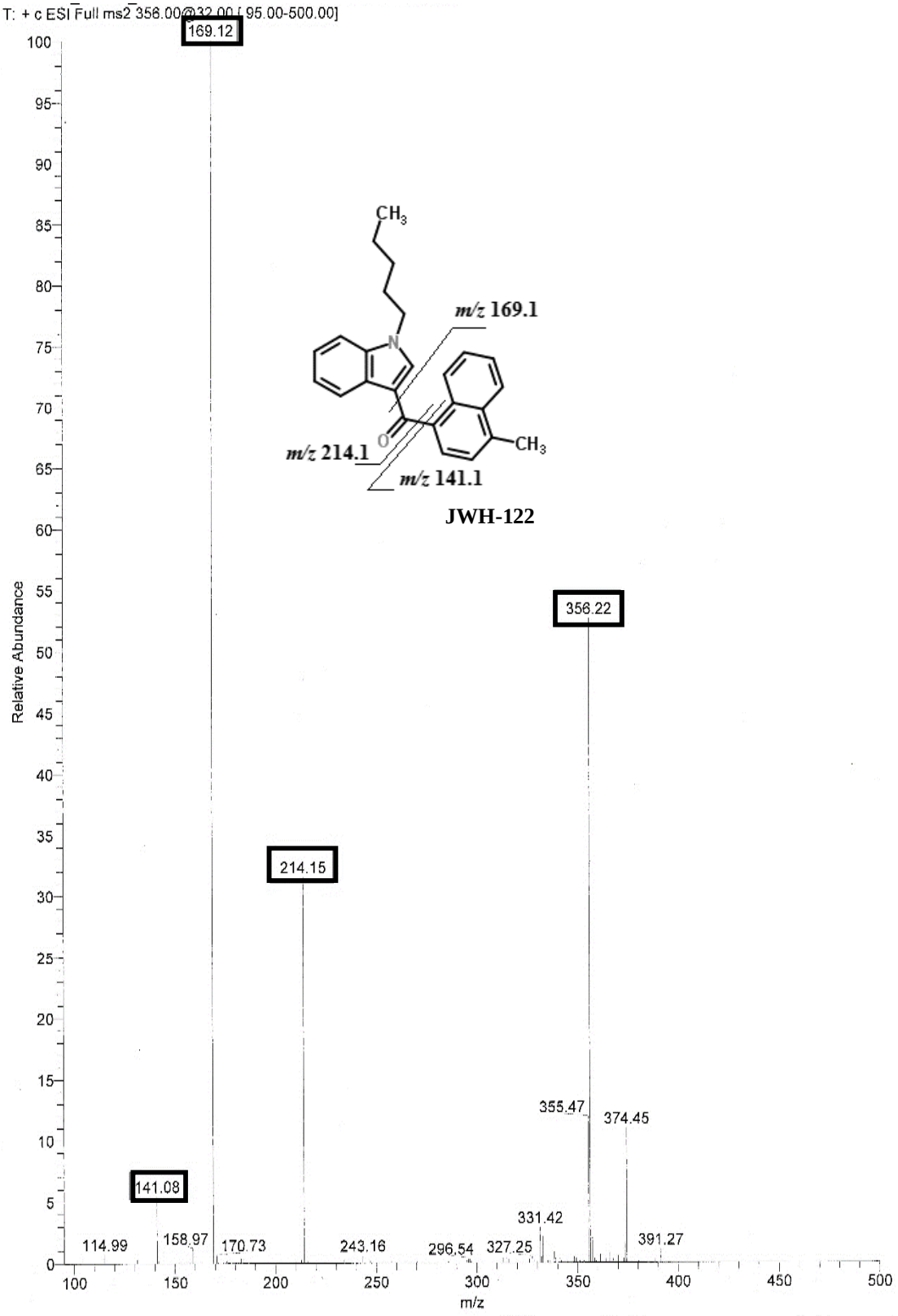

Figure 20 - Mass spectra of the fragmentation ions at m/z 356.2 (JWH-122)

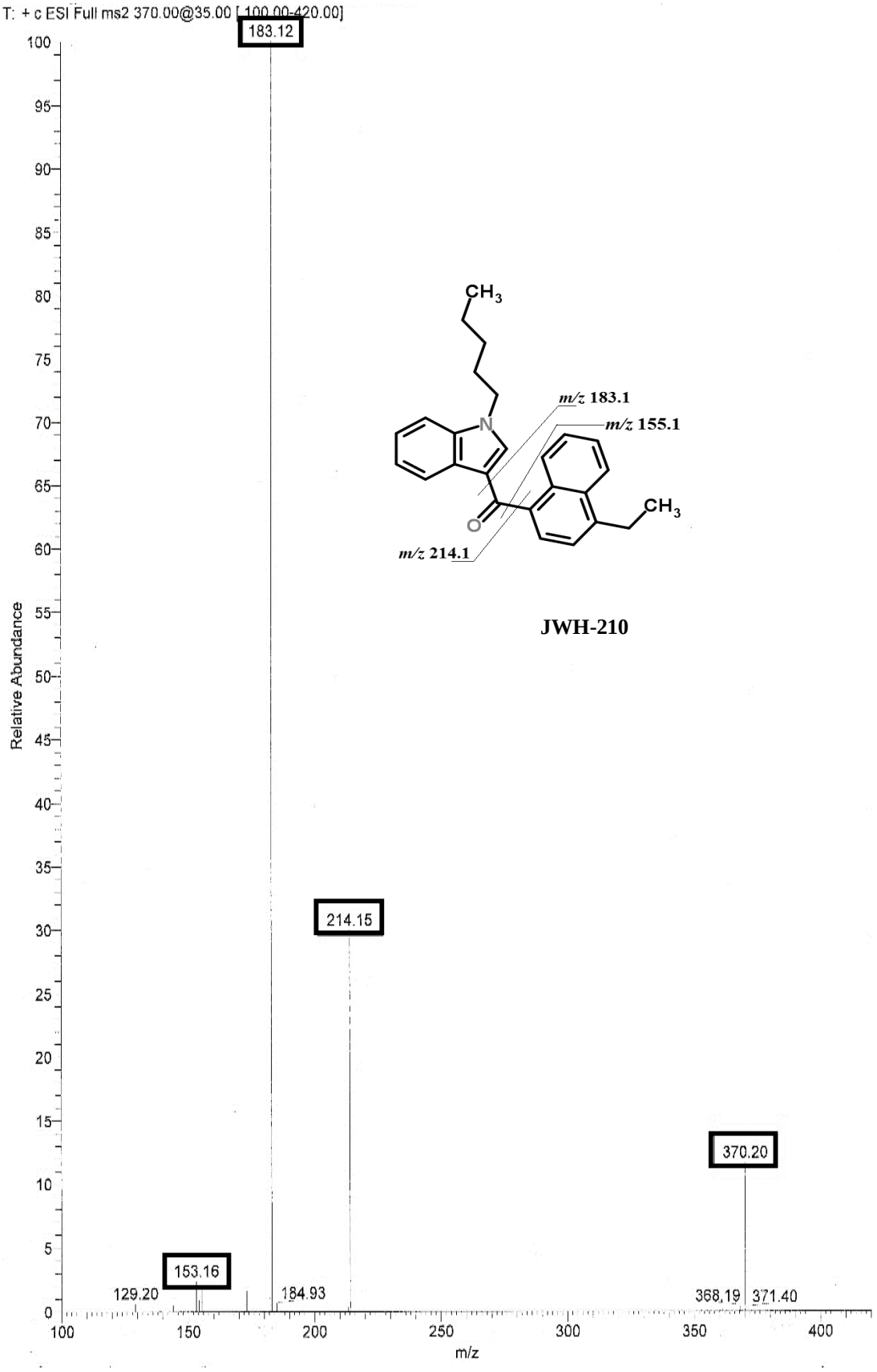

Figure 21- Mass spectra from the fragmentation of the precursor ions m/z 370.3 (JWH-210) – Sample 1

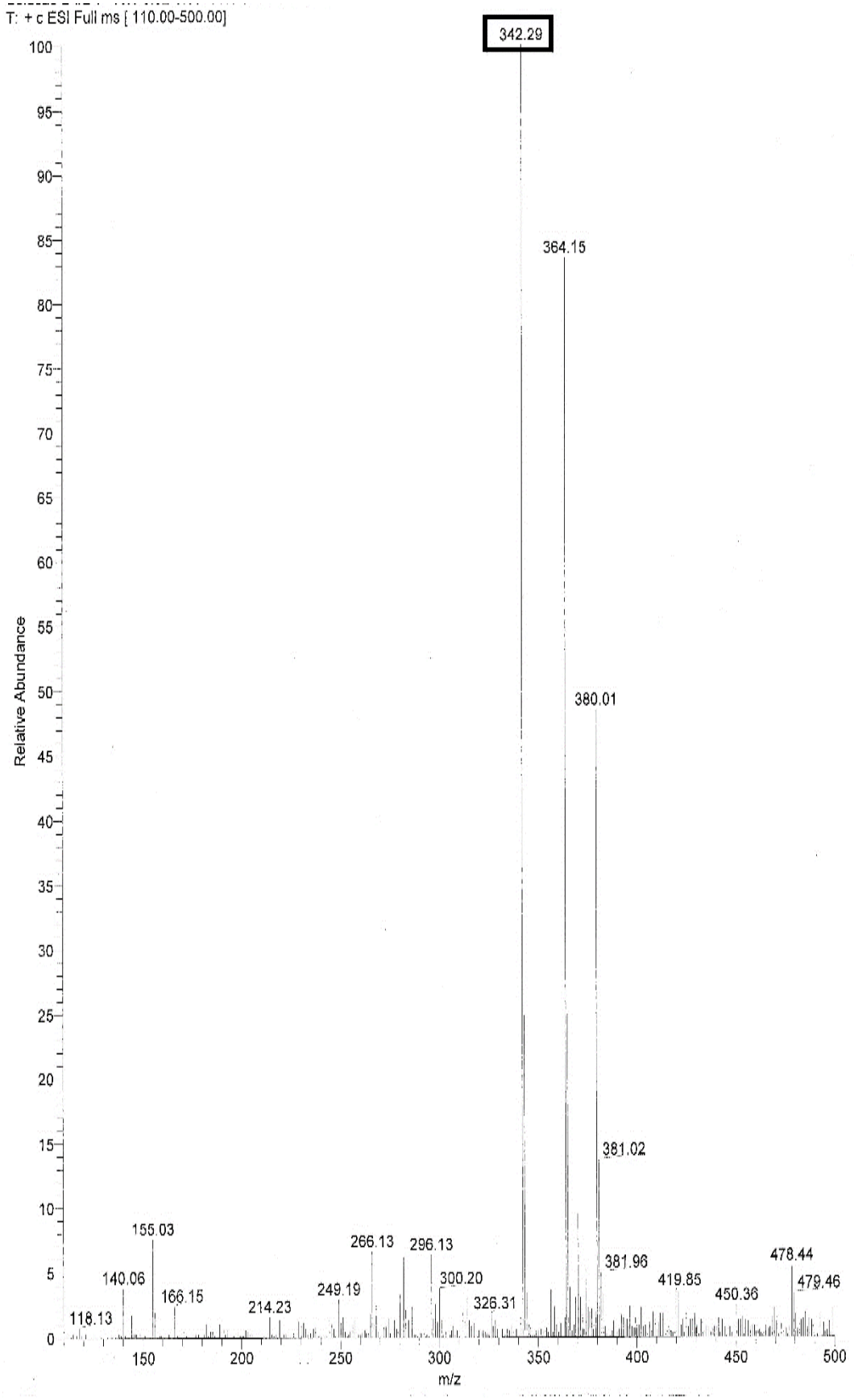

Figure 22 - Full MS spectra from the sample 2, containing the molecular ion at m/z 342.2 (JWH-018)

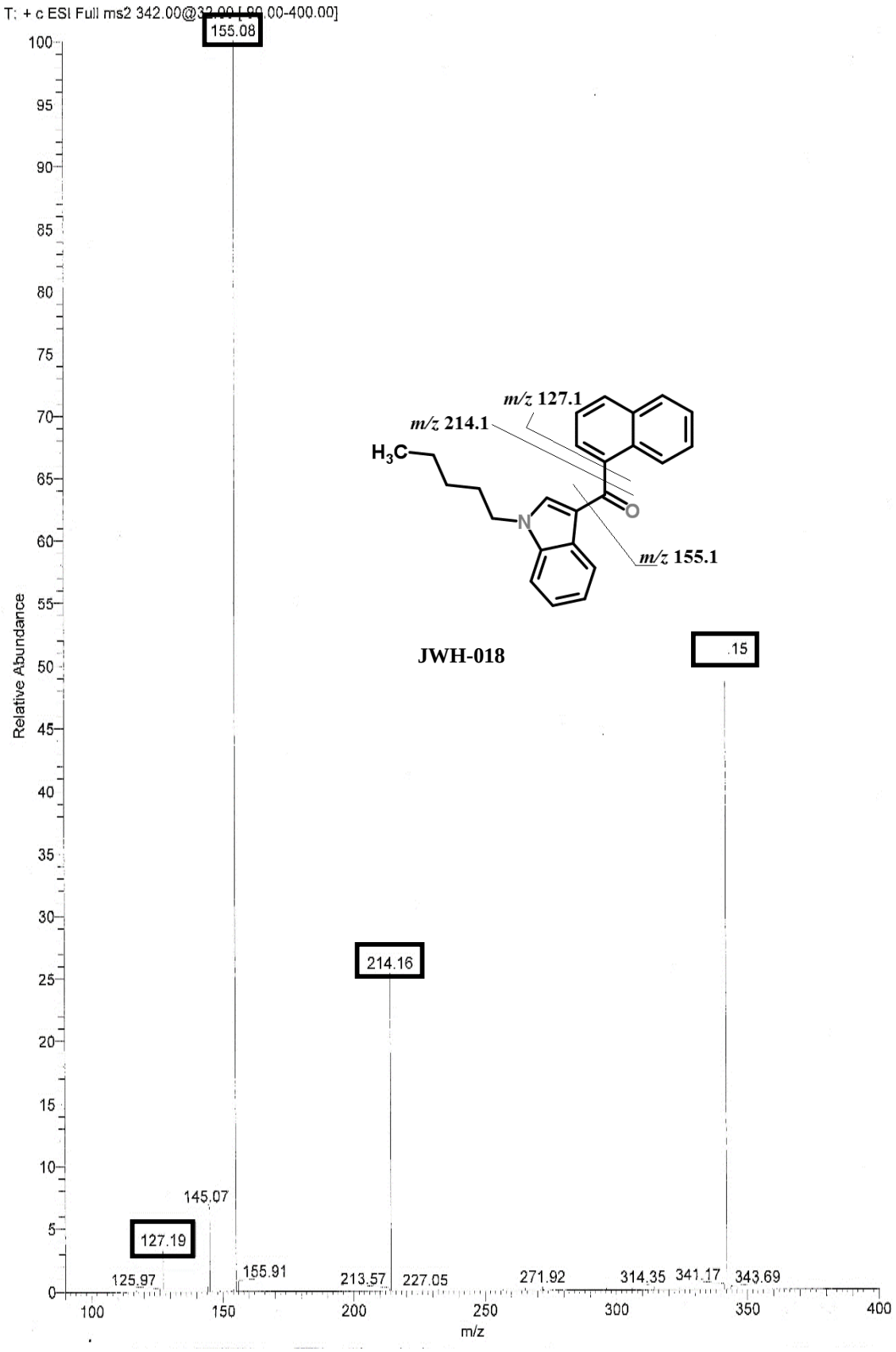

Figure 23 - Mass spectra from the fragmentation of the ion at m/z 356.2 (JWH-018) – Sample 2

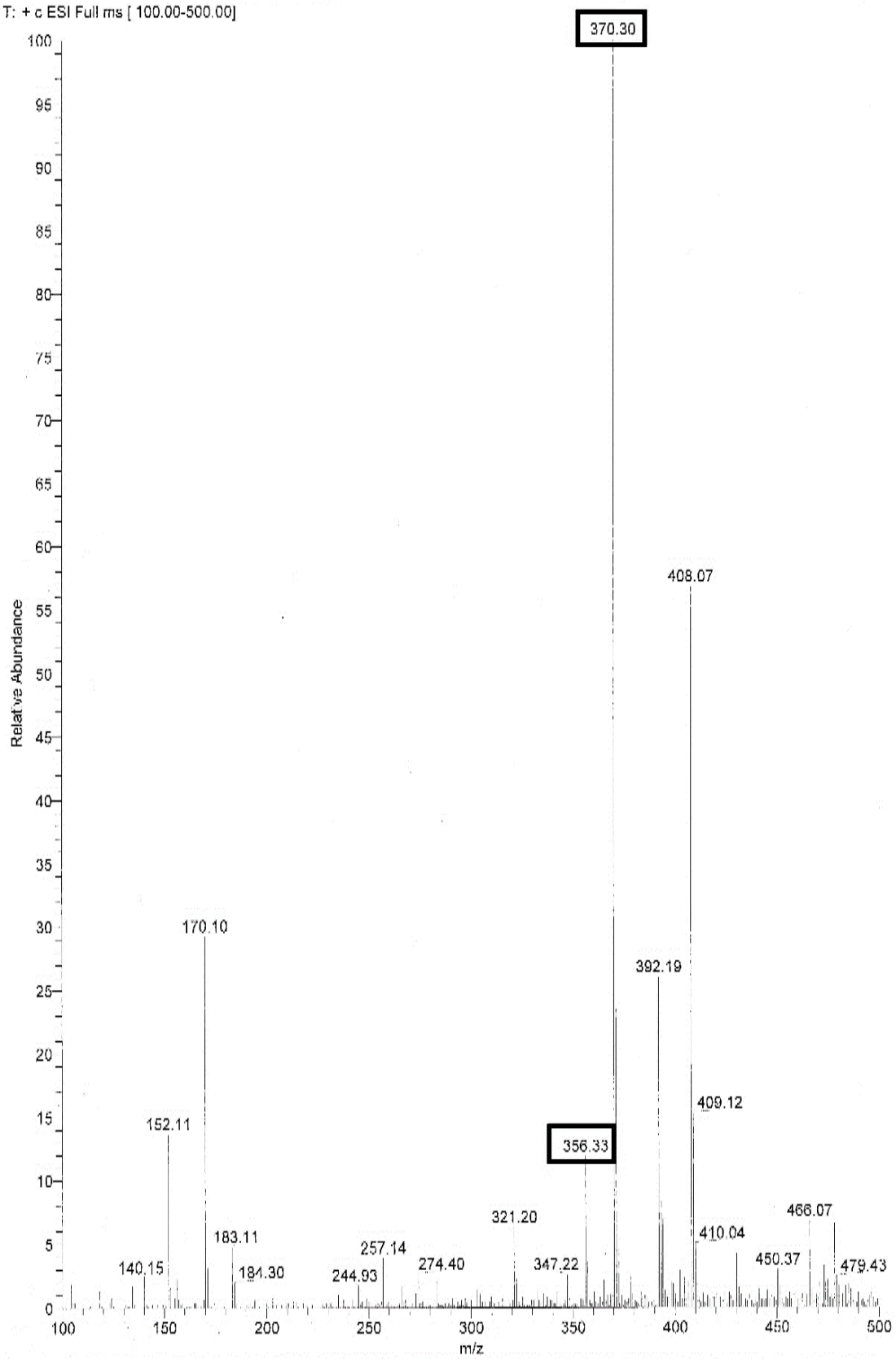

Figure 24 - Full MS spectra from the sample 3, containing the molecular ion at m/z 356.2 (JWH-122) and m/z 370.3 (JWH-210)

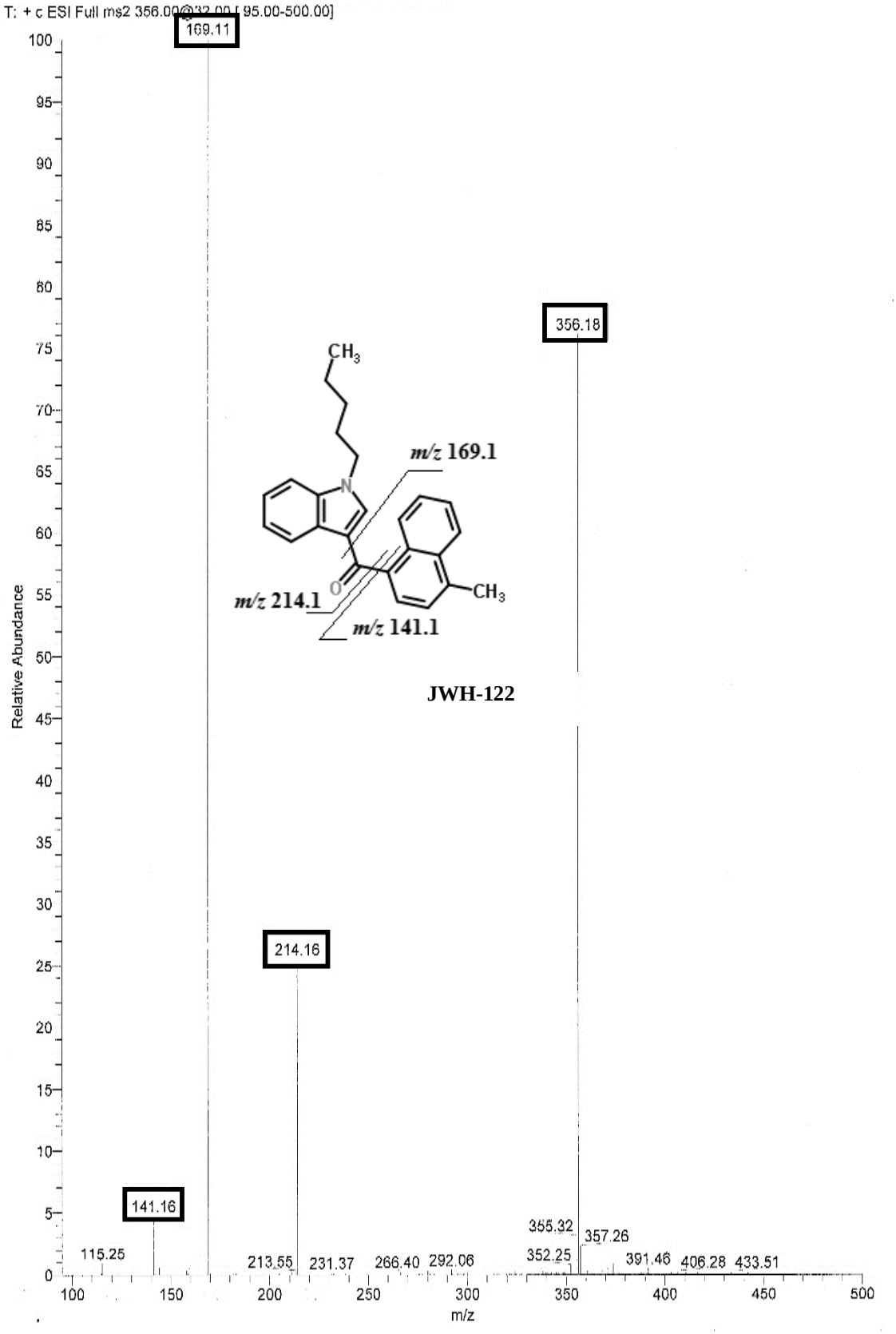

Figure 25 - Mass spectra from the fragmentation of ion at m/z 356.2 (JWH-122) – Sample 3

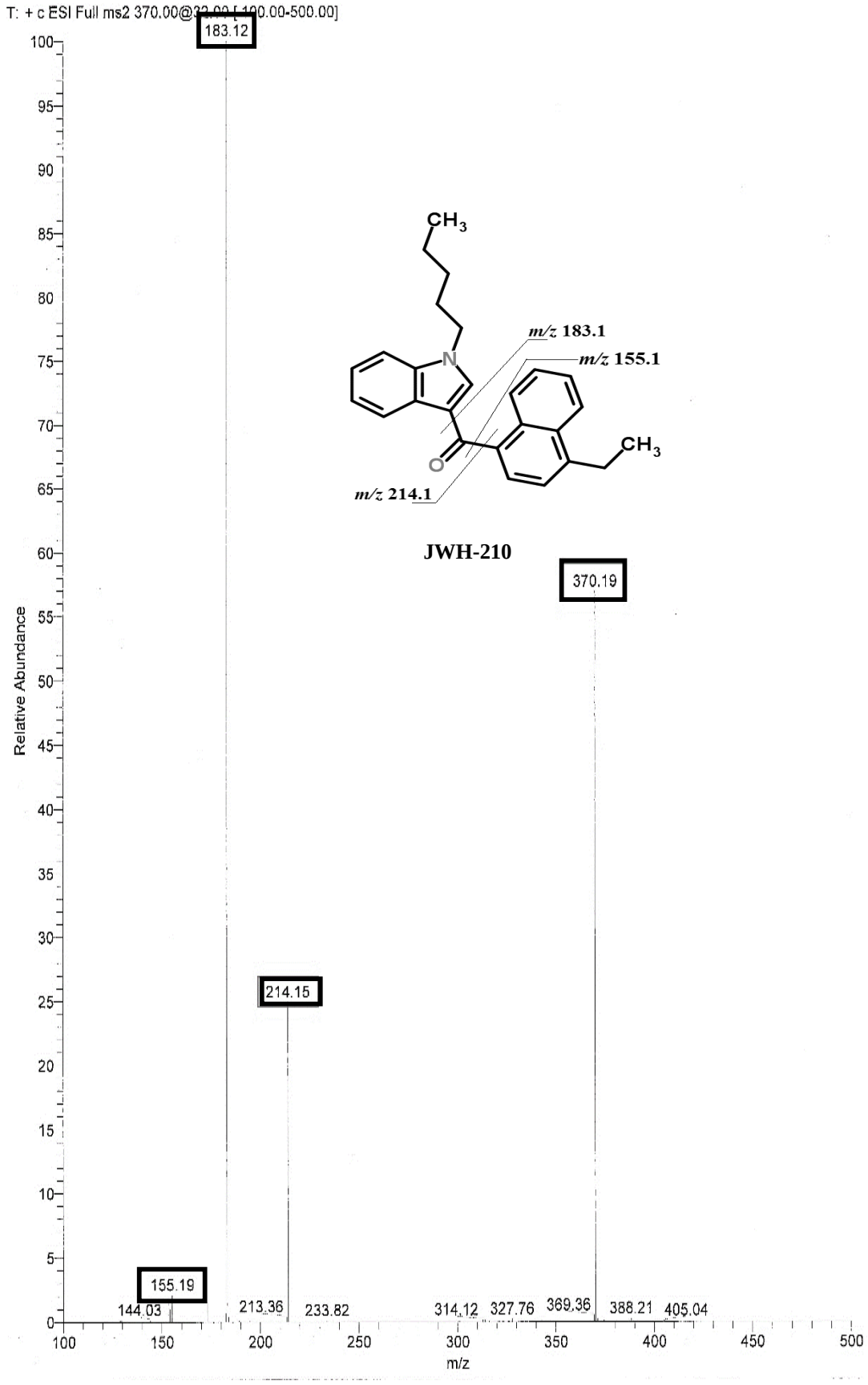

Figure 26 - Mass spectra from the fragmentation at m/z 370.3 (JWH-210) – Sample 3