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Determination of the main compounds of Ayahuasca and the study of their cytotoxicity in dopaminergic neurons

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Para a minha âncora, a minha mãe, com amor

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Resumo Alargado

Ayahuasca é uma bebida psicoativa tradicionalmente formada pela decocção de folhas de *Psychotria viridis* e caules/talos de *Banisteriopsis caapi*. Originalmente consumida por indígenas da região amazónica com propósitos ritualísticos e medicinais. Porém, com o passar dos anos, o seu consumo tem-se alastrado pelos países ocidentais e quem a consome defende que as suas propriedades dão origem a um crescimento pessoal e sentem que é uma forma de obterem conexão espiritual. Não obstante, como os compostos que a constituem têm propriedades alucinogénias e psicoativas, o consumo indevido para usos recreativos também tem aumentado.

A *P. viridis* é maioritariamente constituída pela dimetiltriptamina (DMT), que quando ingerida é facilmente degradada pela ação das monoamina-oxidases (MAO) presentes no intestino e fígado, sendo que o seu efeito é rápido, não sendo capaz de atingir chegar o sistema nervoso central. Por outro lado, os alcaloides de beta-carbolinas (maioritariamente harmina, harmalina e tetrahidroharmina) constituem a *B. caapi*. Estes compostos têm como principal mecanismo de ação a inibição da atividade das MAOs. Desta forma, a DMT consegue facilmente atingir a corrente sanguínea sem sofrer degradação pelas MAOs, podendo atravessar a barreira hemato-encefálica, chegando assim ao sistema nervoso central. Este mecanismo sinergético da DMT com as beta-carbolinas constitui o efeito alucinogénio e psicoativo da bebida ayahuasca. Apesar de estar reportado como principal mecanismo, muito pouco se sabe ainda sobre como atuam estes compostos a nível celular, sendo que a maior parte dos estudos realizados são *in vivo* e raramente estudam a ação conjunta destes compostos químicos. Desta forma, é fundamental, desenvolver estudos que tentem perceber os efeitos proeminentes destes compostos a nível bioquímico celular.

Para além da bebida tradicional, entre outras combinações, também se consome a *P. viridis* com a *Peganum harmala L*, pois esta última também é rica em alcalóides beta-carbolínicos.

Pelo já referido anteriormente, torna-se importante para a Toxicologia Clínica e as Ciências Forenses desenvolver métodos que permitam a deteção e quantificação destes compostos, quer em amostras biológicas, quer nas próprias amostras de chá, de forma a averiguar o conteúdo e a quantidade inicial consumida destes compostos.

Assim, um dos objetivos do presente trabalho centrou-se em desenvolver um método para a determinação destes compostos em amostras comerciais de ayahuasca, utilizando como técnica de preparação de amostras a extração a em fase sólida (SPE) e como técnica analítica a cromatografia gasosa acoplada a espectrometria de massa (GC-MS). O padrão interno usado foi a promazina. O processo de extração foi otimizado de modo a obter a maior quantidade possível de compostos e reduzir a presença de interferentes.

O método desenvolvido foi totalmente validado de acordo com critérios internacionais, tais como os da *Food and Drug Administration* (FDA) e do *Scientific Working Group for Forensic*

Toxicology (SGWTOX). Os parâmetros de validação estudados foram a seletividade, linearidade, precisão e exatidão, limites de quantificação (LLOQ) e recuperação.

Para todos os compostos foi obtida uma linearidade de 0,2 a 20 µg/mL, exceto para a DMT que foi de 0,04-4 µg/mL, sendo que os coeficientes de determinação foram superiores a 0,99, em todos os casos. Relativamente à precisão intra-dia os valores de coeficiente de variação (CV) foram inferiores a 11 % para todos os casos. No que respeita à precisão inter-dia, o estudo foi efetuado ao longo de 5 dias e os CVs obtidos foram inferiores a 10 %, sendo que para a HMN o LLOQ foi de 17,21%. As recuperações obtidas rondaram valores de 44% a 80%, considerando-se valores aceitáveis para a técnica de extração selecionada.

O método validado mostrou ser reprodutível, com boa sensibilidade, e fácil de aplicar a amostras reais pelo que permite a sua utilização na rotina laboratorial nas áreas da Toxicologia Clínica e Forense.

Por outro lado o presente trabalho teve ainda como objetivo avaliar os efeitos citotóxicos em células dopaminérgicas de rato, destes compostos individualmente e quando presentes nos chás de ayahuasca. Para alcançar este objetivo recorreu-se ao kit "CCK-8" que avalia a viabilidade celular aquando da exposição destas células com os compostos em estudo ou com os referidos chás. Em paralelo, e para avaliar o potencial efeito dos compostos no metabolismo proteico, foi feita a quantificação de proteína total, de células expostas a concentrações selecionadas dos compostos em estudo. Verificou-se, que as concentrações mais elevadas dos compostos individuais (HMN THH e HLOL a 10 µM e DMT a 1 µM) exercem um efeito neurotóxico, à exceção da DMT que em nenhuma das concentrações estudadas induziu diminuição da viabilidade celular estatisticamente significativa. Relativamente às bebidas, de um modo geral, todas elas sugerem efeitos citotóxicos para as células usadas, quer a uma concentração de 16 µM quer para uma de 80 µM (*P. harmala*); 2 e 10 µM (*B. caapi* e *DC AB*) e a 1 µM (*M. tenuiflora* e *P.viridis*).

Este é o primeiro estudo a avaliar a citotoxicidade dos compostos da Ayahuasca e de chás comerciais em células dopaminérgicas. No entanto, serão necessários mais estudos para avaliar os efeitos crónicos destes compostos e os mecanismos celulares, bem como o desenvolvimento de métodos que permitam aa determinação destes compostos em amostras biológicas.

Palavras-chave: Ayahuasca, DMT; beta-carbolinas; citotoxicidade.

Abstract

Ayahuasca is a psychoactive beverage prepared traditionally from a mixture of the leaves and stems of *Psychotria viridis* and *Banisteriopsis caapi*, respectively, being originally consumed by indigenous Amazonian tribes for ritual and medicinal purposes. Over the years, its use has spread to other populations as a source of personal growth and spiritual connection. Also, the recreational use of the isolated compounds has become prominent. The main compounds of this tea-like preparation are N,N-dimethyltryptamine (DMT) and ß-Carbolines (ß-CA) or harmala alkaloids, such as: harmine, tetrahydroharmine and harmaline. The latter are monoamine-oxidase (MAO) inhibitors, thus allowing DMT to exert its psychoactive and hallucinogenic effects on the central nervous system (CNS). Although consumers defend its use, its metabolic effects and those on the CNS are not fully understood yet. The majority of studies regarding the effects of this beverage as a whole or as individual compounds are based on *in vivo*, clinical trials or even on surveys.

Therefore, one of the objectives of this work was to develop an analytical method using gas-chromatography coupled to a mass spectrometry (GC-MS) was developed to identify such compounds on five varieties of available commercial teas (*P. viridis, B. caapi; P. harmala; Mimosa tenuiflora and DC AB*). The developed method was fully validated in line with international guidelines for bioanalytical method validation. Linearity was obtained in a range of 0.2-20 µg/mL for all compounds, except for DMT (0.04-4 µg/mL), with determination coefficients above 0.99. In respect to precision and accuracy, the obtained coefficients of variation (CVs) were within the acceptable values (≤ 20 % for lowest limit of quantification (LLOQ) and ≤ for other concentrations), both for intra- and inter-day. Recoveries ranged from 37 – 97 %. The method was considered suitable for quantify such compounds on real tea samples and *P. viridis* presented the highest DMT content, while *P. harmala* presented the highest content of ß-CA. Alongside, the *in vitro* toxicity caused by DMT and ß-Carbolines on N27 rat dopaminergic neurons was evaluated, as well as the toxicity of harmalol, the main metabolite of harmaline. Likewise, all of the teas prepared were tested in cells. Overall, the results show that at the highest concentrations studied all compounds individually and when combined in the tea mixture exert neurotoxicity on N27 dopaminergic cells in a dose-dependent manner.

This is the first study to investigate cytotoxicity of Ayahuasca compounds and commercial teas on dopaminergic cells and use GC-MS to quantify these compounds in five different teas, as well as two tea mixtures.

Keywords: Ayahuasca teas; DMT, beta-carbolines, cytotoxicity.

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Chapter I - Introduction

Chapter II – Experimental Developments

Acronyms

% CTR	Percentage of control
5-HT receptors	5-hydroxytryptamine receptors
ANOVA	One-way analysis of variance
BCA	Bicinchoninic acid
B-CA	Beta-carbolines alkaloids
BIAS	Relative error
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCK-8	Cell counting kit-8
CNS	Central nervous system
CTR	Control
CV	Coefficient of variation
DI	Deionized
DMSO	Dimethyl Sulfoxide
DMT	N,N-dimethyltriptamine
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GC	Gas chromatography
GC-IT-MS	Gas chromatography coupled to ion trap mass spectrometry
GC-MS	Gas chromatography coupled to mass spectrometry
GC-NPD	Gas chromatography coupled to nitrogen phosphorous detector
HLOL	Harmalol
HML	Harmaline
HMN	Harmine
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography coupled to fluorescence detector
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LD_{50}	Lethal dose 50

LLE	Liquid-liquid extraction
LLOQ	Lowest limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MAO	Monoamine oxidase
MeOH	Methanol
MS	Mass spectrometry
N27	Immortalized rat mesencephalic dopaminergic neurons
NaCl	Sodium chloride
PBS	Phosphate-buffer Saline
PD	Parkinson's Disease
PRZ	Promazine
QC	Quality control
R^2	Determination coefficient
RIPA	Radioimmunoprecipitation assay buffer
RPMI-1640	*Roswell Park Memorial Institute 1640 Medium*
SD	Standard deviation
SEM	Standard error of mean
SGWTOX	Scientific Working Group of Forensic Toxicology
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
THH	Tetrahydroharmine
UHPLC-MS/MS	Ultra-high-pressure liquid chromatography- tandem mass spectrometry
WADA	World Anti-Doping Agency
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt

Overview and Objectives

The aims of the present dissertation were:

- The development of an analytical method to quantify the major compounds of Ayahuasca from commercial teas;
- The cytotoxicity study of these compounds in rat dopaminergic neurons cell line (N27 cells);

Taking into account that the purpose of this dissertation is the submission for publication, the present work is divided into two chapters.

Chapter I addresses the physical-chemical and toxicological aspects of each compound, and also its effects as tea components. Moreover, this chapter reviews analytical methods available to detect such compounds, but also focuses on the available literature regarding *in vitro* studies of the same compounds or ayahuasca teas.

Chapter II presents the entire experimental developments of this work, both for the analytical approach to detect DMT and B-CAs on ayahuasca teas and the cytotoxic effect of the selected compounds on N27 cells, a dopaminergic cell line currently used *in vitro* to study Parkinson's

Chapter I - Introduction

1. Contextualization

Ayahuasca is an entheogenic beverage that has been consumed originally by South American population, more specifically by Amazonian indigenous groups for centuries in spiritual rituals and ceremonies in hopes of obtaining a source of further knowledge and divine invigoration (Andrade et al., 2018; Gaujac, Dempster, Navickiene, Brandt, & Andrade, 2013; Labate & Feeney, 2012).

The term "*Ayahuasca*" has a Quechua origin, in which "aya" means "spirit" and "waska" means "vine", that is to say "vine of the souls" (McKenna, 2004). Although sometimes known as Ayahuasca, it can also be referred as *hoasca*, *caapi*, *daime*, *yagé*, *natema* and several other local names in Brazil, Bolivia, Equator and Peru (Callaway et al., 1999) . The word itself can either be applied to the beverage or the vine that is used to produce the beverage (Grob et al., 1996).

Over time, specific churches devoted to the consumption of ayahuasca were created, in order to hold shamanic ceremonies to non-indigenous Amazonian population. The most dominant are *Santo Daime* and *União do Vegetal* (UDV), nonetheless there are others. These religions are a fusion of Christianism, Spiritualism and other religions with African-Brazilian bases. The diffusion of these religious through Northern America, Europe and Asia as also led to an increase of ayahuasca consumption, and thus its concern for public health, toxicity and possible therapeutic potential have risen (Anderson et al. 2012; Labate and Feeney 2012; Malcolm and Lee 2017; Pic-Taylor et al. 2015).

The beverage itself is a tea-like/decoction preparation that commonly consists in a brew mixture of *Banisteriopsis caapi* vine with *Psychotria viridis* leaves (dos Santos et al., 2017). Nevertheless, there are analogues which can be used for the same purpose, for example for *P. viridis* there are *Psychotria carthagenensis, Psychotria carthagenesis, Brugmansia suaveolens, Nicotiana tabacum, Malouetia tamarquina, Tabernaemontana spp., Brunfelsia, spp., Datura suaveolens, Iochroma fuchsioides, Juanulloa spp*., etc. (Ramachandran et al., 2018). In *B. caapi*'s case, other than the natural anolgue *Peganum harmala* (Syrian Rue), there are also some synthetic compounds, such as, Harmine freebase/HCl, Tetrahydroharmine freebase/HCl and Moclobemide (Frison, Favretto, Zancanaro, Fazzin, & Ferrara, 2008; Gaujac et al., 2013; Sklerov, Levine, Moore, King, & Fowler, 2005). In addition, some of the compounds analogues used in the blending of ayahuasca can have different psychotropic substances, such as nicotine and caffeine for example (Morales-García et al., 2017).

A scientific analysis of the *B. caapi*, a vine from the Malpighiaceaea family, showed that it is rich in ß-carbolines alkaloids (B-CA) essentially harmine (HMN), tetrahydroharmine (THH) and harmaline (HML) (McKenna, 1999; Morales-García et al., 2017). Diversely, *P. viridis* belongs to the Rubiaceous family, and contains N,N-dimethyltryptamine (DMT) (Callaway et al., 1999; Rivier & Lindgren, 1972). The chemical structures of ayahuasca compounds are shown in figure 1. Depending on the origin and development of the plants used in ayahuasca beverage preparation, the chemical composition may differ, both, in quantity and quality. This is not the only reason for the different compositions, the preparation procedure of the beverage, done by the religious or non-religious groups, can also impact on ayahuasca's ultimate effect (da Motta et al., 2018). Moreover, depending on the dose of these alkaloids, can also trigger an effect on their own (Frison et al., 2008).

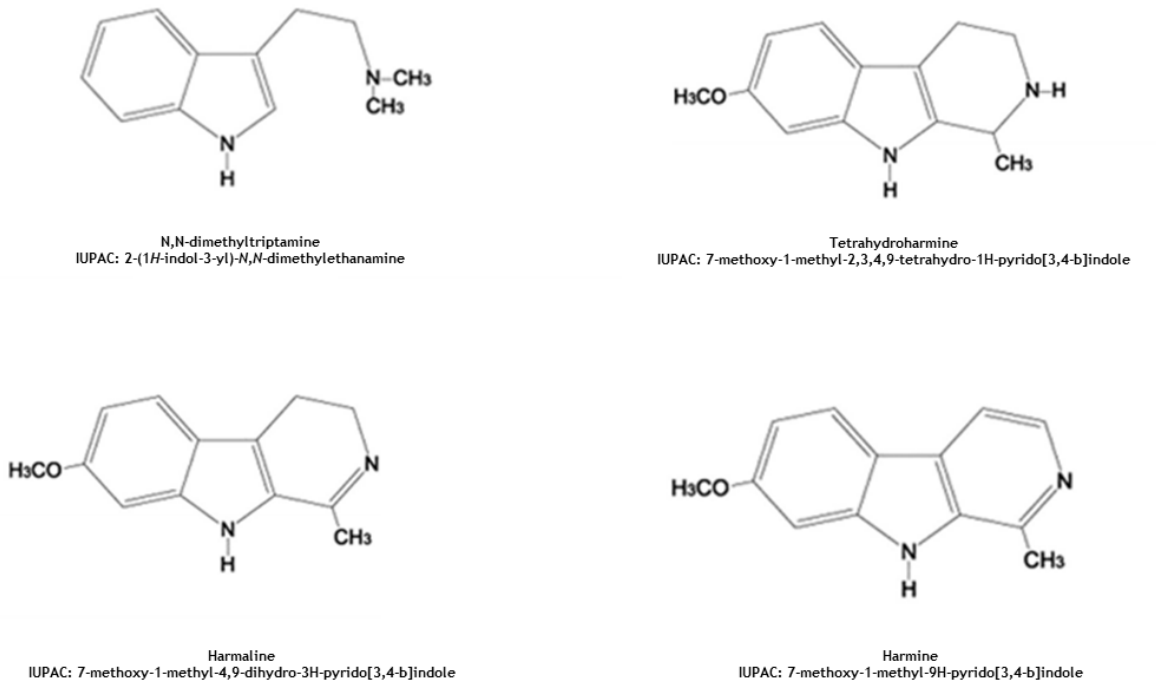

Figure 1 - Chemical structures of DMT; THH; HML and HMN (Adapted from dos Santos & Hallak (2019).

2. N,N-dimethyltryptamine

DMT is a simple molecule of low molecular weight (188.27 g/mol) and hydrophobic character (log P=2.573) (L. P. Cameron & Olson, 2018). It is similar to naturally occurring molecules in the body, such as serotonin and melatonin, rapidly crossing the blood-brain barrier (Pajouhesh

& Lenz, 2005). This compound has high affinity for some neuroreceptors, binding to them and triggering very robust responses (L. P. Cameron & Olson, 2018). DMT is the main psychoactive component of ayahuasca, and can be present in a large number of plants (L. P. Cameron & Olson, 2018; Halpern, 2004; Ott, 1993). As previously stated, DMT is more common in the leaves of *P. viridis*, where the concentration of DMT varies between 0.1% and 0.66% of the dry weight of these same leaves, depending also on the plant and the time of the day in which they are collected (Callaway, Brito, & Neves, 2005; Gable, 2007). Generally, each preparation of ayahuasca can contain between 8.8 mg and 42 mg DMT, causing hallucinogenic effects with doses higher than 0.2 mg/kg (L. P. Cameron & Olson, 2018; Gable, 2007). Intraperitoneal LD_{50} in rats is reported as 47 mg /kg. In the case of intravenous administration, the LD_{50} in rats is 32 mg/kg, while in humans it is estimated to be approximately 1.6 mg/Kg (Gable, 2007). With regard to DMT when ingested on ayahuasca preparations, the LD_{50} estimated is 8 mg/Kg, since is necessary to consider that not all quantity consumed will be bioavailable(Gable, 2007).

2.1. DMT Pharmacokinetics

The effects of DMT vary greatly depending on how it is administered. Smoking is the preferred route for its recreational consumption, although the intravenous route is widely used (Cakic, Potkonyak, & Marshall, 2010; L. P. Cameron & Olson, 2018). When consuming by the latter, the psychoactive effects of this substance are rapid, reaching a maximum concentration 5 minutes after injection, which decreases in the next 30 minutes (L. P. Cameron & Olson, 2018). DMT is rapidly metabolized by the enzyme MAO-A present in the liver, the half-life of this substance being approximately 5 to 15 minutes (Barker, Monti, & Christian, 1981; Sitaram, Lockett, Talomsin, Blackman, & McLeod, 1987). Due to this rapid metabolization, only about 1.8% of the dose of DMT injected into the bloodstream can be measured. Likewise, only 0.16% of the injected dose is detected in urine (L. P. Cameron & Olson, 2018). When this substance is smoked or inflated, psychoactive effects also manifest themselves identically (Barbosa, Mizumoto, Bogenschutz, & Strassman, 2012; L. P. Cameron & Olson, 2018; Gable, 2007). They begin to manifest immediately, peaking in just a few minutes and disappearing about 30 minutes later. However, data on DMT consumed in the smoked form remains scarce (Cakic et al., 2010). However, when taken orally DMT is rapidly degraded by MAO-A present in the intestine and liver, preventing its access to the bloodstream (Barbosa et al., 2012; Rafael G dos Santos, Balthazar, Bouso, & Hallak, 2016; Jordi Riba, McIlhenny, Valle, Bouso, & Barker, 2012; Jordi Riba et al., 2003). As previously mentioned, wwhenever DMT is ingested along with MAO-A inhibitors, such as the case of ayahuasca tea admixtures, it is able to access the bloodstream and rapidly reach the brain exerting its psychoactive effects (L. P. Cameron & Olson, 2018; Gable, 2007). The main metabolites of this mixture identified were indoleacetic acid, 2-methyl-1,2,3,4-tetrahydro-ß-carboline, DMT-N oxide, N-methyltriptamine, 1,2,3,4- hydro-ß-carboline and tryptamine their major metabolites (Appel, West, Rolandi, Alici, & Pechersky, 1999; C.

Cameron et al., 2015; Gatch, Rutledge, Carbonaro, & Forster, 2009; Helsley, Fiorella, Rabin, & Winter, 1998; Karila et al., 2015; R. L. Smith, Canton, Barrett, & Sanders-Bush, 1998).

2.2. DMT Pharmacodynamics

DMT has high affinity for receptors that are part of the serotonergic system. Many of its effects are due to interactions with such receptors (L. P. Cameron & Olson, 2018). 5-HT 1A receptors are coupled to Gi proteins that mediate inhibitory neurotransmission, and are usually expressed in serotonergic neurons, as well as in specific cells of the cortical and subcortical regions (Pazos, Probst, & Palacios, 1987; Sotelo, Cholley, El Mestikawy, Gozlan, & Hamon, 1990). DMT binds with some affinity to this type of receptors, acting as an agonist (Keiser et al., 2009). Agonists of this group's receptors, such as DMT, have been demonstrated to have antidepressant and anxiolytic activity (Domínguez-Clavé et al., 2016; Rafael G Dos Santos et al., 2016). Possibly, these effects are a result of the desensitisation of these receptors during the chronic consumption of these same substances (L. P. Cameron & Olson, 2018). In contrast, 5-HT2A receptors are coupled to the Gq protein and mediates excitatory neurotransmission, been the most well characterized receptors to date (L. P. Cameron & Olson, 2018). DMT leads to the stimulation of this type of receptor, acting as an agonist (L. P. Cameron & Olson, 2018; R. L. Smith et al., 1998). According to literature, the hallucinogenic effects of DMT are due to this agonist effect on 5-HT2A18 receptors (Carbonaro et al., 2015). Studies conducted by Aghajanian and collaborators (G.K Aghajanian & Marek, 1997; George K. Aghajanian & Marek, 1999), showed that DMT is responsible for the stimulation of 5-HT2A receptors, leading to an increased response produced by spontaneous excitatory post-synaptic currents in cortical pyramidal neurons.

In another study, Mckenna and coworkers (Mckenna, Repke, Lo, & Peroutka, 1990), found that DMT has small methyl groups that are responsible for a high affinity towards 5-HT2A receptors. However, over time, this group of receptors is no less sensitive to DMT, which may justify the fact that the human body does not develop tolerance to this substance (L. P. Cameron & Olson, 2018). Another type of serotonergic receptor coupled to Gq proteins is 5-HT2C. In these receptors, DMT only acts with lower affinity, as a partial agonist of the 5-HT2C22 receptor (L. P. Cameron & Olson, 2018). However, over time, 5-HT2C receptors lose sensitivity to DMT (Carbonaro et al., 2015). The affinity of DMT for other serotonergic receptors, such as 5-HT6, 5-HT7 and 5-HT1D, are also described (Heuring & Peroutka, 1997; Keiser et al., 2009; Pierce & Peroutka, 1989). However, further studies are required to understand the possible effects of this affinity. A study by Bunzow *et al*. (Bunzow et al., 2001) suggested that DMT also interacts with the Trace Amine-Associated Receptor 1 (TAAR1) receptor group. In this study, they resorted to the HEK293 cell line expressing TAAR1, and demonstrated that DMT activates TAAR1 by increasing cAMP production (Bunzow et al., 2001).

DMT has also affinity for the sigma-1 receptor. Nonetheless, this affinity is about one hundred times less than that of the above 5-HT 2A receptors. Nevertheless, this substance is one of the few known endogenous agonists of such receptor (Saavedra et al., 1972). Szabo and (Szabo et al., 2016) have recently shown that DMT triggers a mechanism in the sigma-1 receptor that protects cortical neurons from the effects of oxidative stress. Sigma-1 receptors are also involved in the treatment of anxiety and depression.(Hayashi, 2015) Given that DMT produces antidepressant responses it is possible that this receptor is involved in the mechanism that triggers these effects (L. P. Cameron & Olson, 2018).

The effects of DMT on cholinergic and dopaminergic systems have also been investigated, but on a smaller scale than the serotonergic system. Therefore the information available is scarce (L. P. Cameron & Olson, 2018). DMT has a reduced binding affinity for dopamine receptors when compared to other substances (Rickli, Moning, Hoener, & Liechti, 2016). Back in the 70s, a study by Smith (T. L. Smith, 1977) suggested that DMT leads to increased dopamine production. Yet, on a different study by Haubrich and coworkers (Haubrich & Wang, 1977) it was showed that dopamine levels in rat brains decreased after DMT administration. In the same study, it was also possible to demonstrate that when DMT was administered to rats, acetylcholine levels decreased in the striatum, but no change was observed in the cortex (Haubrich & Wang, 1977). In a more recent study, where 18 volunteers were administered between 0.6 and 0.85 mg DMT per kg body weight, it was possible to verify that DMT exerts an agonist effect on dopamine receptors (Jordi Riba et al., 2002).

Also the serotonin transporter (SERT) and the vesicular monoamine transporter (VMAT) are affected by DMT. Sangiah and colleagues (Sangiah, Gomez, & Domino, 1979) observed rat brain slices and found that there is accumulation of DMT in them through an active transport mechanism. In another study by Cozzi and fellow researchers (Cozzi et al., 2009), it was possible to conclude that DMT acts as a substrate for both transporters.

2.3. Adverse effects of DMT

When DMT is consumed, some physical effects such as nausea, vomiting and diarrhea are common (L. P. Cameron & Olson, 2018; Gable, 2007). Also increased heart rate and blood pressure were observed (Strassman & Qualls, 1994). A study by Riba *et al.* (J Riba et al., 2001) showed that an oral dose of 1 mg/kg DMT is sufficient for the occurrence of these effects. Otherwise, if DMT is administered intravenously, only doses between 0.1 and 0.2 mg/kg are required. Another study showed that 2 minutes after intravenous administration, systolic blood pressure increased by about 35 mmHg and diastolic blood pressure by about 30 mmHg (Strassman & Qualls, 1994). In the same study there was also an increase in heart rate by about 26 beats per minute (Strassman & Qualls, 1994).

Other symptoms commonly manifested when consuming DMT are visual hallucinations and delirium (L. P. Cameron & Olson, 2018; Gable, 2007). DMT can cause emotional distress and may lead to psychosis or even schizophrenia (L. P. Cameron & Olson, 2018; Gable, 2007). Studies have shown that in patients with schizophrenia the amount of DMT in urine and blood is above normal (Checkley, Murray, Oon, Rodnight, & Birley, 1980; Lipinski, Mandel, Ahn, Vanden Heuvel, & Walker, 1974). Years later, Ciprian-Ollivier *et al*. (Ciprian-Ollivier & Cetkovich-Bakmas, 1997) constructed the hypothesis that the DMT would lead to a gradual degradation of the cognitive processes. However, these findings are somewhat controversial, since another study has concluded that increased levels of DMT have a calming effect and suppress psychotic activity (Jacob & Presti, 2005). Also the sensation of relaxation has been described in the literature. Gillin *et al*. (Gillin JC, Kaplan J, Stillman R, 1976) underwent a clinical trial with intramuscular administration of DMT in healthy patients and verified that a great part of the participants had a feeling of relaxation. The same results were verified in another study by Strassman and Qualls (Strassman & Qualls, 1994) with intravenous administration of DMT in healthy patients. Other studies also indicate that the action of DMT on serotonergic neurons result in depression and anxiety (De Lima Osório et al., 2011; Fortunato et al., 2010, 2009; R.G. Santos, Landeira-Fernandez, Strassman, Motta, & Cruz, 2007). However, there is, once again some controversy in these results, since other studies suggest that DMT has anti-depressant properties and increase the positive mood (Rafael G. dos Santos et al., 2016; Gable, 2007; Gillin JC, Kaplan J, Stillman R, 1976; Strassman & Qualls, 1994).

Psychedelic substances are commonly associated with dependence, but this does not apply to DMT, considering that to the present date there are no reports in the literature that this substance shows withdrawal symptoms when it is not consumed (L. P. Cameron & Olson, 2018; Gable, 2007).

3. β-Carbolines alkaloids

B-CA derive from tryptophan amino acid, presenting a heterocyclic and dehydrogenated structure. They are synthetized through a condensation of indolamines along with aldehydes or α.keto acids (Kim, Sablin, & Ramsay, 1997; McKenna, 2004). According to Moura and coworkers (Moura, Richter, Boeira, Pêgas Henriques, & Saffi, 2007), B-CA have a broad spectrum of action on human organs and can be found not only in animals, but also in many plants and fungi (McKenna & Towers, 1984). Besides, it can function as endogenous compounds in some mammalian species (McKenna, 2004).

Harmaline, was first isolated from the seeds and roots of *Peganum harmala L* (Zygophyllaceae), being stated as the major compound present (5.6% w/w on the seeds). However, this plant also contains HMN, HLOL and THH, mainly on the seeds and roots (Moloudizargari, Mikaili,

Aghajanshakeri, Asghari, & Shayegh, 2013). In this part of this plant, the quantity of these chemicals varies between 2-5% (Asgarpanah et al., 2012).

In 1999, Ott decided to evaluate the combined effects of DMT and HMN, on his own body. So he started with 20 mg of DMT and 40 mg of HMN, and started to increase HMN doses', having discovered that 1.5 mg/kg of this compound can exert effects combined with DMT, whereas when DMT was tested alone little or any effects were observed (Ott, 1999).

One of B-CA's mechanism is the reversible inhibition of monoamine oxidase (MAO) activity. These enzymes, present in the mitochondria membrane are of two types: MAO-A and MAO-B. Their main functions are the oxidation of endogenous and exogenous amine substrates as mono and/or polyamines, but also modifying amino acids within proteins (Gaweska & Fitzpatrick, 2011; Liester & Prickett, 2012). They can be found in the brain, kidney, spleen and intestines (Orlefors et al., 2003). In the brain MAO-B is more abundant then MAO-A, but both are responsible for the oxidative deamination of several neurotransmitters, such as dopamine, serotonin and norepinephrine (Brierley & Davidson, 2012; Lewis, Miller, & Lea, 2007). Although, it has been reported the specificity of these compounds to MAO-A (Domínguez-Clavé et al., 2016).

On the one hand, B-CA can suffer a N-methylation reaction on the second position and the obtained compound from this is an analogue of 1-methyl-4-phenylpyridinium ion (MPP+), which can have cytotoxic mitochondrial effects, since this product accumulation can lead to oxidative stress, which blocks the complex I of the mitochondrial electrons transport chain (Boulton, Keane, Morris, McNeil, & Manning, 2012). Diversely, some researchers suggested the anti-oxidant properties of B-CA against reactive oxygen species (ROS), possibly reducing cell damage (Kim et al., 1997).

When comparing the effects of harmine with other drugs (LSD and mescaline) in humans and animals, Naranjo found that the oral administration (20-50 mg), harmine psychedelic effects began after 20-30 minutes, lasting up to 6-8 hours. Having its greatest effect at half an hour to 1 hour. The same did not occur using intra muscular application (10-20 mg) (Naranjo Plutarco, 1959b). In this case, the effects started 5-10 minutes lasting up to 3-5 hours, having its maximal effect at 30 minutes after administration. Comparing to the other drugs the effects were very much alike on a qualitatively basis, but with different magnitude (Naranjo Plutarco, 1959b). While harmine was described as the most "horrible" and "paranoid", causing mydriasis, salivation, lacrimation, hyperthermia, hyperglycemia hypotension and others. All of the compounds tested caused aggressive behavior in both humans and animals, as well as psychedelic and emotional effects (Naranjo 1959).

Later studies found that harmaline psychedelics effects were only achieved when intra venous doses were higher than 1mg/kg, and its effects were practically immediate after injection; or orally higher than 4 mg/kg, having effects after about one hour later (Naranjo, 1967). Some of

the subjective effects reported by the author were nausea and vomiting, having different types of vision, feeling like floating on air. Although this discover was essential as a source on the potential effects of B-CAs, it is important to consider the method was archaic compared to today's technology and methodology. Despite all existing researches, more studies of these B-CA need to be performed to understand the depths of its action mechanism and biochemical effects on the human body.

Since the interest of this compounds is in hands with the increase of ayahuasca consumptions, and because DMT is responsible for the psychedelic effects when it is ingested, the majority of publications regarding the pharmacokinetics and dynamics of isolated B-CA remains scarce in literature. Yet, it is already understood how DMT and B-CA behave synergistically.

4. Methods of quantification of ayahuasca

As the consumption of ayahuasca tea and similar plants increase, new challenges and development of new techniques in the forensic and toxicological fields become of utmost importance. Considering one of the objectives of this dissertation, a compilation of various existing and published procedures for ayahuasca quantification. Although the review contains analysis on biological specimens such as urine and plasma, the focus is ayahuasca teas or preparations.

The first work developed for the quantification of DMT and B-CA alkaloids was performed by Yritia *et al.* (Yritia et al., 2002). In this work the DMT present in plasma was extracted using a liquid-liquid extraction (LLE) with n-pentane and was quantified using a gas chromatograph coupled to a nitrogen-phosphorus detector. Retentions of 74% and a quantification limit (LOQ) of 1.6 ng/ml were obtained. On the other hand, harmine, harmaline and tetrahydroharmine, as well as harmine and harmaline O-demethylation metabolites were quantified in the plasma using high-performance liquid chromatography (HPLC) coupled to fluorescence detector having previously performed a solid phase (SPE). Recoveries above 87% and a LOQ of 0.5 ng/ml for harmine, 0.3 ng/mL for harmaline, 0.3 ng/mL for harmol and harmalol and 1.0 ng/mL for THH were obtained. In both processes a good linearity was observed in the concentration ranges evaluated for DMT (2.5-50 ng/mL) and for ß-carbolines (0.3-100 ng/mL). Later, in 2008, Pires *et al.* (Pires et al., 2009) developed a new method for the simultaneous quantification of DMT and B-CA alkaloids. The alkaloids were extracted by SPE (C18) and quantified by gas chromatography with nitrogen-phosphorus detection. The method was linear in the concentration range of 0.02 to 4.0 mg/mL (r^2> 0.99), with LOQ being 0.02 mg/mL. In 2012, Oliveira *et al.* (Oliveira et al., 2012) quantified the constituents of ayahuasca (DMT and B-CA) in human plasma. For this purpose, the analytes were extracted by SPE (C18) and quantified using a liquid chromatography coupled to mass spectrometry (LC-MS/MS), with LOQs of less than 0.5 ng/mL for all analytes. The following year, Gaujac *et al.* (Gaujac et al., 2013)

combined a solid-phase microextraction technique (SPME) in headspace mode with gas chromatography coupled to mass spectrometry (GC-IT-MS) in order to quantify DMT. The method showed accuracy values between 71% and 109% and showed good linearity (1.56 to 300 mg / L, r^2 = 0.9975). The LOQ was 9.5 mg/L and the limit of detection (LOD) was 0.78 mg/L. Finally, in 2014, Pichini *et al*. (Pichini et al., 2014) have developed a method for the detection of various substances, namely DMT, using ultra-high pressure liquid chromatography tandem mass spectrometry in hair samples. Initially, the hair was washed with methyl alcohol and diethyl ether and subsequent addition of internal standards. The samples were then treated with VMA-T M3 for 1 h at 100 ° C and after cooling, 100 µL of M3 extract were diluted with 400 µL of water and 10 µL were injected into the apparatus. A reverse phase column maintained at room temperature was used and elution was performed in linear gradient with 0.3% formic acid in acetonitrile ammonium formate (5 mM pH 3). The method was linear from LOQ (0.03-0.05 ng/mg) to 10 ng/mg hair. Recovery was obtained between 79.6% and 97.4%.

Table 1 – Bioanalytical procedures for the determination and quantification of the major compounds of ayahuasca in biological samples and ayahuasca teas or preparations.

Analyte	Matrix	Sample preparation	Detection mode	Stationary and mobile phase	Recovery (%)	LOD; LOQ	Concentrations of the compounds	Reference
DMT, HMN, HML, THH, HMNOL and HLOL	Plasma	LLE (n-pentane) (DMT); SPE (HMN, HML, THH and THH O-demethylation metabolites)	GC-NPD (DMT); HPLC-FLD (HMN, HML, THH and THH O-demethylation metabolites)	GC-NPD: 5% phenyl-methylsilicone capillary column (12m x 30.2mm x 0.33µm film thickness); Helium (DMT); HPLC-FLD: Kromasil 100 C_{18} (5 µm, 150 x 34 mm) and Solvent A: mixture ammonium acetate buffer (50 mM, pH 8.0) (63:37 v/v) and acetonitrile–methanol (20:30 v/v) and Solvent B: mixture of acetonitrile- methanol (20:30 v/v) (HMN, HML and THH); Solvent A: mixture ammonium acetate buffer (50 mM, pH 6.3)	74 (DMT); > 87 HMN, HML, THH and THH O-demethylation metabolites)	--; 1.6 ng/mL (DMT), 0.5 ng/mL (HMN), 0.3 ng/mL (HML), 0.3 ng/mL (harmol and harmala) and 1.0 ng/mL (THH)	0.53 mg/mL (DMT); 0.9 mg/mL (HMN); 0.06 mg/mL (HML); 0.72 mg/mL (THH)	(Yritia et al., 2002)

				(73:27 v/v) and acetonitrile–methanol (20:30 v/v); Solvent B: acetonitrile–methanol (20:30 v/v) (harmol and HLOL)				
DMT, THH, HML and HMN	Plasma	SPE (C_{18})	LC–MS/MS	Phenomenex Synergi Hydro-RP80A (50 x 2.0 mm, 4 µm) and Solvent A: mixture of aqueous solution of ammonium formate (5 mmol/L) with formic acid (0.1%); Solvent B: methanol and formic acid (0.1%)	88.4-107.7	0.1 ng/mL; 0.2 - 0.4 ng/mL	1.2-19.8 ng/mL (DMT); 1.0-15.6 ng/mL (HMN); 2.7-15.7 ng/mL (HML) and 27.1-71.4 ng/mL (THH)	(Oliveira et al., 2012)
DMT	Hair	Hydrolysis (M3 reagent)	UHPLC-MS/MS	Acquity UHPLC HSS C_{18} column (2.1 mm x 150 mm, 1.8 µm) and solvent A: formic acid in acetonitrile (0.3%), Solvent B: ammonium formate (5 mM, pH 3)	79.6-97.4	0.01-0.02 ng/mg; 0.03-0.05 ng/mg	5.6 ng/mg	(Pichini et al., 2014)

DMT, THH HML and HMN	Ayahuasca preparations	SPE (C_{18})	GC-NPD	HP Ultra-2 fused-silica capillary column (25 m × 0.2mm× 0.33 µm) and Solvent A: formic acid in acetonitrile (0.3%); solvent B: ammonium formate (5 mM, pH 3)	68.4-99	0.01 mg/mL; 0.02 mg/mL	0.31-0.73 mg/mL (DMT); 0.37-0.83 mg/mL (HMN); 0.64-1.72 mg/mL (HML) and 0.21-0.67 mg/mL (THH)	(Pires et al., 2009)
DMT	Ayahuasca beverages	SPME (polydimethylsiloxane /divinylbenzene fiber)	GC-IT-MS	Supelco SLB-5 ms capillary column (30m x 0.25 mm, 0.25 mm film thickness)	71-109	0,78 mg/L; 9.5 mg/L	0.17-1.14 mg/mL	(Gaujac et al., 2013)

DMT: N, N-dimethyltryptamine; HMN: Harmine; HML: Harmaline; THH: tetrahydroharmine; HLOL: Harmalol; LOD: Limit of detection; LOQ: Limit of quantification; LLE: liquid-liquid extraction; GC-NPD: Gas chromatography coupled to Nitrogen Phosphorous Detector; SPE: solid-phase extraction; HPLC-FLD: High-performance liquid chromatography coupled to fluorescence detection; LC–MS/MS: liquid chromatography coupled to tandem mass spectrometry; UHPLC-MS/MS: ultra-high-pressure liquid chromatography tandem mass spectrometry; SPME: solid-phase microextraction; GC-IT-MS: Gas chromatography coupled to ion trap mass spectrometry

5. I*n vivo* and *in vitro* studies of ayahuasca compounds

For many years, studies on ayahuasca or its chemical compounds have been focused on humans, mainly done by inquiries or clinical trials. Therefore, the results obtained are subjective and little is known about biochemical and physiological effects. Some of the symptoms reported after constant ayahuasca consumption are feelings of confidence and optimism, plus on a psychiatric level, a reduction on the lack of concentration, sleep deprivation, irritability and depression were reported (Barbosa, Cazorla, Giglio, & Strassman, 2009; Barbosa, Giglio, & Dalgalarrondo, 2005). On a recent study, which main goal was to understand the possible reproductive effects of the beverage in male Wistar rats (A. de F. A. Santos, Vieira, Pic-Taylor, & Caldas, 2017), researchers used doses of 1x, 2x, 4x, and 8x the dose used in a ritual. The content of dose 1x were 0.146 mg/mL of DMT, 0.12 mg/mL of harmaline and 1.56 mg/mL of harmine. It was observed, that only the animals exposed to the 8x the dose, suffered from stress, "vocalization during the gavage procedure", piloerection, tremors and weight loss. A decrease on the will to eat, was mostly observed on animals treated with 4x or 8x the dose. Indeed two animals treated with the 8x dose died. They found that such results indicate that higher exposure doses could be representative of chronic toxicity in rats. Moreover, at the 4x the dose, it was observed a reproductive toxic effect and for the 8x dose, a reduction on testis size, of male rats was observed, without further morphological changes.

Regarding *in vitro* studies concerning the effects, or therapeutics potential of ayahuasca and/or its major compounds are scarce. Moreover, to best of our knowledge, no *in vitro* study has been done evaluating the effects of traditional ayahuasca (*B. caapi* + *P. viridis*), or other plants used as analogues, such as *P. harmala* or *M. tenuiflora*. In addition, the published works only focus on one of the compounds, being unable to transmit the overall ayahuasca effects. Still, we tried to make a synthetic review on what has been published so far.

In 2010, Samoylenko and coworkers (Samoylenko et al., 2010), conducted a study with different mammalian cellular lines, including, human cancer cell lines (SK-MEL, KB, BT-549 and HepG2), non-human primate kidney fibroblasts (VERO) and pig kidney's epithelial cells (LLC-PK11). The cytotoxicity was tested against different enzymes at a concentration of 100 µg/mL, namely acetylcholinesterase (AChE), butylcholinesterase (BuChE) and catechol-O-methyl transferase (COMT). No negative effects on SK-MEL, BT-549 and monkey's VERO cell line were observed (Samoylenko et al., 2010). These authors have also studied the ability of MAO inhibition by HMN and HML, concluding that these compounds may have therapeutic potential on the treatment of PD. (Samoylenko et al., 2010).

On a different study using just *B. caapi* alkaloids, conducted by Morales-García and collaborators, it was found that HMN, THH and HML harmaline can stimulate adult neurogenesis, which is the mechanism that develops new functional neurons from progenitor cells. This was

grounded on the fact that these B-CA have an anti-depressant effect and are connected to the capacity of this drugs to stimulate neurogenesis. It was concluded that these chemical substances can promote proliferation, migration and differentiation of progenitor cells from the sub ventricular zone to the sub granular zone, which are the main areas of the brain where neurogenesis happens (Morales-García et al., 2017).

6. Conclusion and further perspectives

Over the years, the consumption of ayahuasca all over the world has increased and not only for the use in shamanic and religious rituals, but also for recreational purposes. Therefore, many psychological studies have been conducted in order to evaluate its effects on a mental and subjective level. Given that, clinical and *in vivo* studies have also increased, evaluating biological effects, being the CNS one of the main targets.

Even though it is known that the components of the beverage, mainly B-CA and DMT, exert their effects by inhibiting MAO-A (by B-CA) and allowing DMT to access the bloodstream, little is known of their effects at the cellular level and few *in vitro* studies have been conducted. The ones considered in this review, describe positive effects on proliferation and development of new brain cells (neurogenesis). Nonetheless, one may wonder about the cytotoxicity associated not only because the beverage is consumed for long periods of time, as the rituals take place twice a month, but also because recreational use of this beverage is becoming more prominent on western civilizations. Thus, it is needed further investigation in this sense.

The main goal of this article was to review *in vitro* studies done with these compounds, and try to understand their effects on cells.

Still, more studies need to be performed, to demystify weather the effects are beneficial or if they can bring up severe and irreversible damages after long exposure/intake.

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Chapter II – Experimental developments

In order to facilitate the reading of this document, and this chapter in particular, at first it is presented the analytical part of the study, where the analytes were studied, quantified and characterized in tea samples, as well as the optimization of the extraction procedure and the results obtained. After this explanation, the cytotoxicity studies perform in this work is presented and explained.

1. Materials and methods

1.1. Reagents and standards

The analytical standards of HMN, HML and THH and DMT were kindly provided by Nal von Minden, GmbH (Regensburg, Germany). The internal standard (IS) promazine (PRZ) was acquired from Sigma Aldrich (Lisbon, Portugal). It is important to notice that the selected IS is not commercially available in Portugal and was chosen due to its structural analogy with the chemical compounds in study, allowing to refine linearity, accuracy and precision, while minimizing uncertainties that could have been induced by the sample and other variations and interference that could lead to impairments on the analysis step (Skoog, West, Holler, & Crouch, 2013). Methanol, dichloromethane, 2-propanol, hexane were obtained from Fisher Scientific (Enzymatic; Santo Antão do Tojal, Portugal) and ammonium hydroxide from T.J. Baker (Deventer, Holland). These chemical were all analytical or chromatographic grade. Hydrochloric acid (37% v/v) from Enzymatic (Santo Antão do Tojal, Portugal). Deionized water (DI) was obtained from a Milli-Q system (Millipore Billerica, MA, USA). Oasis® MCX Solid Phase extraction cartridges (3 cm^3/60mg) were obtained from Waters (Milford, MA, USA).

1.2. Preparation of the solutions

Working solutions of THH, HMN and HML were prepared by dilution of the stock solutions (1mg/mL) with methanol to the final concentration of 100 µg/mL and 10 µg/mL. Regarding DMT, the concentration of the stock solution was 100 µg/mL, and then serial dilutions were prepared, to the final concentrations of 10 µg/mL and 1 µg/mL (working solutions).
The concentration of the IS was 1 µg/mL. All of the solutions were stored protected from light at 4 °C.

1.3. Instrumentation and chromatographic conditions

Chromatography was operated on a gas chromatographer model HP7890B equipped with a model a mass spectrometer model 5977A from Agilent Technologies. Data was acquired in the *selected ion monitoring* (SIM) mode using the ChemStation from Agilent Technologies. The separation of the analytes was achieved using a capillary column (30 m × 0.25 mm; 0.25 µm I.D.) packed with 5% de phenylmethylsiloxane (HP-5MS) supplied by Agilent Technologies. Carrier gas was helium set at a constant flow rate of 0.8 mL/min. The injection volume was 2 µL in split mode at a ratio of 5:1. The temperatures of the injector and detector were set at 250 °C and 280 °C, respectively. Ion source temperature was set at 230 °C and quadrupole at 150 °C. The oven temperature started at 90°C for 3 min increasing 15°C/min up to 300°C and held for 8 min. The total separation run took 25 min. The mass spectrometer was operated at 70 eV with a filament of 300 µA in the positive electron ionization mode. Three ions for each analyte and one for the IS were chosen taking into account the selectivity and abundance, in order to maximize the signal-to-noise ratio in matrix extracts. Table 1 summarizes retention times and in what conditions they were detected. SCAN chromatograms are in the attachments.

Table 1 - Retention times and selected ions for the identification of the ß-Carbolines and DMT.

Analyte	Retention time (min)	Quantifying ion (m/z)	Qualifying ion (m/z)	Dwell time (µs)
DMT	12.14	58	130 188	50
THH	14.80	201	172 216	50
HML	15.04	213	198 214	50
HMN	15.35	169	197 212	50
PRZ*	15.84	284	-	50

*Internal Standard; DMT: N,N-Dimethyltryptamine; THH: tetrahydroharmine; HML: harmaline; HMN: harmine; PRZ: promazine

1.4. Sample preparation

In this study, samples were prepared as a liquid extract based on a recipe kindly provided by Dr. Nicolás Fernández (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires Argentina). The plants used were *B. caapi*, *P. viridis, M. tenuiflora; P. harmala* and *DC AB* (commercial name) and were kindly supplied from Nal von Minden, GmbH (Regensburg,

Germany). All plants were weighed as follows: 0.215 g; 0.212 g; 0.213 g; 0.210 g and 0.208 g. Firstly, 250 mL of ultra-pure water was added to 5 different shot flasks and brought up to a boil (100 °C). The plants were then added to the correspondent flask were the boiling process was maintained during 6 h. Nonetheless, as water was evaporating and to avoid total water loss, 2h after the initial time, 150 mL of the same water was added to each flask. The same happened after 4 h of the initial time, but this time 50 mL of ultra-pure water was added and the temperature was reduced to 60 °C until the process finish. The final volume obtained for each tea was approximately 200 mL, from this 50 mL of each sample was put into falcon tubes so that the samples could suffer a process of freeze-drying to posterior cell incubation and testing. For extraction procedure and compounds determination, 2 mL of each were stored in amber glass containers.

1.5. Tea extraction

For this study the extraction was performed with solid phase extraction (SPE) using Oasis® MCX3cc 60mg. SPE procedure conditions were previously optimized (see section 2.1) and so the final conditions were as described herein. Previous to extraction procedure, 500 µL of each tea sample was diluted with 4 mL of phosphate buffer (pH=5) and spiked with 25 µL of IS. SPE cartridges were conditioned with 2 mL of methanol and 2 mL of DI water. After the sample passed through the cartridge, a washing step was done in the following order: 2mL of DI water, 2 mL of hydrochloric acid (0.1 M) and 2 mL hexane. Following this step, the columns were dried under full vacuum for 10 min. Subsequently, the analytes of interest were eluted with 2 mL of a mixture of dichloromethane:isopropanol:ammonium hydroxide (80:20:2 v/v). The resulting extracts were evaporated to dryness under a steam of nitrogen. The remaining residues were dissolved in 50 µL of methanol and vortex mixed and, 2 µL was injected into the GC-MS system in SIM mode.

1.6. Validation procedure

The described method was fully validated in a five-day validation protocol according to the Food and Drug Administration (FDA) and the Scientific Working Group of Forensic Toxicology SGWTOX guidelines (SWGTOX, 2013; U.S. Department of Health and Human Services & Food and Drug Administration, 2018). This study included parameters such as selectivity, linearity, intermediate precision and accuracy, intra and inter-day precision and accuracy, absolute recovery, limits and calibration curves.

1.7. Freeze-drying of the beverages samples

After the sample was prepared and let to cool at room temperature, 50 mL of each sample was collected into falcon tubes and stored at -20 °C for 12 h and the stored at -80 °C for 24 h. Then samples were put into the freeze-drier (Savant, Novalyphe-NL500). This processes assures that all water molecules suffer sublimation, that is, the conversion of liquid water directly to water in the gaseous state. The process occurred for 120 h.

2. Results and discussion

2.1. Optimization of the extraction procedure

In order to optimize the extraction procedure, a total of four different techniques were tested. These techniques were chosen based on the available literature for similar compounds, since literature for the target analytes is scarce (Fernández, Cabanillas, Olivera, & Quiroga, 2019). The elution step was the studied parameter, using different solvents and different concentration levels. For this assay, the samples were spiked with compounds at the lower calibration concentration (C1) and IS was added after extraction. The four techniques evaluated (n=3) are summarized table 2.

Table 2 - Different elution solvents and concentration levels used in solid phase extraction (SPE).

Protocol	Sample (Volume)	Dilution of the sample	Packaging	Washing	Elution
T1	250 µL of DI water spiked with 0.2 µg/mL (B-CA mix.) and 0.02 µg/mL (DMT)	4 mL of phosphate buffer (0.1M), pH=5	2 mL of MeOH + 2 mL of DI water	2 mL DI water + 2 mL HCl (0.1 M) + 2 mL of hexane	Dicholoromethane:isopropanol:ammonium hydroxide [80:20:2]
T2					Methanol:isopropanol:ammonium hydroxide [40:60:0,25]
T3					Methanol:isopropanol:ammonium hydroxide [65,5:32,5:2]
T4					Methanol:ammonium hydroxide [98:2]

B-CA: Beta-Carbolines; DI: deionized water; MeOH: methanol; HCl: hydrochloric acid

When comparing all of the tested elution solvents and different proportions (Figure 1), better results were observed when using T1. Regarding T2, which uses methanol: isopropanol: ammonium hydroxide [40:60:0.25], this revealed low relative peak areas for DMT and THH, and great relative peak areas for HMN and HM, although a large standard deviation is observed for HMN. Despite obtaining good relative peak area values for DMT and HML, on T3 very poor results were achieved for the remaining compounds. Finally, T4, did show good relative peak area values for DMT, HMN and HML, except for THH. For this reason, overall T1 was the one that showed greater recoveries for most target compounds, as well as better coefficient of variation (CV), and the selected extraction procedure to be applied in the present method.

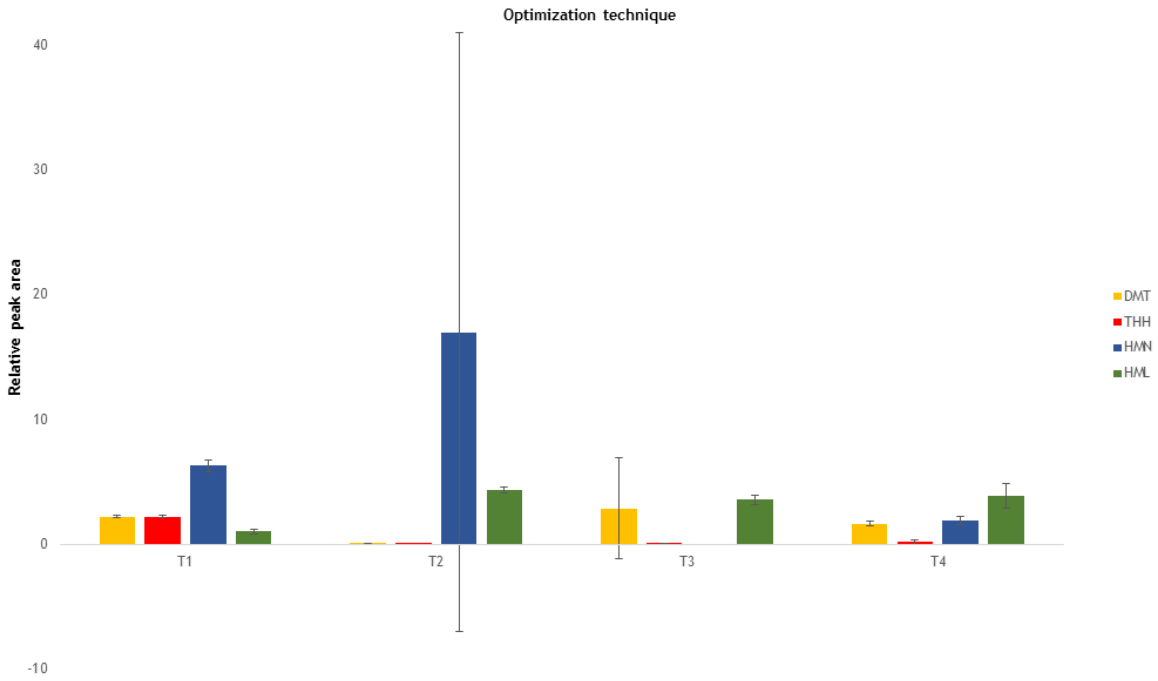

Figure 1 - Evaluation of different solvents and proportions at the extraction technique

2.2. Selectivity

Selectivity is the ability of an analytical method to detect the target compounds while evaluating the presence of potential endogenous interferences (U.S. Department of Health and Human Services & Food and Drug Administration, 2018). This parameter was studied in order to verify the presence of other drugs of abuse (cocaine, opiates, cannabinoids and amphetamines) that the users of ayahuasca may consume, as well as caffeine and nicotine.

The described method was considered selective due to no interferences having been observed at the retention time and respective monitored ions. The criteria for the identification of the analytes was established according to the World Anti-Doping Agency recommendations (World Anti-Doping Agency (WADA), 2010).

2.3. Linearity and limits

To evaluate the method's linearity, calibration curves were prepared ranging from 0.2 µg/mL to 20 µg/mL for all compounds, except for DMT that ranged from 0.04 µg/mL to 5 µg/mL. Seven calibrators (six in the case of DMT), were prepared by spiking DI water with the working solutions. The results were expressed by plotting the peak area ratio between each analyte and the IS with the correspondent concentration.

In order to respect the chosen guidelines criteria, accuracy of the calibrator had a relative error (BIAS) within ± 15% of the nominal concentrations, whereas a value within ± 20% was accepted for the lowest limit of quantification (LLOQ) and a coefficient of variation (CV) equal or lower than 20 %. Moreover, a determination coefficient (R^2) value equal or above 0.99 for linearity is acceptable (mean values). For all other calibrators, CV's accuracy within ± 15 % interval is accepted (U.S. Department of Health and Human Services & Food and Drug Administration, 2018). Results are summarized in table 3.

The LLOQ is defined as the lowest concentration of analyte that could be measured and quantified with adequate precision and accuracy i.e. CV ≤ 20 % and a BIAS within ± 20% (mean values) (U.S. Department of Health and Human Services & Food and Drug Administration, 2018). The LLOQs obtained were 0.2 µg/mL for all compounds, except for DMT where a LLOQ of 0.04 µg/mL was achieved. These results were considered satisfactory, when comparing to other studies. Gaujac and coworkers (Gaujac et al., 2013), obtained a LLOQ for DMT of 9.5 mg/L, which was higher than the value presented in this study. On a different study by Pires coworkers (Pires et al., 2009), the reported LLOQ for all the compounds (DMT, HMN, HML, THH) were 0.02 mg/mL, when using 0.5 mL (double of the volume used herein). However, NPD was used as a detector, which is a less sensitive method than MS. The remaining publication on quantification of these compounds are applied to biological specimens, making it impossible to compare our results (Oliveira et al., 2012; Pichini et al., 2014; Yritia et al., 2002).

The limit of detection (LOD) is usually considered as the lowest concentration of the analyte that could be distinguished from the background noise (U.S. Department of Health and Human Services & Food and Drug Administration, 2018). The LOD was not systematically evaluated, hence considered the same as the LLOQ.

Table 3 - Linearity data (n=5).

Analyte	Weighting factor	Linear range (µg/mL)	Linearity*		R^{2}*	LLOQ (µg/mL)
			m	b		
DMT	1/x	0,04-4	60.9308 ± 1.9890	-2.7790 ± 0.0443	0,9967 ± 0,0017	0.04
THH	1/x	0.2-20	38.4787 ± 54.9862	-10.6541 ± 15.8236	0.9972 ± 0.0007	0.2
HML	1/x	0.2-20	4.0246 ± 0.6677	-0.7049 ± 0.0009	0.9960 ± 0.0015	0.2
HMN	1/x	0.2-20	44.8453 ± 21.8932	-3.236 ± 5.1266	0.9971 ± 0.0007	0.2

m: slope; b: intercept; *Mean values ± standard deviation; DMT: N,N-Dimethyltryptamine; THH: tetrahydroharmine; HML: harmaline; HMN: harmine

2.4. Intra- and inter-day precision and accuracy

Quality control (QC) samples were prepared to guarantee the liability of the developed method. Three different levels of concentrations were used for each analyte (0.8, 8 and 16 µg/mL), except for DMT (0.06, 0.8 and 4 µg/mL), samples were performed in triplicates (n=3) and evaluated along the 5 day validation protocol. To validate the method, the same criteria for CV and RE were applied. Once again, CV values were found to be lower than 15 % and a BIAS within ± 15% (mean values).

Intermediate precision and accuracy was calculated by analyzing the QC samples (3 different concentrations levels within the linearity reach). The obtained CVs were lower than 15 %, and a BIAS of ± 9.6 % (Table 4).

Intra-day precision and accuracy were evaluated by analyzing on the same day (n=6) spiked samples with DMT, HMN, HML and THH at three different concentrations. The CVs were lower than 11 % at the evaluated concentrations and a mean BIAS value within ± 13% (Table 5).

Finally, to determine inter-day precision and accuracy, the seven concentration levels for all compounds (6 for DMT) were evaluated with a 5-day period. It was possible to observe that CVs were typically lower than 14%, except for LLOQs, for which a value of 17.21% was obtained for HMN. (Table 6).

Table 4 - Intermediate precision and accuracy (n=15).

Analyte	Spiked concentration (µg/mL)	Measured concentration* (µg/mL)	CV (%)	BIAS* (%)
DMT	0.06	0.054 ± 0.001	2.06	-9.6
	0.8	0.77 ± 0.100	13.13	-4.3
	4	3.97 ± 0.174	4.39	-0.9
THH	0.8	0.79 ± 0.889	11.29	-1.59
	8	8.14 ± 0.559	6.87	1.78
	16	18.34 ± 9.752	15.22	14.65
HMN	0.8	0.78 ± 0.076	9.74	-2.6
	8	7.66 ± 0.733	9.57	-4.3
	16	16.28 ± 1.407	8.64	1.7
HML	0.8	0.74 ± 0.040	5.45	-7.69
	8	8.37± 0.661	7.89	4.67
	16	16.69 ± 0.915	5.48	4.30

CV: coefficient of variation: BIAS: relative error [(measured concentration-spiked concentration/spiked concentration) × 100)]; *Mean values ± standard deviation.

Table 5 - Intra-day precision and accuracy (n=5)

Analyte	Nominal concentration (µg/mL)	Measured* (µg/mL)	CV (%)	BIAS (%)
DMT	0.4	0.04±0.004	9.04	3.20
	0.5	0.46±0.054	11.69	-7.26
	4	4.00±0.346	8.64	1.34
THH	0.4	0.37 ± 0.019	5.07	-7.35
	1	0.89 ± 0.021	2.32	-11.49
	20	22.63 ± 0.301	1.33	13.14
HMN	0.2	0.18 ± 0.004	1.84	-10.00
	0.4	0.44 ± 0.018	4.06	10.07
	1	1.03 ± 0.096	9.31	3.17
	20	20.29 ± 1.301	6.41	1.44
HML	0.4	0.36 ± 0.019	5.21	-10.30
	1	0.92 ± 0.046	4.97	-8.11
	20	20.31 ± 2.108	10.38	1.54

Table 6 - Inter-day precision and accuracy (n=5).

Analyte	Nominal concentration (µg/mL)	Measured concentration* (µg/mL)	CV (%)	BIAS (%)
DMT	0.04	0.053 ± 0.001	1.68	15.03
	0.1	0.096 ± 0.003	3.06	-3.65
	1	0.954 ± 0.065	6.86	-4.62
	3	2.923 ± 0.286	9.77	-2.57
	5	5.08 ± 0.191	3.77	1.59
THH	0.2	0.219 ± 0.002	0.70	9,50
	0.4	0.440 ± 0.030	6.79	9.93
	1	0.869 ± 0.015	1.68	-13.10
	2.5	2.279 ± 0.1845	8.11	-8.84
	5	4.811 ± 0.259	5.39	-3.76
	10	10.048 ± 0.382	3.81	0.48
	20	20.426 ± 0.206	1.01	2.13
HMN	0.2	0.198 ± 0.034	17.21	-0.73
	0.4	0.404 ± 0.031	7.59	1.01
	1	1.029 ± 0.088	8.55	2.93
	2.5	2.465 ± 0.225	9.14	-1.39
	5	4.772 ± 0.513	10.76	-4.56
	10	10.194 ± 0.589	5.78	1.94
	20	19.980 ± 0.306	1.53	-0.09
HML	0.2	0.218 ± 0.001	0.39	9.00
	0.4	0.377 ± 0.001	2.58	-5.73
	1	0.920 ± 0.101	10.82	-7.02
	2.5	2.457 ± 0.349	14.19	-1.74
	5	4.488 ± 0.071	1.62	-10.24
	10	10.565 ± 0.363	3.44	5.65
	20	20.046 ± 0.051	0.25	0.23

*Mean values ± standard deviation

2.5 Extraction recovery

The recovery studies were performed by spiking samples at the three concentrations for the B-CA 0.4; 8 and 16 µg/mL, except for DMT (0.1; 0.8 and 4 µg/mL) each one in triplicate. These samples were divided into two groups. In the first one, all analytes were spiked before SPE procedure, in the second group analytes were added to the eluates after SPE procedure. In all cases, IS was only added after extraction. The second group is known as neat standards, where the percentage of recovery is 100 %. Therefore, absolute recovery (%) can be determined by comparing the peak ratio between the compounds and IS peak areas at each concentration by comparing the two sets of samples. Absolute recoveries ranged from 37% to 97 % for all analytes, and are shown in Table 7.

Table 7 - *Recoveries of the compounds (n=3)*

Analyte	Concentration (μg/mL)	Recovery* (%)
DMT	0.1	63.05 ± 4.47
	0.8	63.26 ± 10.10
	4	57.23 ± 5.63
THH	0.4	47.24 ± 2.31
	8	57.03 ± 1.64
	16	58.25 ± 7.87
HML	0.4	37.17 ± 5.30
	8	46.55 ± 5.42
	16	48.76 ± 7.31
HMN	0.4	71.61 ± 3.93
	8	97.74 ± 12.70
	16	80.87 ± 6.24

*Mean values ± standard deviation; DMT: N,N-Dimethyltryptamine; THH: tetrahydroharmine; HML: harmaline; HMN: harmine

On a study by Gaujac and coworkers, the determination of only DMT on ayahuasca teas obtained good results for absolute recovery of DMT, values ranging between 71 to 109 %, in our case values were a little inferior ranging from 47 to 63 %, however, the technique of extraction used herein was SPME, which explains such good results for recovery. This studied was only performed for DMT, hence no comparison can be done with the B-CAs (Gaujac et al., 2013).

To best of our knowledge, there is another study available where both DMT and B-CAs were quantified. In this research, Pires and coworkers (Pires et al., 2009) were able to obtain good recovery values: for DMT ranged from 78 to 89%; for HMN ranged from 70 to 87%; for HML ranged from 68 to 95 % and finally for THH recoveries ranged from 84 to 99 %. The extraction technique used was the same as ours (SPE), however, sample extraction procedure had differences on the buffer used and cartridge, while they used C_{18}, while we used MCX columns.

In spite of all the mentioned differences, there is one worth mentioned that is the differences in tea preparation, which are all dependent on the amount of plants used, final volume amongst other.

2.6. Method Applicability

After validation, the herein described method was evaluated in terms of applicability on authentic tea samples. An aliquot of each one of the extract samples was analyzed by GC-MS in full scan mode, in order to confirm the identity of the extract's chemical compounds. The spectrum of each peak was compared with the spectra available in the Wiley-MS and NIST libraries. (Attachments).

After evaluating the content of DMT, THH, HMN and HML, these were quantified with the validated method and the results are summarized on table 8.

Table 8 - Concentrations of N,N-Dimethyltryptamine; tetrahydroharmine; harmaline; and harmine found in *P. viridis, B. caapi, P. harmala, M. tenuiflora* and *DC AB* (n=2).

	Concentration (µg/mL)*			
	DMT	**THH**	**HML**	**HMN**
P. viridis	2.06 ± 0.12	-	0.2 ± 0.21	-
B. caapi		1.12 ± 0.01	0.99 ± 0.21	12.81 ± 0.46
P. harmala	-	7.46 ± 0.01	26.95 ± 0.21	24.27 ± 0.46
M. tenuiflora	1.70 ± 0.12	-	-	0.74 ± 0.46
DC AB	-	1.11 ± 0.01	0.30 ± 0.21	0.20± 0.46

*Mean values ± standard deviation; DMT: N,N-Dimethyltryptamine; THH: tetrahydroharmine; HML: harmaline; HMN: harmine

P. harmala tea possesses the highest concentrations of B-CA, followed by *B. caapi*. By contrast, *DC AB* tea is the one presenting the lowest concentrations of compounds.

It is possible to confirm that DMT is present in two tea varieties, i.e., *P. viridis* and *M. tenuiflora*, having the same as major compound. On the other hand, in the remaining tea varieties, only B-CA are present. However, the major compound is different in each tea. While in *DC AB*, the THH reveals as the major compound, in *B. caapi* the most concentrated compound is HMN. On the other hand, HML is the preponderant compound in *P. harmala* tea. The latter is in accordance with the available literature. Pires and coworkers (Pires et al., 2009), quantified ayahuasca preparations, verifying also HML as the most abundant.

The HML concentrations ranged from 0.20 µg/mL (*P. Viridis*) to 26.95 µg/mL (*P. harmala*). Also, HMN concentrations ranged considerably between teas, 0.20 µg/mL (*DC AB*) to 24.27 µg/mL (*P. harmala*). The remaining compounds do not present such a wide range of concentrations as the latter.

To best of our knowledge, the available literature on the quantification of DMT, THH, HMN and HML in ayahuasca preparations or teas remains scarce, with only two publications on this subject. In 2009, the quantification of the same compounds in ayahuasca preparations, the major compound was HML, however the authors obtained the preparations already made and the varieties of the plants were not discriminated. On the other hand, the compound detected in a minor amount was THH. However, it is not possible to make direct comparisons, since these four compounds were quantified on the same preparation, which does not happen in the present work, where none of the teas presented simultaneously the four compounds. In 2013, Gaujac and coworkers (Gaujac et al., 2013) only dedicated their work to the determination of DMT in ayahuasca tea. The concentrations varied between 0.17mg/mL and 1.14mg/mL, which are much higher than those obtained in the present work. Nevertheless, these concentrations, as well as the ones presented by Pires and colleagues (Pires et al., 2009) varied in function of the volume of tea and the amount of leaves used to prepare the beverages of ayahuasca. These

authors do not describe the volume and the weight used, for this reason the comparison is not possible.

The following lines describe the methodology used on the cytotoxic studies performed on N27 cell, as well as the protein quantification. Moreover, the respective results and discussion are also presented.

3. Cell Lines and Culture

3.1. Immortalized rat mesencephalic dopaminergic neurons (N27)

N27 neural cell line were grown in cell culture dishes with RPMI-1640 medium. To guarantee optimal culture conditions and to avoid bacterial contamination, cell medium was complemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Portugal) and a mixture of antibiotics (100 U/mL penicillin and 100 µg /mL streptomycin). Cells were maintained at 37°C in a humidified atmosphere incubator of 5% CO_2/95% air. For all experiments, cells were trypsinized with a solution of trypsin/EDTA (0.004g of trypsin in 0.1g of EDTA dissolved in 500 mL of PBS 1X) and medium was renewed, until cells reached a confluence of approximately 85-90%. Afterwards, cells were counted using tryptan-blue assay and seeded with 1×10^4 cells/well density in 96-well culture plates. To treat cells with the compounds, they first were let to grow in 96-wells or 6 wells culture plate until a confluence of approximately 40-50% was reached.

3.2. Cell Treatments

3.2.1. Preparation of the working solutions of DMT, THH, Harmine, Harmaline and Harmalol

The stock solutions of THH, HMN, and HLOL were prepared in 1mL of DMSO, at a concentration of 500 µM. From this stock, the final working solutions were prepared with different concentrations of 10 µM, 2 µM, 0.4 µM, 0.08 µM, 0.016 µM, 0.0032 µM and 0.00064 µM. As for HML, the stock solution was prepared in 1 mL of DMSO to a final concentration of 930 µM. From this stock, the final working solutions were prepared with different concentrations: 80 µM, 16 µM, 3.2 µM, 0.64 µM, 0.128 µM, 0.0256 µM and 0.00512 µM. Finally, DMT stock's solution was prepared in 1 mL of DMSO with a final concentration of 50 µM. The following concentrations were applied to cells: 1 µM, 0.2 µM, 0.04 µM and 0.00008 µM. All working solutions were prepared with NaCl 0.9%.

3.2.2. Preparation of tea extracts working solutions

After the step mentioned in section 1.7, tea extracts were dissolved in DMSO. The same principle of serial dilutions was applied, only this time, two concentrations were studied for each tea extract and for the mixtures of *P. harmala* + *B. caapi* and *P. viridis* + *B. caapi*. These concentrations were chosen based on the results obtained and described on section 5.1., that is, the highest and second highest concentration that induce cell death.

3.2.3 Treatments

To evaluate cells viability, using the CCK-8 assay, N27 cells were treated individually with the previously mentioned concentrations (section 3.2.1) of DMT, THH, HMN, HML and HMLOL for 24 hrs. The same was done for each tea extracts, as well as the mixture.
As for total protein extraction and quantification, cells were plated in 6-wells plate at a density of 2×10^5 cells/well, and exposed to the lowest (0.00064 µM for THH, HMN and HLOL; 0.00512 µM for HML; 0.00008 µM for DMT) and highest (10 µM for THH, HMN and HLOL; 80 µM for HML; 1 µM for DMT) concentrations of each one of the compounds for 24 hrs.
Each experimental condition was performed with 5 replicates and in three independent experiments (n=3).

3.2.4. CCK-8 assay, theoretical fundament

Cell counting kit-8 or CCK-8 is a colorimetric assay that allows the determination of the number of viable cells in cell proliferation and cytotoxic assays. It uses a slightly yellow water-soluble tetrazolium salt, WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt]), that is reduced by cell's dehydrogenases in the presence of an electron mediator, producing an orange colored product (WST-formazan) that is soluble in the culture medium. The quantity of formazan dye formed due to the dehydrogenases reaction is directly proportional to the number of living cells. Absorbance is measured at 450 nm.

3.2.5. Experimental Procedure of CCK-8 assay

Test was performed following the manufactures' instructions. After the incubation period of 24 hrs with the compounds, the culture medium was removed and 5 µL of CCK-8 solution was added to each well, alongside with 95 µL of culture medium. Then, cells were stored in the incubator with 5% CO_2 at 37°C, protected from the light, for 3 h. After this time, the absorbance was measured at 450 nm in a microplate spectrophotometer (xMArk™ Microplate Absorbance Spectrophotometer, BIO-RAD).

3.4. Total Protein

3.4.1. Total Protein Extraction

Followed the 24 hrs of N27 cells exposure to the highest and lowest concentrations of the each compound, cell medium was removed and cells extract were collected. 1 mL/well of PBS 1X was added and using a cell scrapper cells started to detach. Afterwards, the resultant suspension was centrifuged at 4000 RPM for 1 min; the supernatant was dismissed and the pellet was suspended in 20 µL of RIPA lysis buffer and cells were let to rest on ice for 15 min. After this period of time, cells were centrifuged at 14000 RCF at 4°C for 20 min; the resulting supernatant was collected to posterior total protein quantification.

3.4.2. Total Protein Quantification, theoretical fundament

This procedure was performed using Pierce™ BCA (bicinchoninic acid) Protein Assay Kit, which uses two reagents: A (containing sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M of sodium hydroxide) and B (containing 4% of cupric sulfate). This method combines the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium. A single Cu^{+} ion binds to two molecules of BCA, forming a purple-colored water-soluble complex, which absorbance can be read at 572 nm (P. K. Smith et al., 1985). Standards concentrations performed by serial dilutions of bovine serum albumin (BSA) protein are prepared, so that protein levels of our sample can be determined with reference to the prepared calibration curve of BSA.

3.4.3. Experimental procedure of total protein quantification

For the calibration curve, 100 µL of BSA (2µg/µL) was added to an eppendorf and serial dilutions were performed from this solution using water, the final concentrations were 1 µg/µL, 0.5 µg/µL, 0.25 µg/µL, 0.125 µg/µL and 0 µg/µL, being this last one prepared only with water to be used as a blank. Then on a different eppendorf, RIPA was diluted 11 times in water (e.g. 50 µL of RIPA + 500 µL of water).

All protein samples (the resulting supernatant obtained from the extraction) were diluted 11 times in water (e.g. 5 µL of protein + 50 µL water).

The estimated volume of BCA reagent need was obtained according to the number of wells used, knowing that each well uses a total volume of 200 µL (for the samples and the calibration curve and each sample is usually done in duplicate); the ratio used is 49 parts of reagent A and 1 part of the B reagent.

In a 96-plate, for the standard curve 25 µL of RIPA + 25 µL of BSA standards was added to each well. In respect to our samples, 25 µL of water + 25 µL of each diluted sample was added to each well. All standard concentrations and samples were performed in duplicates. Following this, 200 µL of the mix (reagent A + B) was added to each well. Then, the plate was incubated for 30 min at 37°C and kept in the dark. After this time, the absorbance was read at 570 nm.

3.5. Statistical Analysis

Experimental conditions were performed in three sets of independent experiments (n), and performed in three of four replicates. The results are presented in % of controls, according to the following expression:

% CTR = (mean values of absorbance after compound's incubation/ mean values of control's absorbance) x 100.

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's Multiple Comparison Test and was considered statistically significant when differences between experimental groups had p values < 0.05. Data are demonstrated as a mean ± standard error of mean (SEM).

4. Results and Discussion of experiments performed on N27 cells

4.1. Cytotoxic evaluation of DMT, HMN, HML, THH and ayahuasca tea preparations

Accordingly to the purpose and objective of this work, N27 cells were incubated with HMN, HML, THH, DMT and the ayahuasca teas, in order to evaluate the effects of these compounds and teas on these cells' viability and proliferation. Once all of the compounds are not water-soluble, they were diluted in DMSO, which worked as a vehicle. However, DMSO on its own can induce cell death at higher percentages (≥ 1-5%) (Zhang et al., 2017), so there was the need to define an adjustable percentage of this organic solvent in order to dismiss its effect on cells' proliferation and viability. It was tested the toxic effect on N27 cells of the highest percentage of DMSO, i.e. 0.2%, that was used as a vehicle. As observed by figure 2 and Table 9, despite existing a decrease in cell viability, when compared to untreated cells (control), no significant difference is observed. Therefore, this percentage of DMSO does not induce cell harm or impairment while evaluating the cytotoxicity of the target compounds at their highest chosen concentration levels. For this reason, it can be assumed that, if a compound or even the teas induce an increase of cell death, this may be caused by the compound itself and not due to the presence of the vehicle.

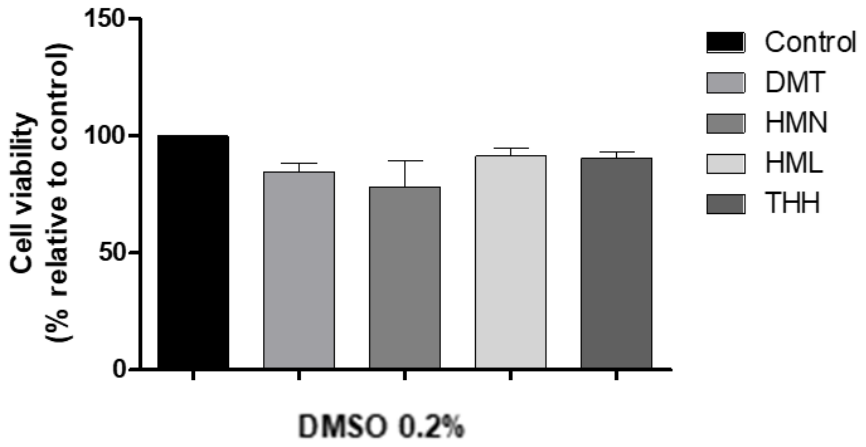

Figure 2 - The effect of 0.2% of DMSO, working as a vehicle on incubation of DMT (1 μM), HMN (10 μM), HML (80 μM), and THH (10 μM) on N27 cell viability (24 hrs); Control culture contains only cells.(n=3, values are shown as mean ± SEM; no significant difference was observed when performing ANOVA followed by Dunnett's Multiple Comparison test).

Table 9 - Relative cell viability (%) of N27 cells treated with vehicle DMSO 0.2% when incubated with DMT (1 μM), HMN (10 μM), HML (80 μM), and THH (10 μM), for 24 hrs; control culture contains only cells (n=3)

	Control (untreated cells)	**DMT (1 *μM*)**	**HMN (10 *μM*)**	**HML (80 *μM*)**	**THH (10 *μM*)**
DMSO 0.2%	100 ± 0.00	84.86 ± 6.29	78.04 ± 20.13	91.40 ± 5.89	90.49 ± 4.36

All values presented as mean ± standard deviation

Regarding the response induced by HMN on N27 cells, a range of concentrations from 0.00064 μM to 10 μM was assessed. It was observed that from 0.00064 μM to 2 μM, cell viability was not affected for the time of cell incubation (24 hrs) and no statistical differences noticed. Nonetheless, for cells incubated with 10 μM, we were able to see a decrease in cell viability to 62.85 ± 4.78 % when compared to control (0 μM) culture (figure 3 A). The same concentrations were studied for THH and the results are similar, that is, when cells were incubated with 10 μM of this compound, a decrease in cell viability is observed (48.25 ± 16.21 %) in comparison to control. For all other concentrations (0.00064 – 2 μM), cell viability was not affected (figure 3 C). As for HML, a range of concentration levels from 0.00512 μM to 80 μM was evaluated. Once

again, only at the highest concentration level, cell viability was affected, namely when cells were cultured with 80 µM of HML. As a whole, all of the concentrations capable reduce cell viability suggest that these compounds may induce neuronal toxicity. Finally, for DMT the concentration levels studied covered were from 0.0008 µM to 1 µM. In this case, for all concentrations no statistical differences were observed when cells were incubated with such compound (Figure 3 D). These results were surprising and perhaps the concentration range studied were extremely low to induce cell death.

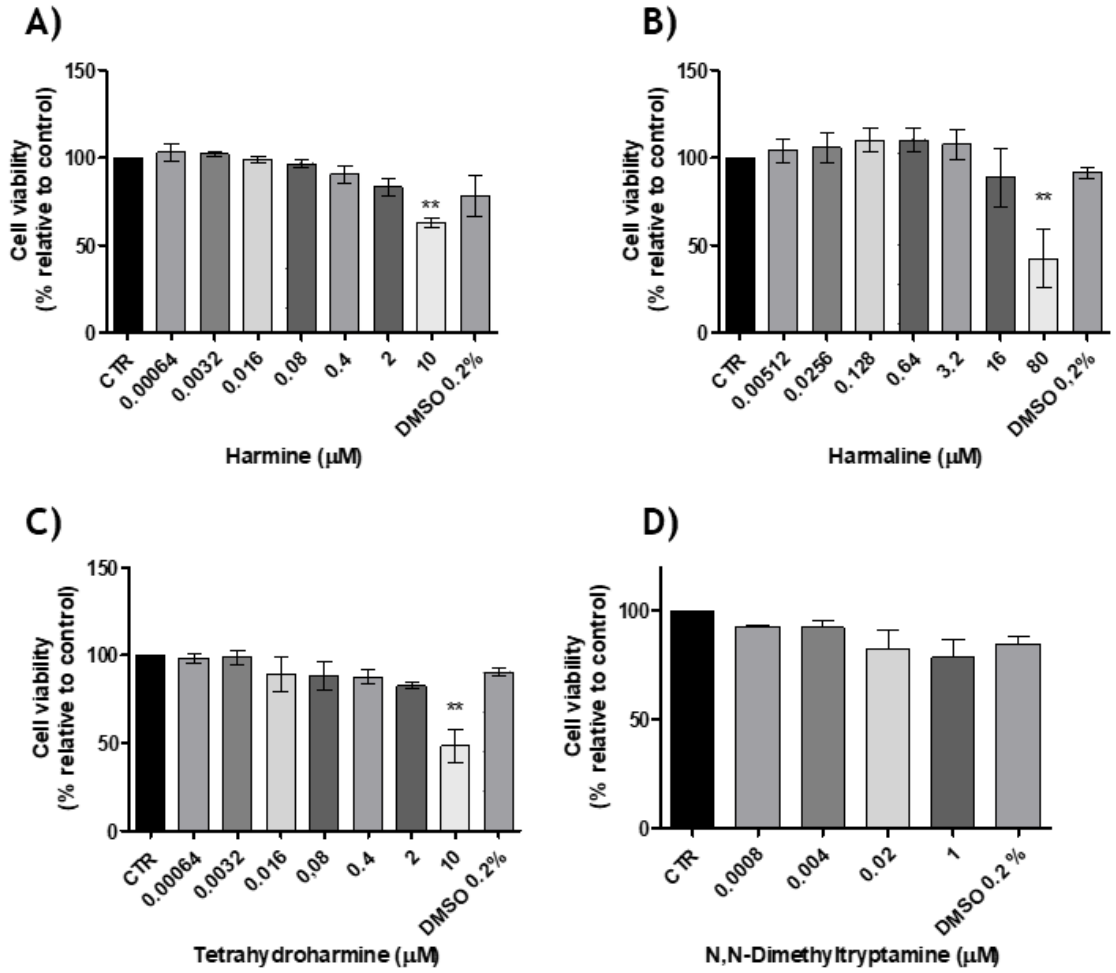

Figure 3 - The effects of A) Harmine, B) Harmaline, C) Tetrahydroharmine and D) N,N-dimethyltryptamine on N27 cell line viability (24hrs), (n=3, values are shown as mean ± SEM ** indicates values that are significantly different from control p <0,01, one-way analysis of variance followed by Bonferroni's Multiple Comparison test).

When ayahuasca tea is consumed, harmalol can be formed by the O-demethylation of harmaline, therefore, high traces of this chemical substance can be found in human plasma (Yritia et al., 2002). Therefore, it is also opportune to assess its effects on a cellular level.

Figure 4 demonstrates the results obtained for HLOL. In spite of the cell viability being not been affected by a range of concentrations between 0.00064 µM to 0.4 µM, the same did not occur to concentrations of 2 µM and 10 µM.

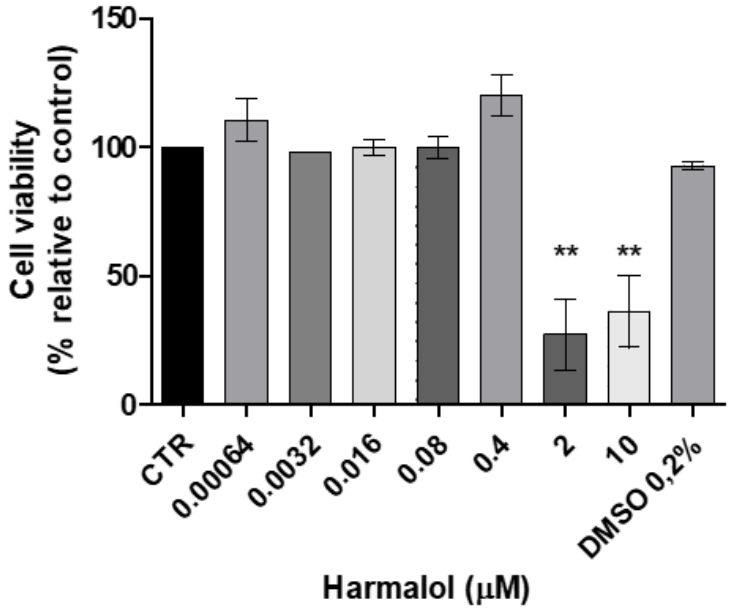

Figure 4 - The effects of Harmalol on N27 cell line viability (24hrs), (n=3, values are shown as mean ± SEM ** indicates values that are significantly different from control p < 0,01, one-way analysis of variance followed by Dunnett's Multiple Comparison test).

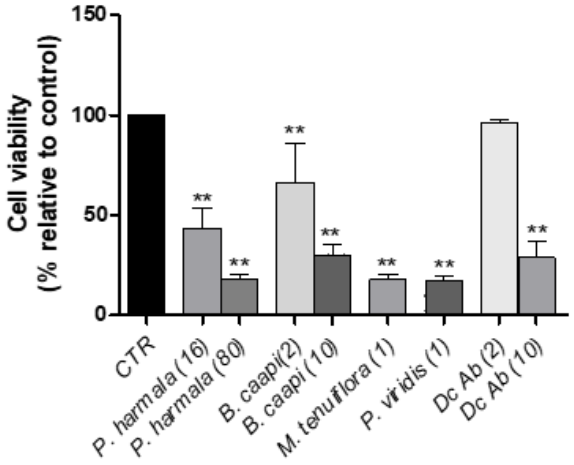

Figure 5 - The effects of *P. harmala* (16 and 80 μM), *B. caapi* (2 and 10 μM), *M.tenuiflora (*1 μM), *P. viridis* (1 μM) and *DC AB* (2 and 10 μM) on N27 cell viability (24h); CTR- control, (n=3, values are shown as mean ± SEM ** indicates values that are significantly different from control p <0,01, one-way analysis of variance followed by Dunnett's Multiple Comparison test).

Altogether these results are interesting to understand their individual behavior on rat dopaminergic cells, however, it is important to note that when ayahuasca tea is consumed a mixture of several compounds are also ingested, or formed by endogenous reactions, as is the case of HLOL, previously mentioned. Hence, the importance of studying the effects of these compounds on the teas as a whole.

In respect to the teas, a total of five tea plants plus two mixtures of plants were incubated with N27 cells to evaluate the effects on cell viability (figure 5). The concentrations were chosen based on the major quantified compound for each plant and the mixtures and at the same concentrations as before.

The evaluation of *P. harmala's* effect on N27 cells, at two different concentrations (16 and 80 µM), showed a significant decrease in cell viability, suggesting a neurotoxic effect. In fact, at the highest concentration studied, the cell viability observed is of 17.72 ± 2.29 % when compared to control culture. Such results may be due to the interaction of all B-CA present in this tea, (THH, HMN and HML), exerting synergetic and negative effects for these cells.

The same happened for *B. caapi*, i.e, when cells were treated with 10 µM (highest concentration) a significant decrease in cell viability was observed. From the quantification results obtained (section 2.7; table 9), this tea presented high content in HMN (12.80 µg/mL), still it also contained low traces of THH (1.12 µg/mL) and HML (0.99 µg/mL). Once again, these results suggest that the possible interaction between these compounds may cause the cytotoxic observed effects on N27 cells.

In the case of *M. tenuiflora*, it is known that DMT is part of its constitution (Frison et al., 2008; Gaujac et al., 2013), still we were able to quantify 0.74 µg/mL of HMN in this tea (section 2.7; table 9). This may suggest the obtained result, when incubation cells with this tea at 1 µM. The same was observed for P. viridis, only this time apart from DMT, the tea was also composed by HML.

Finally, for *DC AB*, it was observed that only when cells were treated at a concentration of 10 µM cell viability decreased.

When exposing N27 cells to the tea mixtures, results showed a significant decrease in cell viability (Figure 6). These results only confirm the suspicion that, in fact, the interaction between DMT (major constituent of *P. viridis*) and the B.CA's (present in *P. harmala* seeds and leaves and in *B. caapi*'s leaves) leads the first to exert its effects. Overall, our results suggest a cell toxicity caused by cooperation of all compounds. Additionally, it is observed that the mixture of *P. viridis* + *P. harmala* has a more prominent neurotoxic effect on the studied cells.

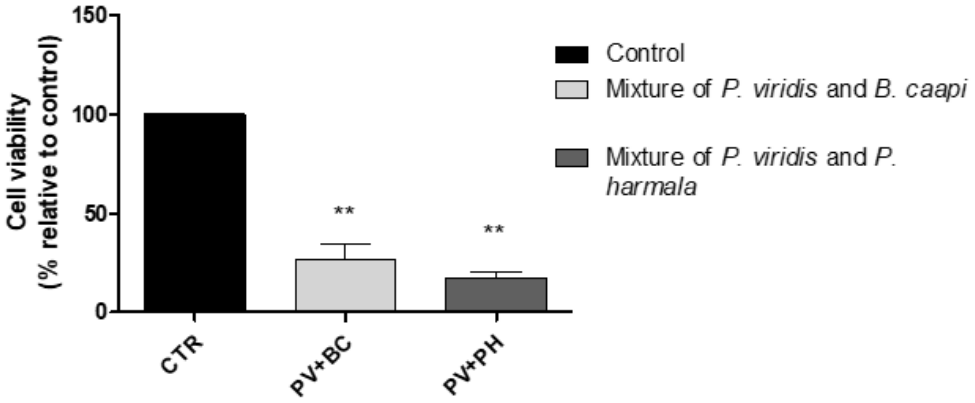

Figure 6 - The effects of tea plant extracts mixtures on N27 cell viability (n=3). Concentrations used were 5 µM and 50 µM; the presented values are presented as mean ± SEM. ** indicates values that are significantly different from control ($p<0,05$, one-way analysis of variance (ANOVA)).

4.2 Total Protein Quantification

In order to understand the effect of the studied compounds in the cellular metabolism of proteins, the total protein levels quantification was performed for the highest and the lowest concentration of each compound individually (Figure 7).

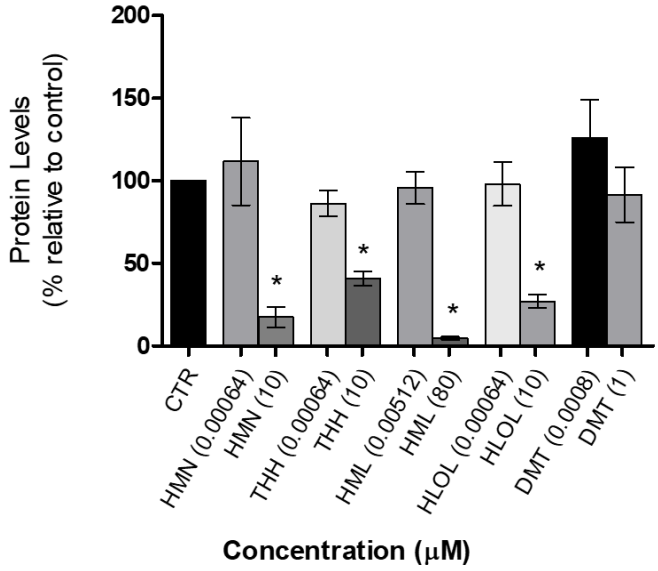

Figure 7 – Protein levels when N27 cells are exposed during 24 hrs to Harmine, Tetrahydroharmine, Harmaline, Harmalol and N,N-dimethyltryptamine (concentrations are in µM) (n=3, values are shown as mean ± SEM ** indicates values that are significantly different from control $p<0,05$, one-way analysis of variance followed by Dunnett's Multiple Comparison test)

The obtained results are in line with the results obtained from the ones obtained from the cytotoxic assays. That is, when cell viability decreases, so does the cellular content in protein,

which by the results from section 4.1, occurs at the highest concentration levels. The contrary is also true, namely at the lowest concentrations studied, protein levels are high and so is the percentage of cell viability, when compared to control cultures. Protein levels on cells are determined both by the amounts that are synthetized and the rates of degradation, which in the latter case there are two possible pathways of how this may occur: the ubiquitin-proteasome system or by degradation mediated by proteolysis in the lysosome (GM, 2000). Although this remains to be evaluated in future studies, the results obtained on the reduction of the total protein content in N27 cells exposed to each compound, may be explained by the putative inhibitory effect on the protein synthesis machinery or activator effect on the protein degradation systems, exerted by each compound. For instance, on a study by Valente and coworkers (Valente et al., 2017), it was shown a relation between the neurotoxic effects caused by two ß-keto amphetamines on SH-SY5Y human dopaminergic cells, and autophagy dysregulation. The results obtained by these authors suggest that the neurotoxicity of such compounds lead to the production of reactive oxygen species and consequently to an activation of autophagy, both in a time and concentration-dependent manner. Despite being different compounds from the compounds studied in this dissertation, one can speculate that, psychoactive compounds may induce autophagy and downregulate protein levels and ultimately induce cellular apoptosis (Valente et al., 2017).

All together the results showed that the ayahuasca teas are dose-dependent when exerting cytotoxicity, since at the lowest concentrations cell viability is not specially affected, but on the contrary the opposite is observed. Furthermore, it is possible to speculate the eventual exposition of these cells to concentrations higher that the ones studied on this work, may also induce cell death. Perhaps due to apoptosis, or mal function on cellular mechanisms. Still, further studies need to be performed in order to evaluate why these compounds reduces N27 cells viability.

5. Conclusions

An analytical method using SPE as extraction technique and GC-MS was developed, optimized and fully validated to quantify and detect ayahuasca beverages major compounds, DMT and the beta-carbolines THH, HMN and HML. Validation processes followed international guidelines from FDA and SWGTOX.

A linearity range of 0.2-20 µg/mL was obtained for all compounds, except for DMT (0.04-6 µg/mL). A LLOQ of 0.2 µg/mL was achieved for the beta-carbolines, except for DMT (0.04 µg/mL), using a low volume of sample (500 µL). Moreover, the method's selectivity and precision and accuracy were considered adequate.

This is the first GC-MS method using SPE that was used to the determination and quantification of these analytes all together. SPE provides low matrix effects and rarely interferences are observed from other used drugs. In addition, its ease in use and operation, allow for better laboratorial results.

Concerning cytotoxicity, the results obtained from the total protein quantification were consistent with the CCK-8 results, since the assays for the highest concentrations, total protein was affected, meaning that protein production could have been affected by the effect of the compounds. Moreover, CCK-8 results obtained for the five teas and the two mixtures suggest that the synergetic effect of compounds present in each plant exert neurotoxicity. Regarding analytical quantification, *P. viridis* presented the highest DMT content, while *P. harmala* presented the highest content of ß-Carbolines.

This is the first study investigating cytotoxicity of Ayahuasca compounds and commercial teas on dopaminergic cells. This is significant since one of the organs affected by the intake of such substances is the brain. Furthermore, not all of the teas include DMT, the same happens for some of the ß-Carbolines. Nonetheless, more studies would be important to assess chronic effects of the compounds and the cellular mechanisms responsible for their cytotoxicity, even the determination of these compounds in biological samples.

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Dissemination

The present dissertation was disseminated in different congresses in the area of toxicology:

Presentations in congress:

ANALYSIS OF THE MAIN COMPONENTS OF AYAHUASCA AND THEIR CYTOTOXICITY IN DOPAMINERGIC CELLS

Ana Y. Simão, Joana Gonçalves, Débora Caramelo, Tiago Rosado, Mário Barroso, José Restolho, Nicolás Fernández, Ana Paula Duarte, Ana Clara Cristóvão, Eugenia Gallardo

57th Annual Meeting of the International Association of Forensic Toxicologists, Birmingham (United Kingdom), Junho 2019 (accepted)

AVALIAÇÃO DA TOXICIDADE *IN VITRO* DA AYAHUASCA: ESTUDO DA TOXICIDADE DAS β-CARBOLINAS EM CÉLULAS DOPAMINÉRGICAS (Poster);

Ana Y. Simão, Eugenia Gallardo, Ana Clara Cristóvão

VI Jornadas de Química e Bioquímica, Covilhã (Portugal), Abril 2019

This Poster exhibition led to winning the 1st place award on this event.

Attachments

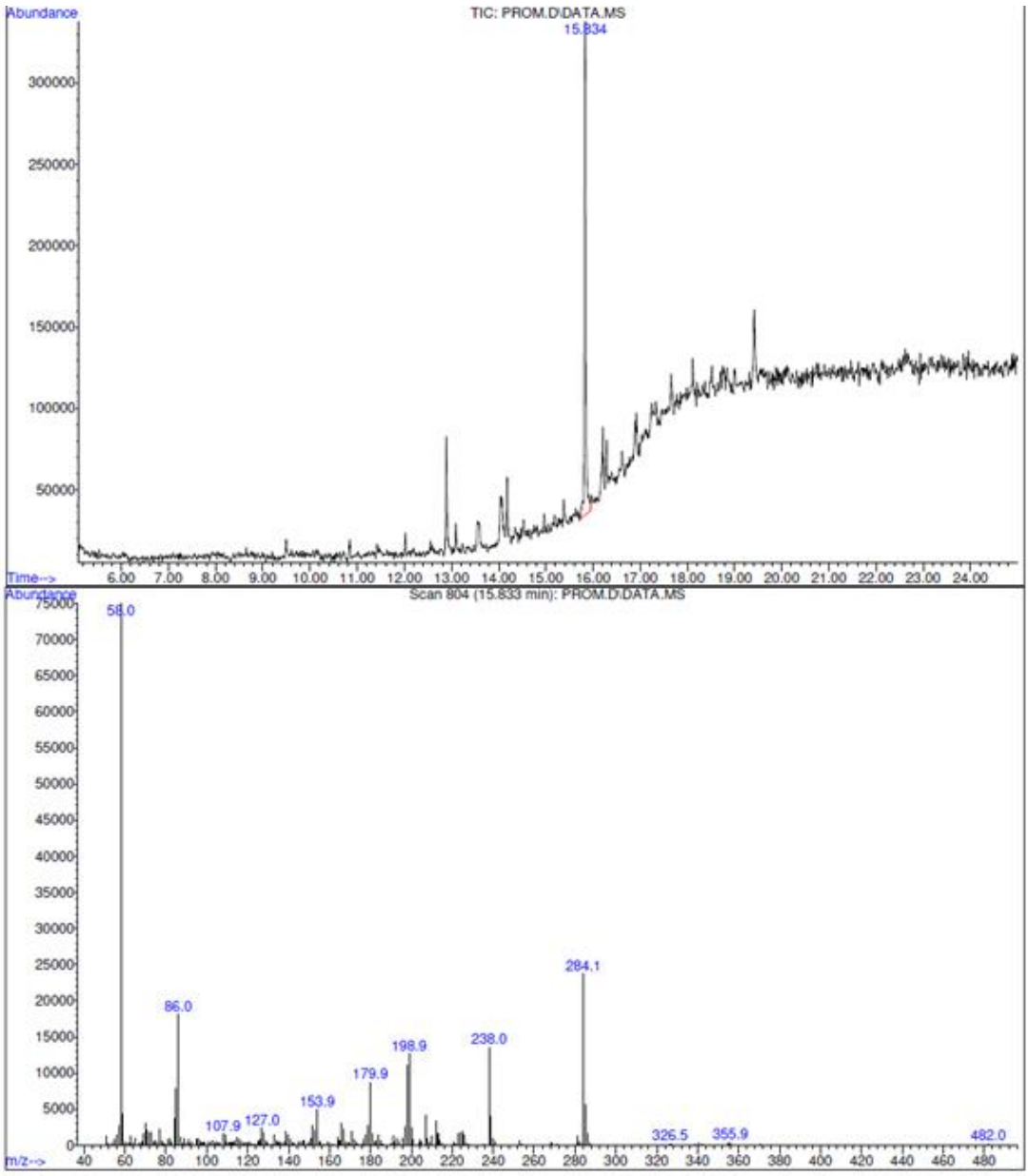

Figure 8 –Chromatogram and mass spectrum of promazine in SCAN mode (RT: 15.84 min)

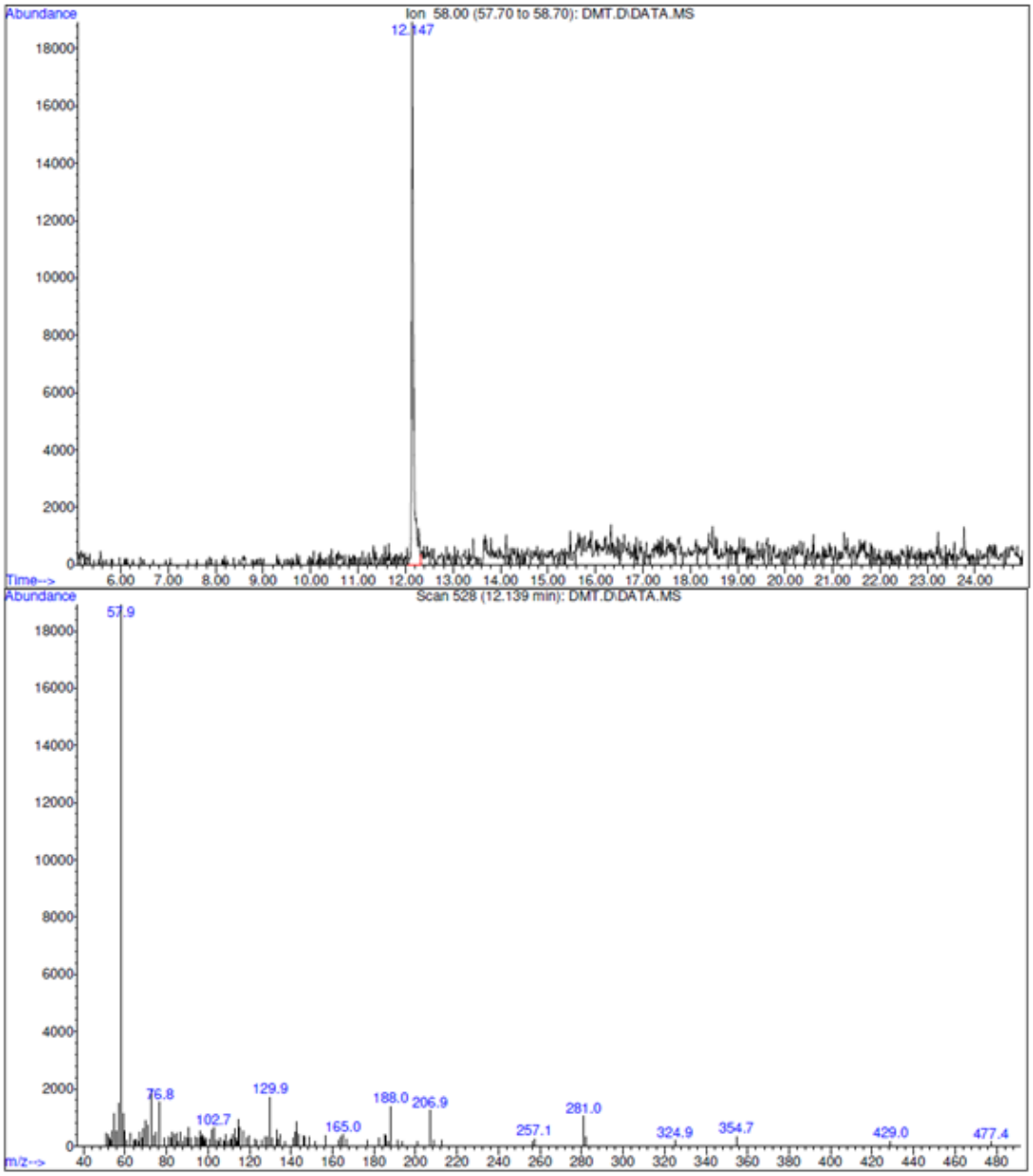

Figure 9 – Chromatogram and mass spectrum of N,N-dimethyltryptamine in SCAN mode (RT=12.14)

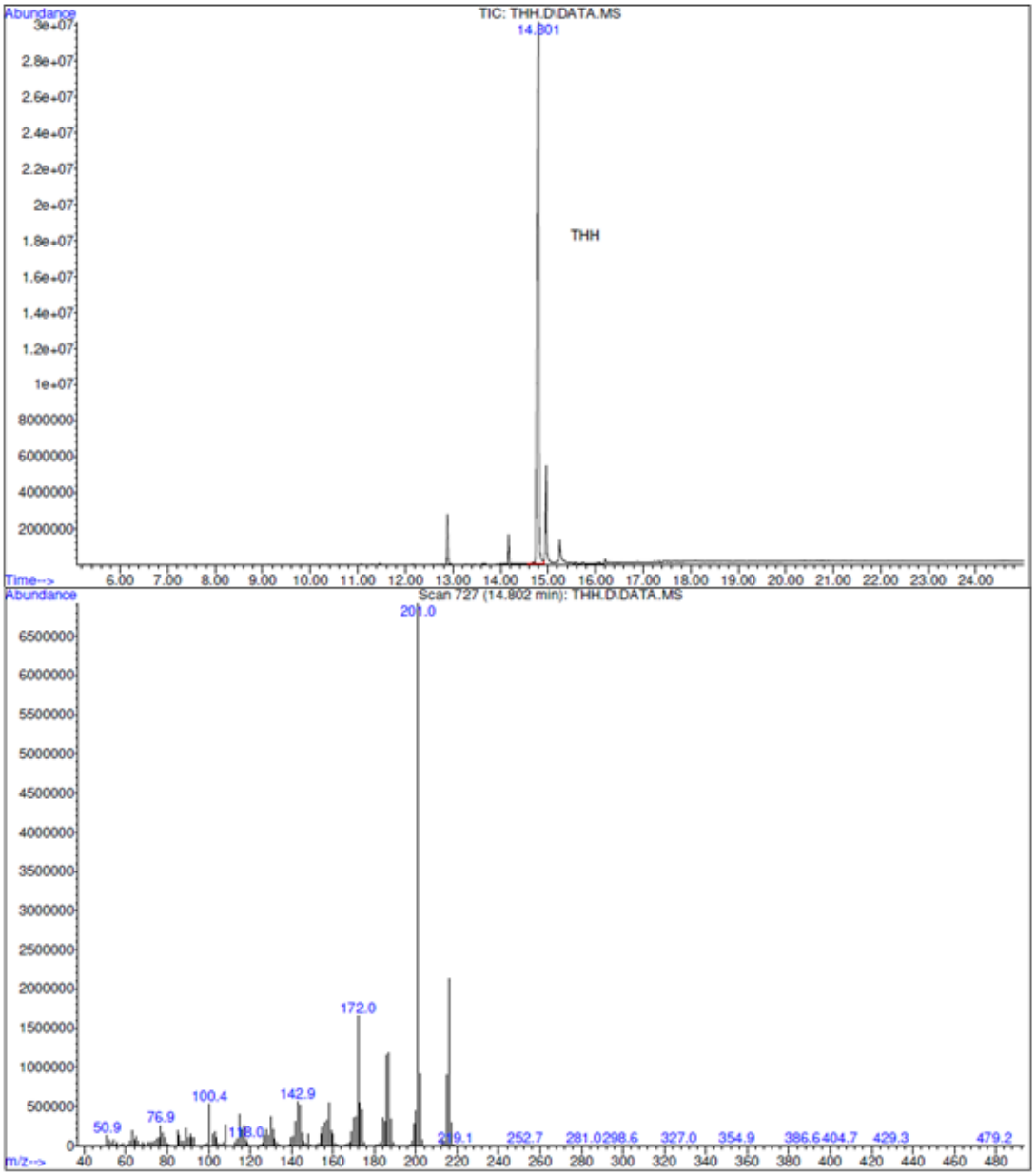

Figure 10 – **C**hromatogram and mass spectrum of tetrahydroharmine in SCAN mode (RT=14.80 min)

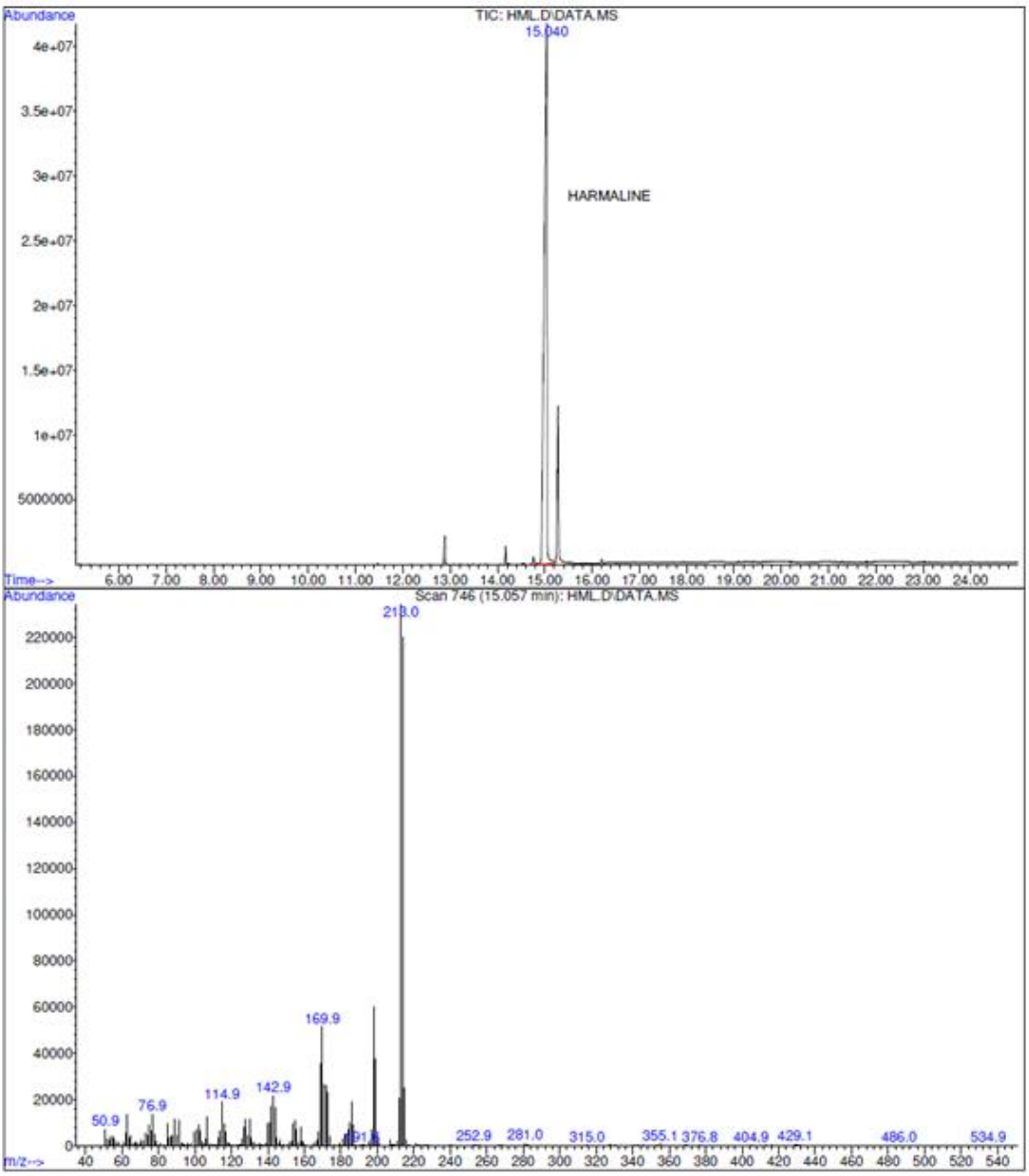

Figure 11 – **C**hromatogram and mass spectrum of harmaline in SCAN mode (RT=15.04 min)

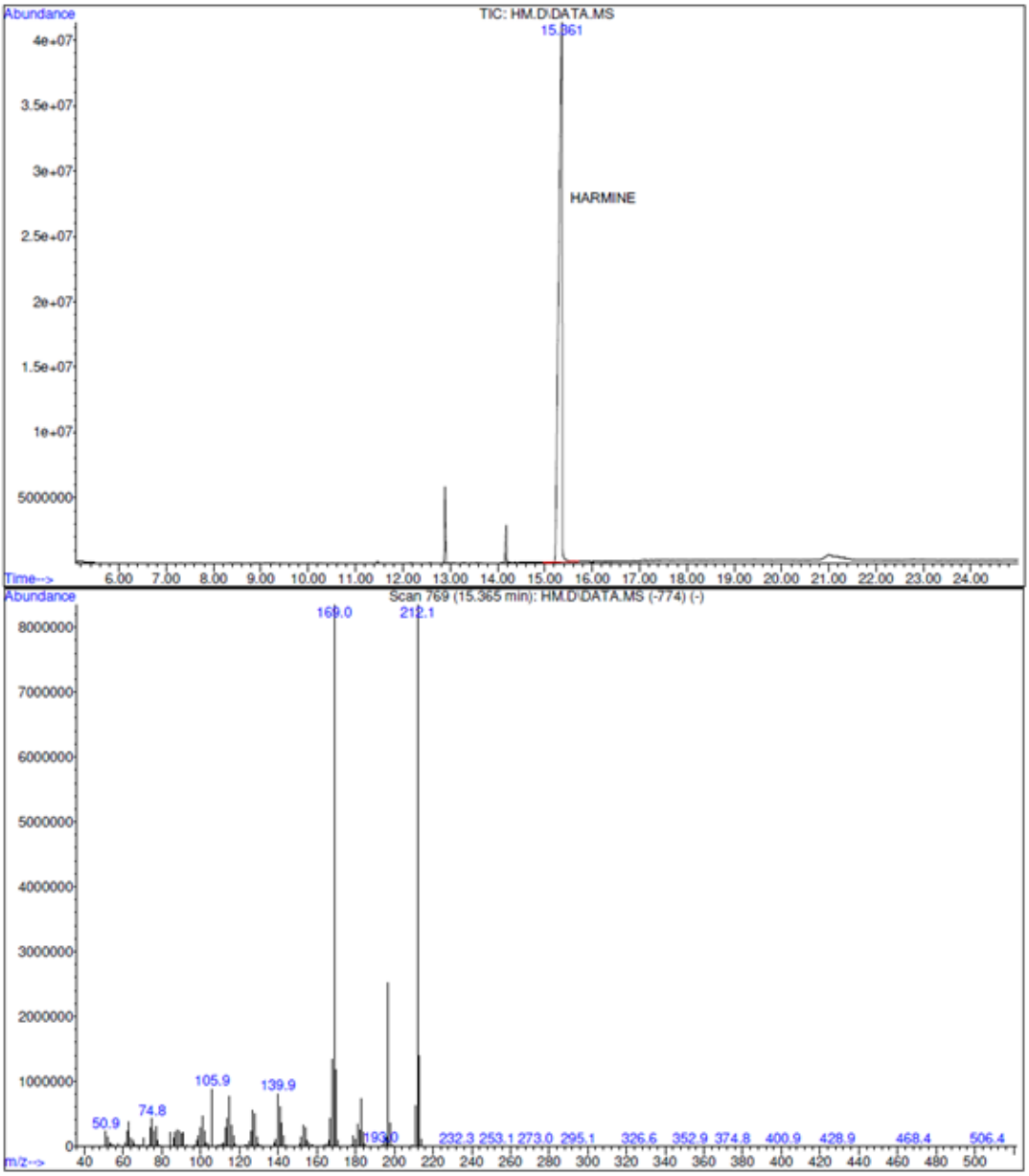

Figure 12 – Chromatogram and mass spectrum of harmine in SCAN mode (RT = 15.36 min)