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Drug abuse patterns in Coimbra recreational nightlife

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ABSTRACT

Background: The use of psychoactive substances among young people, in recreational spaces, has been a subject of growing concern. The uncontrolled consumption of these substances, associated to risk behaviors, morbility and morbidity, corresponds to a heavy burden for the modern society.

Objective: The main objective of this study was to analyze the drug abuse patterns in Coimbra recreational nightlife.

Methods: The study was applied in the form of the questionnaire and the collection of biological samples from volunteers gathered at nighttime recreational spaces in Coimbra. Each blood and oral fluid sample was qualitatively and quantitatively analyzed according to each psychoactive substance reported to be used by the interviewed volunteer. Ethanol was also evaluated in exhaled air and oral fluid. A GC-MS methodology was developed for the measurement of Δ9-tetrahydrocannabinol (THC), and standard methods were used for the other psychoactive substances analyzed. The data were gathered by a non-randomized process of accidental sampling, and quantitative analyzes using the statistics tests of Mann-Whitney, Kruskal-Wallis, Pearson Chi-Square, Odd's Ratio.

Results: 78 young adults, between 18 to 30 years, participated in the study. 26,92% were females and 73,08% males. The majority of them were students (75,64%). Alcohol was the psychoactive substance more used (87,2%), followed by tobacco (64,1%), cannabis (19,2%), cocaine and amphetamines (2,6%) and finally LSD and smartshop substances (1,3%). Males used more psychoactive substances than females, both in quantity and in diversity. In relation to risk behaviors or health problems, the cocaine users had the highest risk of both types of problems, following cannabis, alcohol and finally tobacco. Relatively to biological samples, the ethanol and Δ9-THC were the compounds quantitatively analyzed in this study due to their high frequency. Ethanol was the substance that had more discordance, as compared to the surveys. The results of Δ9-THC had more concordance with answers of the surveys.

Conclusion: Alcohol, tobacco and cannabis were the psychoactive substances more used in recreational spaces. Alcohol is the major substance used among young people, usually in an excessive way. Exhaled air reveal to be the best method for analyzing ethanol. The Δ9-THC method validated in the present study, showed to be selective, linear, efficient, and precise.

Keywords: psychoactive substances, alcohol, THC, recreational nightlife.

RESUMO

Introdução: O consumo de substâncias psicoativas entre jovens, em espaços de lazer, tem sido um tema de crescente preocupação. O consumo incontrolado destas substâncias, associadas a comportamentos de risco, morbilidade e morbidade constituem uma tarefa árdua para a sociedade moderna.

Objetivo: O objetivo principal deste estudo foi analisar os padrões de consumo recreativo, de substâncias psicoativas, na cidade de Coimbra.

Métodos: O estudo foi efetuado sob a forma de um questionário acompanhado de uma amostra biológica, colhida a jovens voluntários que se encontravam em espaços recreativos noturnos, na cidade de Coimbra. Cada amostra de sangue e fluido oral foi qualitativamente e quantitativamente analisada de acordo com o tipo de substância psicoativa consumida, reportada pelo participante. O etanol foi também colhido sob a forma de ar exalado e fluido oral. Para analisar Δ9-tetrahydrocannabinol desenvolveu-se uma metodologia analítica, enquanto que as restantes substâncias foram analisadas segundo metodologias padrão. A recolha de dados foi efetuada segundo um processo não aleatório de amostra acidental, e a sua análise foi realizada quantitativamente recorrendo a testes estatísticos de Mann-Whitney, Kruskal-Wallis, Pearson Chi-Square e Odd's Ratio.

Resultados: 78 jovens adultos, entre 18 e 30 anos, participaram neste estudo. 26,92% eram do género feminino e 73,08% do género masculino. A maioria dos participantes eram estudantes (75,64%). O álcool foi a substância psicoativa mais consumida (87,2%), seguindo o tabaco (64,1%), canábis (19,2%), cocaína e anfetaminas (2,6%) e finalmente LSD e substâncias vendidas em smartshop (1,3%). Os participantes do género masculino consomem mais substâncias psicoativas do que os do género feminino, tanto em quantidade como em diversidade. Em relação aos riscos comportamentais ou de saúde, os consumidores de cocaína apresentavam maior risco de possuírem ambos os problemas, seguindo os consumidores de cannabis, álcool e por último tabaco. Relativamente às amostras biológicas, o etanol e o Δ9-THC foram as substâncias analisadas quantitativamente neste estudo, devido á sua maior frequência. O etanol foi a substância que teve maior discordância, quando comparado com as respostas dos questionário, enquanto que os resultados do Δ9-THC mostraram-se mais concordantes.

Conclusão: Álcool, tabaco e cannabis são as substâncias mais consumidas em espaços recreativos noturnos. O álcool é a substancia mais consumida pelos jovens de uma forma exacerbada. A análise ao álcool por ar exalado foi aquele que obteve resultados mais

fidedignos. O método de Δ9-THC validado neste estudo mostrou ser seletivo, linear, eficiente e preciso.

Palavras-chave: Substâncias psicoativas, álcool, THC, ambiente recreativo noturno

ABBREVIATION LIST

% CV - Coefficient of variation

Δ^9 THC - Δ^9 tetrahydrocannabinol

AD - Alcohol dehydrogenase

ADH - Antidiuretic hormone

ALDH - Aldehyde dehydrogenase

ARS-IP - Administração Regional de Saúde do Norte

AWS - Alcohol withdrawal syndrome

BAC - Blood alcohol concentration

BSTFA - Bis(trimethylsilyl)trifluoroacetamide

cAMP - Cyclic adenosine monophosphate

CB receptor - Cannabinoid receptor

CB1/2 - Cannabinoid receptor type 1 and 2

CNS - Central nervous system

CSF - Cerebrospinal fluid

CYP2E1 - Cytochrome P450 2E1

DA - Dopamine

DJ - Disc jockey

DSM - Diagnostic and statistical manual of mental disorders

EEG - Electroencephalogram

EMCDDA - European monitoring centre for drugs and drug addiction

GABA - Gamma-aminobutyric acid

GC-FID - Gas chromatography flame ionization detector

GC-MS - Gas chromatography-mass spectrometry

GHB - Gamma-Hydroxybutyric acid

HCl - Hydrochloric acid

$HClO_4$ - Perchloric Acid

I.S. - Internal standard

K2CO3 - Potassium carbonate

LLE - Liquid-liquid extraction

LLOQ - Lower limit of quantification

LOD - Limit of detection

LSD - Lysergic acid diethylamide

Max - Maximum

MDA - 3,4-Methylenedioxyamphetamine

MDMA - 3,4-methylenedioxy-N-methylamphetamine

MEOS - Microssomal ethanol-oxidizing system

Min - Minimum

MSTFA - N-Methyl-N-trimethylsilyltrifluoroacetamide

N2 - Nitrogen gas

NA - Nucleus accumbens

NaOH - Sodium hydroxide

NDMA - N -methyl-Daspartate

NH4CO3 - Ammonium carbonate

NH4OH - Ammonium hydroxide

NIST - National institute of standards and technology

PCP - Phencyclidine

pH - Potential of hydrogen

pKa - Acid dissociation constant

PKA - Protein kinase A

r^2 - Coefficient of determination

S - Slope

SD - Standard deviation

SPE - Solid phase extraction

SPSS - Software Package for Social Sciences

STI - Sexually Transmitted Infections

THC - Tetrahydrocannabinol

TMCS - Trimethylchlorosilane

Vs - Versus

VTA - Ventral tegmental area

σ - Standard deviation

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PART I:

Introduction

1. Drug use among young people

In the last few years, most of the consumption of alcohol and illicit drugs among young people in Europe, occurs in night-time recreational activities, typically with friends, either in formal recreational settings such as pubs, bars and nightclubs, or in informal settings such as on the street and at home. Much of party-goers young people's drug use occurs on weekends and during holiday periods (Calafat et al., 2012). A wide range of diverse studies have shown that the levels of drug use among young people are higher in adolescents frequenting night clubs than among young people in the general population (Figure 1) (EMCDDA, 2010).The annual European Report (EMCDDA) shows that high levels of cocaine use occurs in regular attendees in clubs and other recreational settings. In 2010, 24% of visitors to pubs in Amsterdam were cocaine users. In Czech Republic, more than 1000 respondents to a online questionnaire, 29% reported having used cocaine in the last 12 months (Figure 1) (EMCDDA, 2012).

Cannabis is another drug very common among adolescents. An estimated 15.4 million young Europeans between 15 to 34 years (11.7% of this age group) used cannabis in the last year, together with alcohol, in nightlife (EMCDDA, 2013).

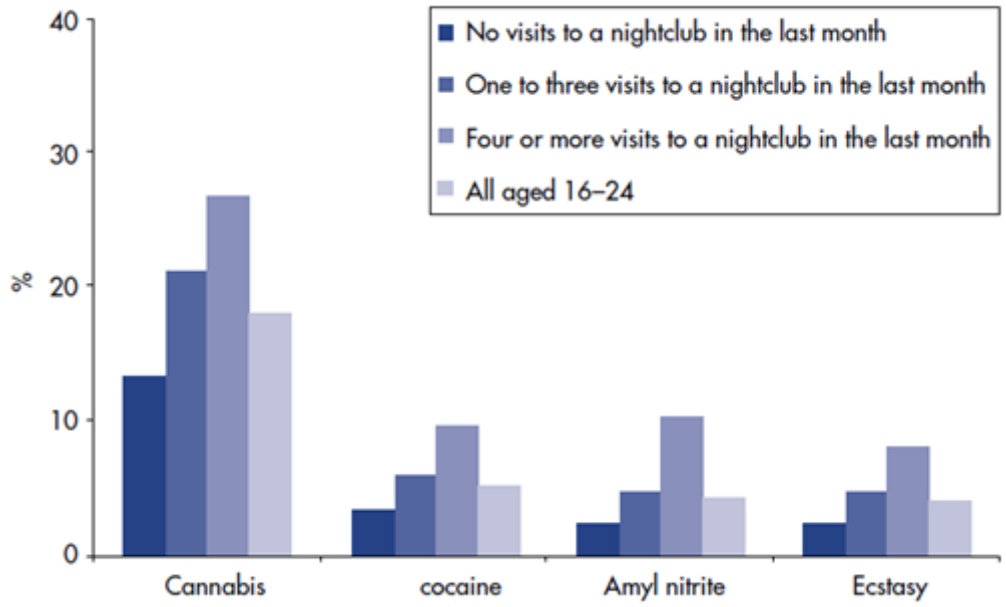

Figure 1. Proportion of 16 to 24 year olds reporting use of the most prevalent drugs in the last year, by frequency of nightclub visits (adapted from EMCDDA, 2012)

. In fact, night recreational settings have been growing importance in young people's life, determining lifestyles and legitimizing behaviors considered necessary for them to experience fun and immediate pleasure. For many adolescents and young adults, fun means being in fashion places, with friends, and enjoys activities related to music and dance. However, in the last few years the recreation nightlife has an intrinsic link with the alcohol and other psychoactive substances. Consequently its use in recreational environments is currently so high that these contexts are considered risk factors for consumption (Lomba et al., 2011). Nowadays, for the majority of young people who participate in recreational environments, substance use are almost the norm. Young Europeans are constructing a new pattern of entertainment, in which going out with friends, mainly on weekends, means drink a lot of alcohol, use drugs, make new experiences, to escape from the social constraints of the rest of the week (Calafat et al., 2003). As recreational settings are increasingly attractive (hegemonic model), almost all focused on psychoactive substances, increasing the risk to individual and collective health and safety, urgent strategies centered on person and self-control should be implemented, once to reduce damage becomes unfeasible. On the other hand, society (parents, institutions and authorities), are gradually assuming the harm related to these contexts as an inevitable consequence of this form of youth fun, devaluing losses in health as well as costs of anti-social behavior and impact that all this might have on the youth future (Calafat, et al., 2004).However, the use of drugs in these recreational places, has been associated with other risk behaviors. At present, violence and use of alcohol and other drugs occurs frequently in recreational settings. The peak time for violent offences is weekend nights and around pubs and clubs. In many countries the majority of violent assaults take place around a pub or club, and almost half of all violence and disorder-related incidents occur on weekend nights (Calafat et al., 2011). Many studies relate violence occurring in nightlife environments, and the use of alcohol and/or illegal drugs. One particularly study shows that 5.2% of the youngsters who go out at night carrying weapons, were involved in polydrug use. Another interesting result is that 11.6% of youngsters attacked or threatened with a weapon, were involved in rows or argument related to substance use. Finally 23% of the youngsters participating in fights, were also involved in rows or arguments related to substance use (Blay et al., 2010). These studies confirm that prevention should be taken especially in problematic young people who engage in polydrug use and who often have fights related to drug consumption.

Another risk behavior, associated with the use of psychoactive substances among young people in night recreational settings, is sexual risk behavior. In Coimbra, it was recently accomplished a survey where were selected teenagers, in the recreational settings of Coimbra. Among these teenagers, 64,52% experienced sex under the effects

of drugs, 40,33% never/seldom used a condom and 9,64% admitted not having used a condom due to being “drunk” or “stoned”. In the same study, 14,60% of these teenagers had engaged in sexual activity due to the consumption of drugs and alcohol, having regretted it later, and 26,61% undertook medical tests for STIs (Sexually Transmitted Infections) (Lomba et al., 2008). Another issue that is in vogue related with drugs and sexual behaviors is the consumption of drugs for increasing the sexual performance. Some young people use alcohol and other drugs in nightlife, with the proposal to find a specific effect on sexuality. The most popular drug used by young people to initiate the sexual encounter, for more unusual or the “hottest” experiences, to increase arousal, and to prolong sex is alcohol, followed by cocaine. The majority of cases were accomplished by women who drank alcohol for increasing arousal, when they want unusual sex or to prolong it (Calafat et al., 2008).

The road traffic crashes is another risk behavior associated with nightlife alcohol and recreational drug use. The big number of road traffic fatalities in Europe, among young people, occurs due to the consumption of alcohol, resulting in an estimated of 17,000 drink driving deaths each year. However, alcohol is not the only substance that can affect driving safety. Cannabis, cocaine, amphetamines and other drugs can influence driving. Private car was the most frequent form of transport used when going out, especially by males and older individuals. Males showed higher levels of drunkenness, drug consumption, traffic risk behaviors and traffic crashes than females (Calafat et al., 2009).

Although the consequences of these risk behaviors are increasingly explicit in the media, inclusive in education of young people, the number of drug users in new generations have maintained all over the years. So, what is happening? Why do young people use drugs?

2. Why do some people use, abuse, and become dependent on drugs?

Many scientists of different areas have frequently asked this question to their audience. The diverse audiences focus on this question because they hope that the answer will provide clues as to what to do about the problem, as they see and experience it. The answer has been investigated, but unfortunately each scientist has different answers. Some of them have created models emphasizing neurobiological or psycho-behavioral or socio-environmental factors and dynamics. There are some factors that explain this question (Pandina and Johnson, 1999).

2.1 The nature and the special properties of psychoactive substances

Psychoactive substances are substances that have the ability to change an individual's consciousness, mood or thinking processes. These occur because they act in the mechanisms of the brain that normally regulate the functions of mood, thoughts and motivations. There are many psychoactive substances, however, that could be divided into three categories according to their sociolegal status. The first category is used as medications for many reasons as: relieving pain, promoting sleep or wakefulness, and relieving mood disorders. The second category are illegal substances that are used to enjoy or benefit from the psychoactive proprieties of the substance. The third group are used as legal substances such as: caffeine, nicotine, tea and alcohol that have stimulant proprieties most often used by actual society (Word Health Organization, 2004).

In recent decades, clandestine laboratories are synthesizing drugs that are chemically and pharmacologically similar to substances that are listed in the Controlled Substances Act, but which themselves are not specifically controlled. This kind of drugs are called "designer drugs" (Karch, 1998).

2.2 The outcomes of drug use

Each drug has different properties that can be used to estimate dependence or to classify its potential hazard. A prototype model for comparing relative hazards associated with the use of 20 drugs representing six major drug classes, has been described, using only two criteria: the quantitative estimates of dependence potential and toxicity (Figure 2) (Pandina and Johnson, 1999).

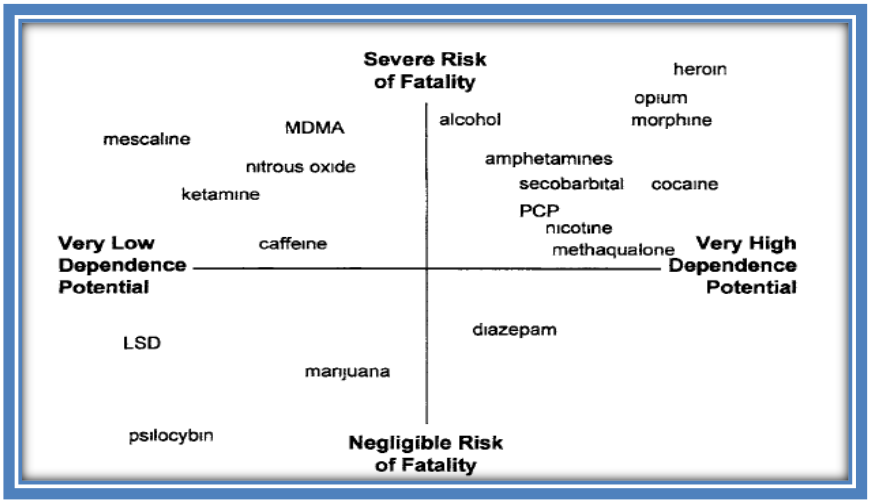

Figure 2. Prototype model for comparing relative hazards, using two criteria: potential dependence and toxicity (adapted from Pandina and Johnson, 1999).

2.3 The dynamics of use behavior

There is a model created by (Pandina and Johnson, 1999) to explain the most common, identifiable sequence and process of the transition to dependency. This model divided use patterns in three developmental stages. The first one is the acquisition stage where the individuals learn about the drugs, the response capacity of biological tissue to drugs, the individual differences and preferences, and the customs of use practices in the local life space.

The second stage is the first experimentation of drugs. Many people experiment the drug, however most of them never progress to initiation from the priming phase for most drugs (e.g. heroin). This experimentation can be done many times and becomes a habit. Thus, use responses become routinized. In this phase, use is under the behavioral control of the individual and it does not dominate the response repertoire, bring few

consequences. When the person becomes dependent on drugs its state reduces to maintain homeostatic stability. In this case, use reacquires its centrality in the life of the individual and dominates the response repertoire.

The third stage occurs when there is the obsessive-compulsive behavior. It is marked by increased cracking and by the decrease in apparent efficacy of a drug to maintain the homeostasis of the organism (Pandina and Johnson, 1999).

2.4 The individual differences in vulnerabilities

This topic shows how individual differences determine vulnerability to drug use. The motivation for the consumption of drugs is essentially based on biological, psychological, and socio-environmental dynamics. The genetic predisposition and the neural adaptation (biological), the personality profile and emotional regulation of the user (psychological) and the peer-group membership and geopolitical climate (socio-environmental) are the main reasons that explain the motivational substrata. However these factors do not act in the single way and each person is unique. It is the answer that explains why some people seem immune or resistant even when they may appear to be at high risk (Pandina and Johnson, 1999).

3. Predisposing factors for drug use

Most studies have identified several predisposing factors associated with drug use in adolescents such as: curiosity, obtaining pleasure (hedonism), relaxation of psychological tensions, facilitating socialization, peer group pressure and rejection, social isolation, family dynamics, low self-esteem, genetic, social, and environmental influences, pleasure to face danger (ordalic behaviour) (Kliewer and Murrelle, 2007). However, it is difficult to ascertain which risk factors or combination of risk factors are most virulent, which are modifiable, and which are specific to drug abuse (Hawkins et al., 1992).

These factors can be divided into two major categories. The first is related to contextual factors (or external factors) that supply legal and normative expectations for behavior. The second category correspond to individual and interpersonal environments (internal factors) such as family, school and peer group influence, that enhance the risk of adolescents initiate or continue the use of drugs (Chakravarthy et al., 2013).

3.1 Curiosity

The main internal factor that is associated with drug use in adolescence is curiosity. It is the major reason for the first use. Curiosity can increase brand recognition and can also prompt experimentation with drug (Wagner and Sundar, 2008). Many teenagers have heard about drugs and they are curious to experience them. They have heard that drugs can be fun, or make a person feel good sensations. Although their family sometimes says exactly the opposite, in this stage of their live they want to see how it really feels (Giusti et al., 2002).

3.2 Peer group influence

Peer group influence has consistently been found to be among the strongest predictors of substance use among young people, more than family influence (Hawkins et al., 1992). The best predictors of adolescents' substance use are the proportion of friends who are users and their friends' tolerance of use. The behavioral and values structure of the peer group, influence the teenager on initial involvement in substance use (Bauman and Ennett, 1996). These peer influences amplify the effects of individual sensation seeking on affect evaluation, increase the attraction to risky behavior, and reduce the perceptions of risk (Romer and Hennessy, 2007).

3.3 Ordalic behaviour

Ordalic behaviour is defined for some authors as “the person who faces the risk is trusting themselves to an external absolute power which will decide the outcome of the risky behaviour” (Ranieri, 2011). The use of psychoactive substances and the ordalic behaviour is closely related. The fear caused by dangerous situations and the satisfaction derived from returning to a state of safety, are the main reasons for drug users, mainly adolescents, wanting to be voluntary exposed to danger situations (Ranieri, 2011).

In the last few years, the Observatory on Youth and Alcohol has faced this problem not only in relation with alcohol but also with the use and abuse of other drugs. An interview realized by this organization shows that the majority of risk behaviors among young people occurs due to the excessive use of alcohol and drugs. The personal risk experiences that are presented in the report refers to: school absenteeism, participation in dangerous motorbike driving and frequent traffic accidents, involving dangerous nightlife called “life in the borders”, sexual harassment, street violence and competition in driving. The largest number of cases answered that they did not know in advance that the behaviour they were engaged was risky. The rest of the cases answered that recognized risk at a later stage. Depending on the severity of the risk episode, young people try to modify their attitudes, beliefs and behaviour. Their decision is also influenced by other peer group members with whom they discussed the risky experience and shared their feelings (Observatory on Youth and Alcohol, 1998).

3.4 Hedonism

Hedonism is a philosophical concept that regards pleasure as the ultimate goal of life. For users of drugs seeking pleasure and avoidance of pain, it is the main reason for the consumption of psychoactive substances. However “Bentham, one of the chief of advocates hedonism, point out that the more we seek pleasure, the less we get it, therefore we should not seek pleasure but seek objects that are pleasurable to us” (Klerman, 1974). The substances as drugs, with abusive potential fulfill the criteria of such objects of pleasure, and the tendency to avoid pain has resulted in a dependent society, more and more hedonistic (Ajai and Singh, 1995). Several researches have revealed that 92% of young students reported use of psychoactive substances for pleasure, indicating that this consumption is about hedonism rather than about escaping from problems (Newbury-Birch et al., 2000). However this pleasure has not reached with the consumption of drugs. The use of psychotropic drugs is slightly negatively related to

happiness, especially the use of hard drugs. People who have never tried drugs are half a point happier than their compatriots who have used them (Figure 3) (Veenhoven, 2003).

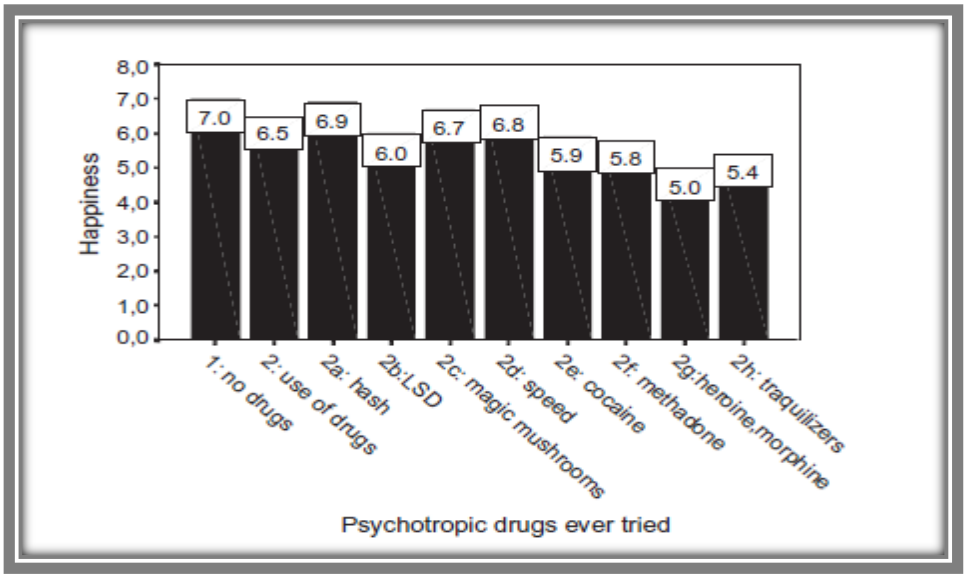

Figure 3. Experience with drugs and happiness among young people (adapted from Veenhoven, 2003).

4. Brain Reward System

4.1 Neuroanatomy of brain reward system

In 1953, the scientists Olds and Milner discovered that rats learned to return to the portions of their environment where they had been given direct electrical stimulation of the septal area of the brain. This stimulation was rewarding and the scientists confirmed that they could train rats to lever-press, by making short pulse trains of septal brain stimulation contingent upon this arbitrary response. Following studies have shown that brain stimulation reward establishes and maintains response habits in patterns very analogous to those established and maintained by natural rewards such as water, food and sexual activity (Wise, 1996). The reason for studying the laboratory reward of brain stimulation is to understand the mechanisms of natural rewards and drug rewards. Such as natural rewards, the drugs of abuse can also influence the brain stimulation and activate directly the reward circuits. This fact helps us to understand better the anatomy and neurochemistry of endogenous reward substrates (Wise, 1980).

The core structure of the brain reward pathway is constituted be two dopamine pathways particularly important for the reward system: the mesocortical and the

mesolimbic. The mesocortical dopamine pathway projects to multiple cortical areas and it is important for many aspects of reward-processing, including hedonic evaluation, comparative valuation, and option-assessment. This pathway projects primarily to prefrontal, cingulate, and entorhinal cortices in rodents, but to the entire cortical mantle in primates (Taber et al., 2012). The mesolimbic dopamine pathway projects primarily from ventral tegmental area (VTA -via the medial forebrain bundle) to nucleus accumbens (NA) and ventral striatum, secondly projects to other limbic areas such as amygdala, olfactory tubercle, septum. This pathway is important for the positive reinforcing effects of both natural rewards and drugs of abuse. It is responsible to modulate the activity of the ventral striatum, a brain region thought to be involved in converting emotion into motivated action and movement (Figure 4) (Koob, 1992).

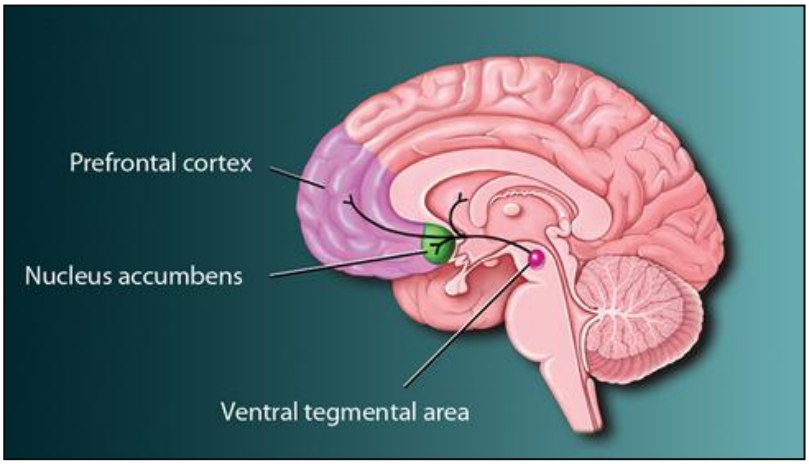

Figure 4. Neuroanatomy of the Brain Reward System (Kibiuk, 2012).

4.2 Molecular physiology of brain reward system

Neuronal communications in the brain occurs through an electrochemical process, with electrical impulses in a neuron modulating the release of neurotransmitters, such as dopamine, serotonin, endogenous opiates, *N* -methyl-Daspartate (NMDA), gamma-aminobutyric acid (GABA), and acetylcholine. These neurotransmitters diffusing across small spaces (called synapse) between adjacent neurons, and binding with proteins (called receptors) on the membranes of the adjacent neurons to modulate electrical signals and other activities. Then, neurotransmitters are deactivated through metabolism or reabsorbed by neurons for reuse (Figure 5) (Kelly et al., 2009).

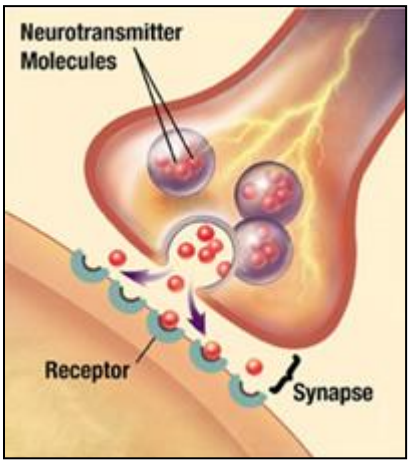

Figure 5. Neurotransmitters mediate communication between adjacent neurons (Foundation for Alternative and Integrative Medicine, 2013).

Psychoactive drugs capitalize on this system, modulating action at the receptor level and altering the manner in which neurons regulate neurotransmitters. When drugs act on this system effects the hormonal action and then other capacities stay affected such as memory, mood, reward, learning and behavior (Kelly et al., 2009). Although drugs of abuse act through separate mechanisms and on various locations in the brain reward system, all of them increase dopamine levels. Dopamine is a main neurotransmitter of the reward pathway, however there are more neurotransmitters such as: serotonin, endogenous opiates and GABA that are closely involved in the brain reward pathway and also modulate dopamine levels (Esch and Stefano, 2004).

Psychoactive drugs could act in the central nervous system as agonists or as antagonists at the receptors for endogenous chemical messengers. In general, most drugs of abuse act as agonist and increase dopamine neurotransmitter levels in the reward pathway. For example, cocaine and amphetamines blocks reuptake of dopamine and stimulate of release (Wise, 1998). Serotonin neurotransmitter is also affected with the use of drugs. This neurotransmitter is mainly involved in the modulation of motivational factors and it appears to regulate dopamine release at the nucleus accumbens. The consumption of alcohol increases the synaptic availability of serotonin with precursor loading, blockade of serotonin reuptake, or blockade of certain serotonin receptor subtypes (Koob et al., 1999). GABA is an inhibitory neurotransmitter and has long been implicated in the modulation of dopaminergic reward systems, playing a role in the mediation of effects of many drugs of abuse such as: alcohol, benzodiazepines and barbiturates. These drugs inhibit the release of GABA and release more dopamine in the reward system (Wallner et al., 2006). Further neurotransmitters are also affected with the

presence of drugs such as: opiates act at receptors for endogenous opioid neurotransmitters; nicotine acts at a subclass nicotinic of acetylcholine receptors; cannabis acts at receptors an endogenous cannabanoid; phencyclidine acts at the N-methyl-D-aspartate subtype of glutamate receptor and caffeine acts at adenosine receptors. (Koob et al.,1999).

4.3 Action of drug abuse in brain reward system

4.3.1 Alcohol

Alcohol is a drug of use/abuse most widely used in our civilization, however it affecting the neurochemical system in diverse areas of the CNS. The severity of its effects depends on the dose, genetic susceptibilities and the type of administration (acute or chronic). Alcohol causes changes in the brain neurochemical dopamine and norepinephrine, especially in VTA (ventral tegmental area). The use of alcohol increases the release of dopamine and norepinephrine. The acute administration of alcohol increases the release of serotonin within the brain, but chronic administration of this drug tends to decrease the amount of serotonin stored in the CNS (Marc and Schuckit, 2006).

Another neurochemical mechanism that has a major impact on the effects of alcohol is GABA. It is the main inhibitory neurotransmitter in the brain and short-term alcohol exposure increases the inhibitory effect of GABAA receptors. Alcohol has been shown to increase the function of glycine receptors in laboratory and also increase inhibitory neurotransmission by increasing the activity of inhibitory neuromodulators such as adenosine (Valenzuela, 1997).

Alcohol induces sedative effects by reducing the major excitatory neurotransmitters in the brain, called amino acids aspartate and glutamate, which act in NMDA receptors. Acute doses of alcohol inhibit both NMDA and non-NMDA receptor activity, potentially resulting in sedation (Valenzuela, 1997).

4.3.2 Opiates

Actually, there are three families of endogenous opioid peptides known: the endorphins, enkephalins, and dynorphins, and three major receptors: mu, kappa, and delta. All opioids have major impact on the receptor mu, more precisely on mu1 and mu2. The activation of these receptors causes analgesia and a feeling reinforcement, having an important impact on the neurotransmitter dopamine, in VTA. Opioids can also mediate acute effects on NMDA, cholinergic, GABA, cannabinoid, and serotonin systems (Marc

and Schuckit, 2006). The use of heroin, the most common opioid of drug abuse, modifies the action of dopamine. Once crossing the blood-brain barrier, heroin is converted to morphine, which acts as a powerful agonist at the mu opioid receptors subtype. This binding inhibits the release of GABA from the nerve terminal, reducing the inhibitory effect of GABA on dopaminergic neurons. The increased action of dopaminergic neurons and the release of dopamine into the synaptic cause a sustained activation of post-synaptic membrane. This continued action leads to the feelings of euphoria associated with heroin use (Figure 6).

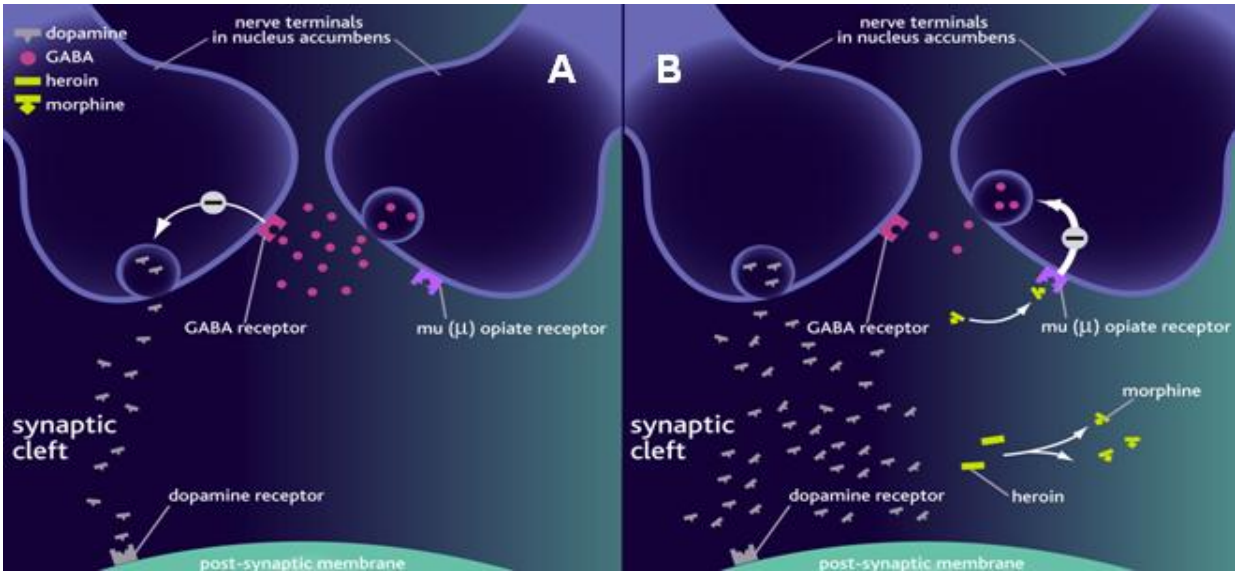

Figure 6. The mechanism of action of heroin (adapted from www.cnsforum.com).

4.3.3 Cannabis

Cannabis has two neuronal cannabinoid receptors: CB1 and CB2 were recently discovered in human cells. Both of these receptor types are coupled through G-proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase. CB1 receptors are also coupled to ion channels through G-proteins, negatively to N-type and P/Q-type calcium channels and positively to A-type and inwardly rectifying potassium channels. Under certain conditions, CB1 receptors may also activate adenylate cyclase through G-proteins (Pertwee, 1999). The activation of CB1 receptors produces a cascade of effects in the second messenger system within the cells, with an impact on dopamine-rich areas (located in nucleus accumbens) as well as opioid, gamma aminobutyric acid (GABA) and glutamate systems (Marc and Schuckit, 2006). The majority of THC effects are mediated through agonistic actions at cannabinoid receptors of the human body. In general, when Δ^9-THC binds CB1 on pre-synaptic nerve terminals in the brain, activates

G- proteins. G-protein activation also activates inwardly, rectifying potassium channels and the MAP kinase signalling pathway. This effect on these pathways causes euphoric feelings associated with cannabis use (Figure 7) (Grotenhermen, 2006).

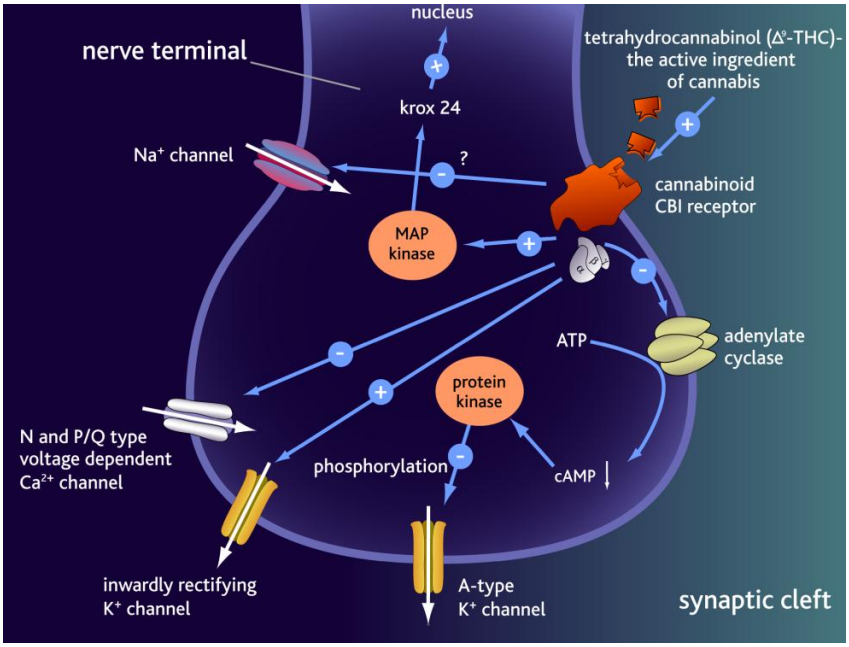

Figure 7. The mechanism of action of cannabis (adapted from www.cnsforum.com).

4.3.4 Cocaine

Cocaine is a strong stimulant drug of abuse derived from leafs of *Erythroxylon coca*. However the same drug has also local anesthetic properties that may be applied in ophthalmology (Dackis and O'Brien, 2001).

During the early years of the last century, it became evident that cocaine was addicting and producing serious medical complications, especially with the availability of cocaine powder for intranasal or intravenous use (Dackis and O'Brien, 2001).

Cocaine affects three major dopaminergic systems: the mesolimbic (ventral tegmental area to nucleus accumbens), the mesocortical (VTA to medial prefrontal cortex and orbitofrontal cortex), and the nigrostriatal. The main synaptic action of cocaine in the reward pathway is to block reuptake of dopamine, norepinephrine, and serotonin, acting through cocaine-binding sites on bioamine uptake transporters (Figure 8) (Brust, 2004). Recent studies have confirmed an increase of 20% or more in dopamine activity in the mesolimbic and mesocortical brain areas in humans that use relevant doses of cocaine. There is also evidence that the level of euphoria experienced with cocaine correlates with the degree of dopamine change in the *corpus striatum* (Marc and Schuckit, 2006).

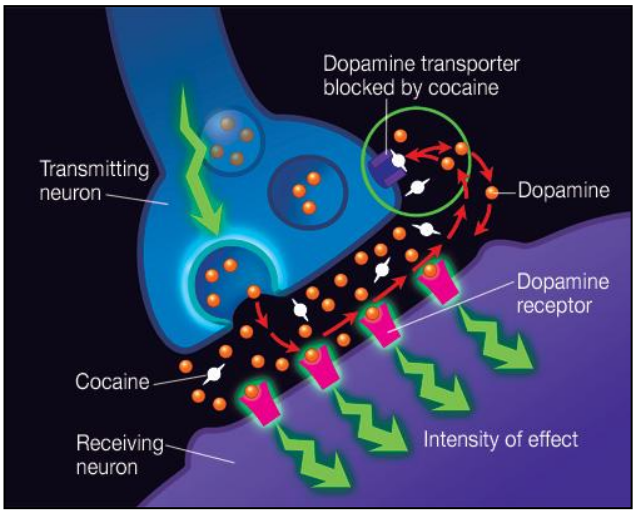

Figure 8. The mechanism of action of cocaine (adapted from Moussa et al., 2006).

5. From pleasure to drug addiction

One of the main reasons for the consumption of drugs is the pleasure that it causes. According to folk psychology, humans tend to repeat behaviors that bring pleasure and relieve suffering. Most drugs of abuse act on ancient and remarkably conserved neural mechanisms, associated with positive emotions that evolved to mediate incentive behavior. Drugs stimulate positive emotions and block bad ones such as: anxiety, low mood, emotional suffering and others. However this pleasure tends to disappear with the continued use of drugs and bad effects in other systems such as memory, mood, reward, learning and behavior begins to appear (Nesse and Berridge, 1997).

A study realized with three different groups of ecstasy users: the first is a group of regular ecstasy users who had taken MDMA (3,4-methylenedioxymethamphetamine) on ten or more occasions; the second is a group of novice ecstasy users who had taken MDMA on fewer than ten previous occasions; and the third is a control group who had never taken MDMA, shows the disappearance of good feelings (good mood and pleasure) with the continued use of ecstasy and the appearance of bad mood and lacks in memory. All three groups reported positive mood at the dance club (on-drug). Nevertheless two days after, the ecstasy users felt significantly more depressed, abnormal, unsociable, unpleasant, and less good tempered, than the controls. The cognitive performance such as verbal recall and visual scanning was significantly reduced on regular users. Memory recall show also worst results compared to regular users (Parrott and Lasky, 1998).

This occurs because persistent drug use induced neuroadaptations in molecular, cellular and neural system levels that are critical in the transition to addition. These

neuroadaptations occur, in the brain, to support the constant presence of drugs and decrease the strong stimulant response caused by drug use. In consequence, psychological function is also changed. Initially, occurs the decreasing of the pleasure and the beginning of the unpleasant withdrawal symptoms (Koob and Moal, 2001; Robinson and Berridge, 2003).

The withdrawals are the symptoms of reversing the development of neuroadaptation to a drug. The drug users feel physical discomfort, psychological pain and other symptoms that differ according to the drug, and discourage people from trying to escape their addiction (Koob and Moal, 2001; Robinson and Berridge, 2003). With the continued consumption of drugs, the drug users become drug abusers, as drug induce sensitization of brain systems and the users start to have a compulsive behaviour to take addictive drugs. Furthermore, drug abusers have dysfunction of frontal cortical systems. The frontal cortical system regulates decision making and has an inhibitory control over behavior. The presence of drugs leads to impaired judgment and promotes impulsivity in addicts (Robinson and Berridge, 2003).

6. Tolerance and sensitization in drug of abuse

Tolerance is a decreased sensitivity to a drug that develops as a result of repeated exposure to it. In this case the drug has fewer effects following repeated exposures. Therefore a higher dose of drug is required to produce the same effect (Figure 9)(Brust, 2004). Tolerance is an expected accompaniment of regular drug use that reflects the adaptation of the receptor site to the presence of the drug. The function of tolerance is to maintain homeostatic balance functioning in spite of the stimulating effects of a foreign substance (drug abuse). Dopamine and other neurotransmitters are present in the brain at higher levels than usual, so the brain tries to adjust, by turning off some of the receptors (locks) into which that molecular key fits. The system may also change the level of neurotransmitters which it produces in order to compensate for the increased levels of whatever drug is being taken. Depending on the drug, these processes can happen in a matter of minutes or over several weeks (Miller and Gold, 1991).

Sensitization or reverse tolerance is an increase in a drug effect that occurs after repeated exposures to the drug. In this case the drug has more effects than that before exposure it. Therefore a lower dose of drug is required to produce the same effect (Figure 9) (Swift and Lewis, 2009). This term is well explained with incentive sensitization theory. It refers to neurobiological changes that occur in brain mesolimbic dopamine systems and other structures that belong to the same brain circuit that mediate the psychological function of incentive salience (“wanting”) (Berridge and Robinson, 2011).

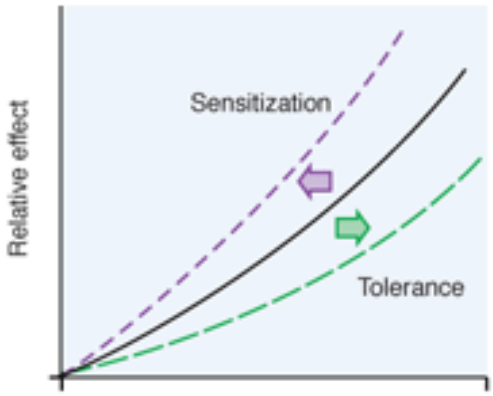

Figure 9. Shifts in a dose-response curve with tolerance and sensitization.

Drug abuse causes tolerance in the body of the drug users by many mechanisms. One of them is natural or innate tolerance. Natural tolerance occurs when preexist inter-individual variations in sensitivity to the drug, before the first administration. It can arise from genetic variation of receptors at which the drug acts or differences among individuals in drug absorption, metabolism, or excretion. Genetic variability is strongly influenced by the environment. Innate tolerance is observed with alcohol. People who have low innate sensitivity, in young adults are of higher risk for alcoholism later (Golan et al., 2012). Whereas innate tolerance has already preexisted in our body, acquired tolerance is determined by an individual's experiences that results from exposure to drug abuse. This kind of tolerance can develop during a single drug exposure (acute tolerance) or during from repeated exposure (chronic tolerance) (Goudie and Young, 1995).

Pharmacologists divide tolerance into two broad categories: dispositional or pharmacokinetic tolerance, which reduce the concentration of a drug or its duration of action in a target system; and functional or pharmacodynamic tolerance, which reduce the sensitivity of drug-sensitive systems to a given drug concentration (Krasnegor, 1978; Goudie and Young, 1995). Dispositional tolerance occurs when the body speeds up the metabolism of the drug in order to eliminate it. There is a physiological change in absorption, distribution, metabolism, or excretion that diminish the concentration of a drug at effectors sites. For example, an increase in the production of enzymes in the liver that breaks down the drug (Krasnegor, 1978). Functional tolerance is described for changes in sensitivity that results from adaptive changes in drug-sensitive systems that diminish the initial effects of a drug. It occurs when the brain learns to compensate for the effects of the drug by using parts of the brain that are not affected. This happens in chronic alcohol and marijuana users. The brain manages to function quite well despite levels of intoxication that would incapacitate people who are less accustomed to the drug. Tolerance of psychoactive drugs is largely functional (Krasnegor, 1978).

6.1 Alcohol

Both types of tolerance occur with the consumption of alcohol. Initially occurs the pharmacokinetic tolerance and then pharmacodynamic tolerance. The pharmacokinetic tolerance is recognizable through a slight increase in both ADH activity and in the liver microssomal ethanol-oxidizing system (MEOS). For this reason, the elimination of alcohol becomes more rapid in chronic users. Functional tolerance result of a direct adaptation of CNS tissues to alcohol, so it is necessary drink more quantity of alcohol for have the same effect (Sommer and Spanagel, 2012).

6.2 Opiates

Tolerance develops rapidly to most opioids, particularly with the more potent analgesics. After repeated uses of opiates, it produces complete tolerance to their euphoric effects, as well as sedation and respiratory depression. In chronic users, it causes pupils constriction, producing the pinpoint pupils, as well as slow bowel function. Because of this differential tolerance to specific opiates effects, long term opiate users have little risk of overdose (Dupont, 1997). Cross-tolerance is also common among the opioids, with a predictable variability depending on the opioid receptor type most prominently affected (Marc and Schuckit, 2006).

6.3 Cannabis

The users of cannabis can develop tolerance, if they consume high doses of cannabis for a sustained period of time. The phrase “Less is more” was the slogan used in 1976 to deter recreational consumption of cannabis: “The less frequently a person users cannabis, the less likely is that person to develop tolerance to the original dose, and the less likely is that person develop cannabis tolerance”. In fact, little tolerance is observed when the doses of cannabis are small, and infrequent for limited duration (Mathre, 1997).

6.4 Cocaine

Cocaine has optimal conditions to develop tolerance, mainly acute tolerance. Due to the short action of cocaine, acute tolerance develops within 24h (Grabowski, 1994).

Cross tolerance occurs when cocaine users consume the drug with other psychostimulants as amphetamines and methamphetamines. Repeated administration of cocaine induced tolerance to the effects of methamphetamines, and repeated exposure to

amphetamines causes tolerance to cocaine. However cross-tolerance is not observed in opiates. Repeated administration of morphine did not induce tolerance to cocaine (Higgins and Katz, 1998).

The main symptoms caused by cocaine tolerance are: progressive increases of irritability, restlessness, hyper vigilance, paranoid and suspicious behavior (Grabowski, 1994).

7. Addiction and physical dependence in drug of abuse

Addiction or dependence (described in the Diagnostic and Statistical Manual of Mental Disorders (DSM) used by the American Psychiatric Association) is defined as a chronic relapsing brain disease that is characterized essentially by compulsive drug seeking and use, despite negative and dangerous effects. It is considered a brain disease because drugs change the brain´s structure and how it works. These brain changes can be long lasting and can lead to many harmful, often self-destructive, behaviors (Mc Lellan et al., 2000).

The physical dependence occurs with the chronic use of any substance, legal or illegal, even when taken as prescribed. It occurs because the body naturally adapts to chronic exposure to a substance, and when that substance is taken away, symptoms can emerge while the body readjusts to the loss of the substance (McLellan et al., 2000). This adaptation can result in withdrawal symptoms when drugs of abuse are discontinued. That means that a person needs a drug to function normally (O´Brien and Volkow, 2006)

The Diagnostic and Statistical Manual of Mental Disorders uses the term "substance use" and “substance dependence” to refer a maladaptive pattern of substance use that leads to clinically significant impairment. Drug abuse is associated to some symptoms such as: failure to fulfill major role obligations; legal problems; use in situations that are physically hazardous; and continued use despite persistent social or interpersonal problems. The term dependence includes symptoms as: drug taking in larger amounts than intended; inability to cut down on drug use; a great deal of time spent in activities necessary to obtain the drug; and continued use despite knowledge of health or social problems caused by the drug (McLellan et al., 2000).

7.1 Alcohol

Dependence of alcohol or chronic alcoholism is characterized by symptoms similar to those found with other forms of drug addiction, but it also presents other typical neurological, psychiatric, and physical disorders. Studies demonstrate a possible

biochemical link between alcoholism and opiates in inducing their respective dependences. The products of alcohol metabolism (ex: isoquinolines) may interact at opiate receptor sites, and causes marked alteration in the cerebrospinal fluid (CSF) content of proopiocortin-related peptides which may play a role in the alcohol-seeking behavior typical of the syndrome (Genazzani et al., 1982).

7.2 Opiates

Physical dependence in opiates is explained by inhibition of the functional activity of the cAMP pathway, indicated by cellular levels of cAMP and cAMP-dependent protein phosphorylation. The cAMP pathway is implicated in neural-specific phenomena, in particular, the regulation of synaptic transmission. With continued opiate exposure, functional activity of the cAMP pathway gradually recovers to normal, and on addition of an opioid receptor antagonist, cAMP levels increase far above control values. These changes in the functional \state of the cAMP pathway are mediated via the induction of adenylyl cyclase and protein kinase A (PKA) in response to chronic administration of opiates. These adaptations in cAMP pathway contribute to opiate dependence, and the activation of the cAMP pathway observed on removal of opiates causes withdrawal symptoms (Figure 10) (Nestler, 2004).

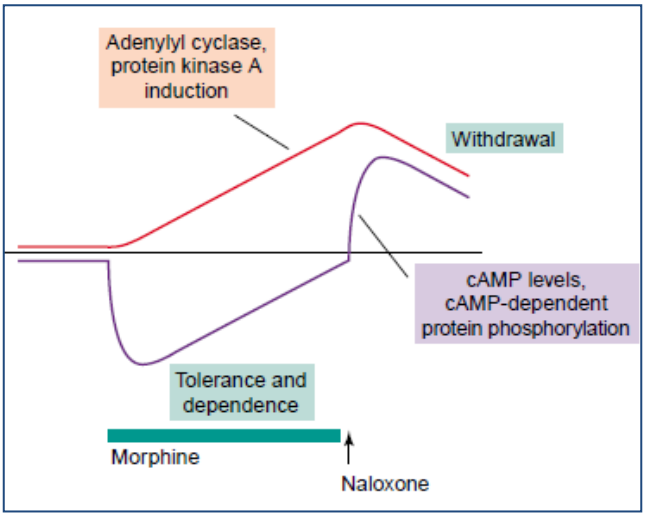

Figure 10. The cAMP pathway in opiate dependence (adapted from Nestler, 2004).

7.3 Cannabis

Cannabis dependence is very similar to other substance dependence disorders, although it is likely to be less severe. Adults seeking treatment for cannabis abuse or dependence average more than 10 years of near-daily use and more than six serious attempts at quitting. Most perceive themselves as unable to stop, and most experience a withdrawal syndrome upon cessation (Budney et al., 2007).

The risk of dependence is higher in identical twins of individuals with cannabinol dependence than it is for fraternal twin pairs, because there is a genetic predisposition toward dependence on cannabinols that influences the CB receptors or cannabinol-metabolizing enzymes. The symptoms of cannabis dependence is characterized by lack of mental drive and focus along with the cognitive impairments that can interfere with job or school performance, impair coordination and judgment important for driving, or contribute to legal problems (Marc and Schuckit, 2006; Budney et al., 2007).

7.4 Cocaine

Cocaine dependence and addiction is typically associated with frequent and intense drug wanting triggered by internal or environmental cues associated with past drug use (Kilts et al., 2001). Cocaine causes psychological and physiological problems that can be associated with dependence. More than 90% of people who are cocaine dependent describe a prominent and disturbing physiological withdrawal syndrome involving depression, somnolence, and intense hunger. These symptoms are experienced for several days following abstinence. While physiological dependence is characterized by an intense craving for cocaine, fatigue and an increase in appetite. The symptoms of psychological dependence are usually characterized by a major change in a person's personality. In cocaine abusers the drug becomes their top priority and they may let other activities they have enjoyed in the past take a back seat. The person may also become obsessed with making sure to always have enough cocaine (Marc and Schuckit, 2006).

8. Withdrawal syndrome in drug of abuse

Withdrawal syndrome results in physical, behavioral as well as motivational symptoms following cessation from the continuous use of an addictive drug. The character and severity of the symptoms depend upon the particular drug, the daily dose, the frequency and the duration of drug use (Koob et al., 1999).

While in several psychoactive substances as: barbiturates, alcohol, stimulants, opiates and benzodiazepines the withdrawal syndrome can cause severe effects, in other psychoactive substances as: caffeine and nicotine the withdrawal effects are less felt. A mild withdrawal is associated with cannabis use, while there is no evidence of withdrawal syndrome related to LSD. The changes in mood and in motivation are some symptoms caused by withdrawal syndrome There are four withdrawal syndromes, with each representing the opposite of the acute effects of the drugs of that class. (U.S. Congress Ofice of Tecnology Assessment, 1993), as explained below.

The depressant withdrawal felt in alcohol, barbiturates or benzodiazepines abusers consist in signs and symptoms such as: anxiety, insomnia, seizures, delirium (more precisely delirium tremens, typically in alcohol withdrawal syndrome), nausea, as well as higher blood pressure and heart rate. (Lowinson et al., 2005).

The main symptoms associated with opiate withdrawal are: discomfort as psychological as physical, dilatation of pupils, stuffy nose and gooseflesh. Although these symptoms are extremely uncomfortable, they are rarely life threatening (Dupont, 1997).

The stimulant withdrawal syndrome felt by cocaine, amphetamine and ecstasy abusers consists basely of sleeping too much, eating too much, and depression. When there is a combination of two different types of drug such as: alcohol and cocaine or heroin and cocaine, the stimulant portion of the withdrawal is treated with education and reassurance, while the depressant and opioid withdrawal is likely to require short-term medications (Marc and Schuckit, 2006).

The withdrawal syndrome felt in cannabinoids and hallucinogens abusers are less clinically relevant (U.S. Congress Ofice of Tecnology Assessment, 1993)

8.1 Alcohol

Alcohol withdrawal syndrome (AWS) can be a life threatening condition, which may occur following abrupt cessation of alcohol intake in individuals whose consumption has been extensive and excessive (Cooper and Vernon, 2012). This syndrome is characterized by a variety of symptoms that depends on the frequency and severity. It is typically related with the amount and duration of alcohol use. Depending on the range of severity, this kind of users narrows essential elements of withdrawal syndrome that might include: drink seeking behaviour; an increase tolerance to alcohol; repeat withdrawal symptoms; repeat relief or avoidance of withdrawal symptoms by further drinking; subjective awareness of a compulsion to drink; replacement of the syndrome after abstinence (Edwards and Gross, 1976).

Although there are many exceptions, the withdrawal syndrome appears 6 to 12 hours after the last alcoholic drink, in individuals that are used to drink about 200-300 g of alcohol a day for several years. This syndrome cannot occur without a high degree of CNS tolerance, but tolerance can exist without clinically manifest withdrawal symptoms. At first, the symptoms are intermittent and mild, causing a little incapacity and one may be experienced without others. With the increase of the dependence, the frequency and the severity of the symptoms are also increased and the patient has severe multiple symptoms every morning on waking (Marc and Schuckit, 2006).

The spectrum of symptoms is ample and includes: tremor, anxiety, insomnia, nausea, sweating, headaches, tinnitus, itching, muscle cramps, palpitations, mood and gastrointestinal disturbance. After that, the patients could develop further complications such as: perceptual distortion, hallucination and grand-mal seizures. The most life-threatening manifestation of AWS is delirium tremens, which affects approximately 5% of alcohol abusers. This symptom occurs within 2–4 days after alcohol cessation. The associated mortality rate of delirium tremens is 1–5% and the condition may result in respiratory and cardiovascular collapse (Marc and Schuckit, 2006; Cooper and Vernon, 2012).

8.2 Opiates

Opiate withdrawal refers to the wide range of symptoms that occur after stopping or dramatically reducing opiate drugs after heavy and prolonged use. One of the reasons commonly given by opiate users for continuing opiate use is the avoidance of withdrawal symptoms. They begin a few hours from the last dose, the peak appears after two to three days, and start to subside after a week. In opiod abusers some withdrawal symptoms will occur several times per day and during the life of a patient before they seek treatment (Gerada and Ashworth, 1997; O'Brien et al., 1998).

The heroin withdrawal causes many symptoms such as: craving, nausea, anorexia, anxiety and restlessness, aching bones and muscles, insomnia or yen sleep, and hot and cold flashes. Therefore there are signs considered comprised such as: tremors, yawning (addiction and dependence), vomiting, diarrhea, perspiration, lacrimination, rhinorrhea, increased respiration rate and depth, goose flesh, mydriasis (dilated pupils), spontaneous orgasm, increased temperature, tachycardia, and increased blood pressure (Gallimberti et al., 1993).

The treatment for opiate withdrawal consists in giving drugs that modify the neurotransmitter system responsible for the behavioural and biochemical opioid withdrawal effects. The usual treatment for opiate withdrawal is replacement with clonidine

or another adrenergic agent. Recently this medication was substituted by buprenorphine, a partial opioid agonist used for the treatment of opioid withdrawal (Kosten and O'Connor, 2003).

8.3 Cannabis

Upon abrupt cessation of high doses of marijuana (cannabis) administration, we can observe withdrawal syndrome (Marc and Schuckit, 2006). It syndrome is characterized by many symptoms as: yawning, anger/aggression, irritability, anxiety, decreased appetite/weight loss, restlessness and sleep difficulty. Other signs have also been reported, but with less frequently such as: depressed mood, stomach pain/physical discomfort, shakiness, and sweating (Vandrey et al., 2005; Budney et al., 2007).

Most symptoms beginning within 24 h of abstinence, peak between day two and six of abstinence and most return to baseline by day 14. Other symptoms as: sleep difficulty, anger/aggression, irritability and physical tension have persisted for three to four days. Strange dreams failed to return to baseline during the 45-day abstinence (Winstock et al., 2009).

The cannabis withdrawal syndrome acts as a negative reinforcement for relapse to cannabis use in individuals trying to abstain. Hence there is a pharmacological treatment aimed at alleviating cannabis withdrawal might prevent relapse and reduce dependence. This medication works as CB receptor agonists that directly suppress the withdrawal syndrome and alleviate symptoms of cannabis withdrawal such as dysphonic mood and irritability (Weinstein and Gorelick, 2011).

8.4 Cocaine

After cessation or reduction in prolonged, heavy use of cocaine, the presence of withdrawal syndrome appears. The symptoms described for cocaine withdrawal does not result in the peripheral signs and symptoms of autonomic instability often seen in other drug withdrawal syndromes. Cocaine withdrawal is mainly associated with significant psychiatric symptoms such as: dysphoria, anhedonia, anergia, anxiety, irritability, boredom, craving (Kampman et al., 2000). Nevertheless, there are other symptoms associated with cocaine withdrawal such as: appetite disturbance, gastrointestinal upset, depression, muscle pain, and tremor (Brower et al., 1988).The symptoms begin within 24h of stopping cocaine, and become most intense for 3–5 days. These symptoms remain at decreasing intensity for many weeks, and perhaps months after cessation of cocaine use (Brower et al., 1988; Marc and Schuckit, 2006).

Cocaine withdrawal is also characterized by electroencephalogram (EEG) changes, usually involving an excess of alpha power along with a decrease in the faster delta and theta frequency bands, with at least one report of a continuation of these findings in some individuals for 6 months. This phenomenon is related to changes in dopamine (DA) transmission, in several brain areas, including the amygdala. Additional changes are also observed in cerebral glucose utilization and changes in the normal hormonal alterations, including prolactin (Alper, 1999).

9. Toxic reactions and overdose in drug of abuse

Toxic reactions caused by psychoactive substances consist in toxic physiological, psychological, behavioral manifestations and life-threatening reactions resulting from intentional and unintentional (accidental) overdose. A drug overdose is the use of drug in an amount that is higher than is normally used, or when the drug is taken in combination with other drugs inclusive alcohol (Khantzian and McKenna, 1979).

All drugs have the potential to produce toxic effects and cause overdose by a variety of mechanisms such as: Toxic effects that are direct and predictable following a drug overdose occur due to the drug itself or it may reflect a change in the metabolism of the drug, resulting in a toxic metabolite; Toxic effects may be direct and predictable following repeated dosing of the drug, are mediated by metabolites, pharmacologic in nature or immunologic in mechanism; Toxic effects may be direct and unpredictable, either following one dose or just a few doses occur due to a idiosyncratic response to drugs and may be immunologic or pharmacologic in mechanism. This type of toxic effects appear in only a very few patients and have no good predictors for their occurrence; The last one are toxic effects that results of some drug interaction or may be caused by a change of tolerance due to temporary drug abstinence (Khantzian and McKenna, 1979; Kjelsberg et al., 1995).

With the exception of using opioid antagonists, overdose conditions are usually treated by supporting the vital signs, so that the body can metabolize and excrete the drugs and return to normal. After that is necessary to control of symptoms regardless of the specific drug involved. If the drugs involved in overdose were depressants, the patient will require control of respirations and bolstering of blood pressure, because the vital signals were depressed. If the drugs involved in overdose were stimulants or opioids, the emergency treatment depends upon the vital sign changes. The mainly treatment in these cases includes a controlling high blood pressure and elevated body temperature. If the drug involved was opioid, the clinical should administer an opioid antagonist (e.g., naloxone). Another general rule is to consider gastric lavage, followed by the possible use

of activated charcoal if there is evidence of recent oral administration of the drugs (Marc and Schuckit, 2006).

9.1 Alcohol

An overdose of alcohol occurs when a person has a blood alcohol concentration (BAC) sufficient to produce impairments that increase the risk of harm. The severity of toxic reaction could vary among individual characteristics such as: age, drinking experience, gender and the amount of drinker food eaten (Li et al., 2003).

There are two forms of ethanol intoxication: pathological intoxication, also called idiosyncratic intoxication and acute alcoholic paranoid state. The acute alcoholic paranoid intoxication consists of sudden extreme excitement, sometimes with delusions, hallucinations, and violent behavior, even homicide. In some cases after minutes to hours, there is amnesia for the episode. The pathological intoxication is characterized by psychological dissociative reactions resulting of paradoxical excitation (Brust, 2004).

Acute overdose with ethanol presents primarily as CNS depression. The most common symptoms of acute alcoholic overdose are: signs of vasodilatation (flushing, tachycardia, hypotension, and hypothermia), depressed consciousness, hyponatremia, hypovolemia, electrolyte imbalance, hypoglycemia and abnormal temperature. In chronic ethanol intoxication is common to appear hepatotoxicity and cardiotoxicity, causing potential cirrhosis and cardiomypathy progressing to failure, respectively (Malcolm and Alkana, 1983; Brust, 2004; Waring et al., 2008).

The treatment of severe ethanol poisoning is similar to that of other depressant drugs. Death is from respiratory depression, and so patients require artificial ventilation in an intensive care unit. In chronic ethanol ingestion is necessary to make the detoxification with employ of chlordiazepoxide, to prevent delerium tremens and other signs of ethanol withdrawal (Brust, 2004).

9.2 Opiates

The opioid overdose is usually an acute, life-threatening event that is most often accidental but that could represent a deliberate suicide. At least one overdose occurs during the course of 50% of heroin users. The user is likely to be found in a semi-comatose condition with evidence of a recent intravenous injection. The risk of overdose depends on many factors such as: the high-quality heroin (increase the risk) or use of a more potent opioid (e.g. fentanyl), the price of the drug (less cost, high risk of overdose), the use of quinine for dilute the drug can decrease the activity of the cardiac pacemaker,

decrease cardiac electrical conductivity, and thereby induce a prolonged cardiac electrical refractory period that increases the risk for ventricular fibrillation (Marc and Schuckit, 2006).

Acute overdose causes depression, especially of the respiratory centre. The symptoms associated with opioids overdose include: reduced levels of consciousness, lethargy, miosis (pinpoint pupils), flaccid muscle tone, cool skin, hypotension, bradycardia, hypothermia, cyanosis, hypoventilation, apnea and coma. Death is usually due to respiratory failure (Darke and Zador, 1996).

Opioid overdose is treated by administration of opioid antagonist such as naloxone or naltrexone. Naloxone is mainly used in the treatment of opioid-overdose-induced respiratory depression, in ultra-rapid detoxification and in combination with buprenorphine for maintenance therapy to prevent intravenous abuse. However the administration of naloxone in opioid-dependent patient has some implications that include: the occurrence of vomiting and aspiration is potentially life threatening, and in patients treated for severe pain with high-dose naloxone may cause catecholamine release and consequently pulmonary edema and cardiac arrhythmias. So the administration of naloxone imply an adequate monitoring of the cardiorespiratory status of the patient (Dorp et al., 2007).

9.3 Cannabis

Cannabis has acute and chronic effects on mental health. Acute effects vary among individuals and the degree and severity of these effects is related to the dosage, method of administration, environment and personality of the user. Chronic effects could cause serious psychiatric illness such as: depression, anxiety, low motivation, psychosis and schizophrenia (Cho et al., 2005).

Although cannabis cause less clinically relevant effects, when used in higher quantities causes adverse toxic reactions. High doses of cannabis cause auditory and visual illusions or hallucinations that consist of flashes of light or color, geometric figures, human faces, or complex pictures. Also, it can cause bizarre illusions include loss of depth perception, the appearance of people talking with their mouths, voices unsynchronized, and “streaking” (moving light sources in a dark environment becoming long streaks as in a time-exposed photograph).The patients can describe fantastic complex hallucinations with extraordinary dilation of subjective time, and there are some reports that propose that marijuana improves night vision. If increasing more the higher doses of cannabis, we could observe confusion, disorientation, anxiety, psychotic depression or excitement, bradycardia and hypotension occur. Nowadays, fatal overdose has not been documented (Hollister, 1986; Brust, 2004; Cho et al., 2005; Calafat et al., 2012).

Cannabis severe symptoms are treated by administration of benzodiazepines, haloperidol or other anxiolytic. This medication is adequate for treatment of anxiety and feature of cannabis withdrawal (Piomelli, 2004).

9.4 Cocaine

Overdose of cocaine occurs in users with the following characteristics: female gender, injectors of cocaine, to have a longer cocaine use careers, to have used more cocaine in the preceding month and preceding 6 months, to have higher levels of cocaine dependence and more extensive polydrug use (Kaye and Darke, 2004).

Cocaine acts in CNS and in cardiovascular system cause biphasic response. In lower doses it tends to improve motor performance and to produce a decrease in heart rate via actions on the vagus nerve. In high doses it causes deterioration in CNS, with subsequent severe tremors and possible convulsions, and in cardiovascular system causes an increased heart rate and vasoconstriction, with a resulting elevation in blood pressure (Marc and Schuckit, 2006). The most common symptoms of overdose are: palpitations, nausea, vomiting, dilated pupils, an increased body temperature, intense sweating, seizures, muscle contractions, arrhythmias due to catecholamine release. Fatal cocaine overdose has also occurred due to brain hemorrhage, stroke and kidney failure, myocardial infarction, hyperthermia, or ventricular arrhythmias (Gerada and Ashworth, 1997; Kaye and Darke, 2004).

The time course of effects differs with the route of administration. Intravenous use of cocaine has an onset of about 3–5 min, with peak effects at 10–20 min, and a fading high by 45 min or less. The risk of overdosing increases when it is administered in a way that causes a rapid increase in brain levels of the drug, as happens with injected cocaine (Kaye and Darke, 2004; Marc and Schuckit, 2006).

Actually there is no specific antidote for cocaine overdose. The treatment options are supportive and symptomatic in nature, and will depend on which clinical features are present. If the patient is unconscious should be ensured the adequate ventilation and volume depletion, cardiac arrhythmias, seizures, hypertension, agitation, and hyperthermia should be managed symptomatically. Benzodiazepines are the medication of choice for the management of patients with agitation, seizures, tachycardia, and hypertension. If hypertension persists after the administration of benzodiazepines specific antihypertensive therapy (e.g. intravenous nitrates or calcium-channel blockers) can be given. Beta-blockers should be avoided due to the risk of coronary vasoconstriction and paradoxical hypertension. However, has been described recently, that the administration of intravenous lipid is an effective treatment for cardiovascular complications associated

with local anaesthetic-induced toxicity. This medication is also acts as an effective antidote to the overdose of lipid-soluble drugs including beta-receptor antagonists, calcium channel blockers and antidepressants (Carrera et al., 2005; Jakkala-Saibaba et al., 2011).

PART II:
General and specific objectives of the thesis

1. Objectives of the thesis

The general objective of this work was to evaluate through surveys and collection/analysis of biological samples, the drug abuse patterns in Coimbra recreational nightlife.

The specific objectives of this work were:

- To investigate the presence of psychoactive substances in biological samples ceded by willing participants of this study;
- To analyze qualitatively and quantitatively the components of these samples by GC-MS, to be implemented in the present studies;
- To confront the answers in the surveys with the chemical results of analyzed samples;
- The final objective is to contribute for a better risk management concerning drug abuse among young people, by comparing their perception of the risk abuse patterns.

PART III:

Experimental Part

Chapter I:

Materials and Methods

Surveys

1. Surveys

1.1 Characterization of the study

This study is observational, descriptive and cross-sectional. Studies with these characteristics allow describing a phenomenon or a concept related to a specific population. This type of study is used to determine the characteristics of a population or a sample. It is also used to assess the prevalence of conditions in a population, since the exposure is measured at the same point in time (Fortin, 1999).

1.2 Study population

The study population comprehends all young adults between 18 to 30 years who attend recreational nightlife, in Coimbra, at least once per week.

1.3 Study sample

To determine the sample of this study, it was decided to use the non-randomized process of accidental sampling. Although this type of process does not ensure that all elements of the population have the same probability of being included in this sample, it facilitates the inclusion of a higher sample size. The data were collected in days of higher affluence, in recreational spaces, from May to September 2013. For this study the inclusion criteria was: to be aged between 18-30 years old and to be in a recreational context at nights of data collection. No exclusion criteria were used for this study.

1.4 Legal and administrative procedures

- Approval by National Commission of Data Protection.
- Approval by ethical commission of Faculty of Pharmacy, and University of Porto.
- Approval by City Hall of Coimbra.
- Approval by ARS-IP.
- Written informed consent by all participants.

1.5 Study variables

In this work it was used, as research tool, a survey validated for Portuguese population. This survey is composed for five sections, which allow defining the following variables:

- **Socio-demographic characteristics:** age; gender; student/non-student; typology of degree; course; residence; type of study; professional status and number of household.

- **Recreational nightlife habits:** frequency of nights out per week and per month; number of recreational spaces frequented per night; money spent per night.

- **Health information:** weight; height; health problems; use of medications with or without prescription; last food intake (in number of hours ago).

- **Problems due to use of psychoactive substances:** health problems related with the use of psychoactive substances; influence of psychoactive substances in friendship relations; driving under psychoactive substances; problems with authority; perception of risk.

- **Drug abuse patterns:** type of psychoactive substances used during the last week; dosage; route of administration; type of psychoactive substances used in the end of the night.

1.6 Collection of surveys

The surveys were made in days of higher affluence towards the door of nightclubs or inside it, as previously agreed with public relations of clubs and pubs in Coimbra. The young adults between 18 to 30 years, who were in nightclubs, were freely invited to participate in this study. After written informed consent, the surveys and biological samples were collected and transported to the Department of Toxicology, Faculty of Pharmacy, University of Porto. All surveys and written informed consent remain stored in a biobanc of this University for at least five years. After this period of time it can be automatically destroyed. All participants that wanted to be informed about the results of toxicological analysis were sent an e-mail with the respective results.

1.7 Statistic treatment

All information collected through the surveys were quantitatively analyzed, using the Software Package for Social Sciences (SPSS®), version 17.0 for Microsoft Windows®. For descriptive analyze of the data, was determined the counts and the frequencies of the nominal variables. For numeric variables, it was used the measures of central tendency (mean, mode and median), the dispersion (standard deviation) and the amplitude variation (minimum and maximum).

Due the short size of the sample, for comparative and inferential analyze, non-parametric tests were used: Mann-Whitney test to compare two independent groups ; Kruskal-Wallis test to compare two or more independent groups. The Pearson Chi-Square test was used with the objective of correlating two independent groups and, when

applicable, the Odd's Ratio. For inferential analysis, it was defined a significance level of 0,05.

Chapter I I
Materials and Methods
Identification and quantification of psychoactive drugs in biological samples

1. Identification and quantification of psychoactive drugs in biological samples

1.1 Ethics statement

This research was approved by the following institutions: National Commission of Data Protection, Ethics Commission of Faculty of Pharmacy University of Porto, City Hall of Coimbra and ARS-IP. A written informed consent was signed by all participants of this study.

1.2 Reagents and standards

All the chemicals used for the ethanol quantification were of analytical grade: ethanol (> 99.9%, Panreac, Barcelona, Spain), 1-propanol (> 99%, Sigma-Aldrich Co., St. Louis,MO), Triton X-100 (Sigma-Aldrich Co.).

The analytical standard ∆9-Tetrahydrocannabinol, molecular mass 314.45 g mol^{-1} and the internal standard benzophenone, molecular mass 182.22 g mol^{-1} were obtained from Sigma-Aldrich (St Louis, MO, USA). Methanol and N,O-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA+1%TMCS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Hexane, acetonitrile and ethyl acetate were purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate and di-potassium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Nitrogen (99.99% purity) and helium (99.99%) were obtained from Gasin (Portugal).

The analytical standard cocaine hydrocloride, molecular mass 339.81 g mol^{-1} and the internal standard benzophenone, molecular mass 182.22 g mol^{-1} were obtained from Sigma-Aldrich (St Louis, MO, USA).

The cocaine screening test was obtained from commercial brand First Check@ Test.

- All the reagents used were of analytical grade or from the highest available grade.

1.3 Biological specimens

Blood and oral fluid samples were collected from young adults attending recreational nightlife, in Coimbra, at least, once per week. Blood samples were collected from the median cubital vein. The collection method used in this study was through a commercial vacuum-sampling system, decreasing the risk of contamination. Initially, a tourniquet was used to distend the vein prior to sampling. After that, the skin was cleaned with disinfectant swabs containing alcohol. In view of the requirement to prevent stress on patients, only 5 to 10 mL of blood was collected. These samples were kept in heparin tubes at 7ºC. The oral fluid samples were collected through a spitting method (around 1-2

mL) for an appropriate collection container and were kept at 7ºC. After the collection, all samples were stored and transported, in refrigerator containers, to the Laboratory of Toxicology, Faculty of Pharmacy University of Porto. For validation of the analytical method for THC, drug-free blood was used, to which was added the appropriate volume of THC standards. All samples were stored at -20 ºC prior to analysis.

1.4 Analysis of ethanol in blood and oral fluid samples

Sixty nine blood samples, 17 oral fluid samples, and 46 exhaled air samples were obtained from these participants. Blood and oral fluid analysis were performed according to a previously published method (Pontes et al., 2009).

1.4.1 Preparation of stock solution

A sock solution containing ethanol was prepared in commercial deionized water from the purchased sample (> 99.9%, Panreac, Barcelona, Spain), at the concentration of 12g/L.

1.4.2 Preparation of calibration standard solution

For blood sample analysis, calibration standard solutions of 0; 0.15; 0.30; 0.60; 1.20; 2.40 g/L were freshly prepared for each analysis from the stock solution of 12g/L ethanol, by adding the appropriate volume to drug-free blood.

For oral fluid sample analysis, calibration standard solutions of 0; 0.0375; 0.075; 0.30; 0.60 g/L were freshly prepared for each analysis from the stock solution of ethanol 12g/L by adding the appropriate volume to drug-free oral fluid.

1.4.3 Preparation of internal standard (IS)

A working solution of 1-propanol was prepared in deionized water from the purchased solution of 1-propanol (> 99%, Sigma-Aldrich Co., St. Louis,MO), at the concentration of 2,2 g/L. This solution was used as internal standard (IS).

- All working and stock solutions were prepared fresh daily and stored at 7 ºC prior use.

1.4.4 Sample preparation for gas-chromatography flame ionization detector

1.4.4.1 Drug-free samples

One drug free blood and oral fluid samples were tested with the same protocol of ethanol blood/oral fluid samples respectively, in order to verify the selectivity of the method.

1.4.4.2 Blood samples

Two hundred microliters of blood samples were mixed with 40 µL of IS. The samples were diluted to 780 µL with the Triton X-100 solution. All samples were vortex mixed and centrifuged at 13.000 rpm for 3 min. 0.5 µL of the supernatant were directly injected into the GC-FID system.

1.4.4.3 Oral fluid samples

Two hundred microliters of oral fluid samples were mixed with 40 µL of IS. The samples were diluted to 780 µL with the Triton X-100 solution. All samples were vortex mixed and centrifuged at 13.000 rpm for 3 min. 0.5 µL of the supernatant were directly injected into the GC-FID system.

1.4.5 Gas-chromatography flame ionization detector conditions

The GC used was a ThermoFinnigan Model Focus GC equipped with a FID. The injection port of the chromatograph was installed with a glass liner (5-mmi.d.) appropriated for split analysis, to prevent the contamination of the GC column with non-volatile material from the tested matrices. For blood and oral fluid samples, the liner was replaced after 50 injections. The analyses were performed under the following chromatographic conditions: Column, CPWax 57 CB (WCOT Fused Silica), 25 m × 0.25 mm i.d., DF = 0.2 µm, from Varian (Palo Alto, CA). The temperature of the FID was 220ºC, and the injector temperature was 220°C. The oven temperature was programmed to 40°C (for 5 min), followed by an increase of 10ºC/min until 150ºC. After that, the oven temperature increase of 2ºC/min until 210ºC. The carrier gas was helium with a flow of 1.5 mL/min. The injection of blood and oral fluid samples were performed by means of a 10 µL Hamilton syringe (Model 701 RN) with a removable needle (needle gauge 22S), cleaned under vacuum between each injection with the Triton X-100 solution. The volume of injection was 0.5 µL, with a ratio of 1:180 min corresponding to a split flow of 120 mL/min for blood and oral fluid samples.

The representative chromatogram was reprocessed using the following retention times for each analyte, presented in Table 1.

Table 1. Retention times of ethanol, and IS analyzed by GC-FID.

Analytes	Retention time (minutes)
Ethanol	3.1
IS	5.2

1.5 Analysis of Δ^9-THC in blood and oral fluid samples

1.5.1 Preparation of stock solution

A 1mg/mL methanolic solution containing Δ^9-THC was prepared from the purchased solution of Δ^9-THC Sigma-Aldrich (St Louis, MO, USA).

1.5.2 Preparation of working solutions

Four working solutions of 10; 100; 1000; 10000 ng/mL were prepared from the stock solution (1mg/mL) in methanol.

1.5.3 Preparation of calibration standard solution

Calibration standard solutions of 0, 1, 5, 10, 25, 50, 100, 500, 1000 ng/mL were freshly prepared for each analysis from working solutions by adding the appropriate volume to drug-free blood.

1.5.4 Preparation of internal standard (IS)

A working solution of benzophenone was prepared in acetonitrile from the purchased solution of benzophenone Sigma-Aldrich (St Louis, MO, USA), at the concentration of 40ug/mL. This solution was used as internal standard (IS).

- All working and stock solutions were prepared fresh daily and stored at -20 ºC prior use.

1.5.5 Sample preparation for gas-chromatography mass spectrometry

1.5.5.1 <u>THC extraction from biological samples</u>

Different methods of LLE and SPE were tested to determine the optimal conditions of extraction. The method described is the one that resulted in higher recoveries. Hence, liquid-liquid extraction (LLE) was performed in falcon tubes of 15 mL. To each falcon 1 mL of blood sample and 50µL of I.S were added. All tubes were vortex mixed and added 1 mL of phosphate buffer (pH 4.1 M). The tubes were vortex mixed again and 5 mL of hexane/ethyl acetate (5/1) were added subsequently. The tubes were shaken on a rotary mixer for 10 min and centrifuged at 2500 rpm for 20 min. The organic layer was transferred to GC vials. A second aliquot of 5 mL hexane/ethyl acetate (5/1) was added to the remaining aqueous layer and the process was repeated to maximize recovery. The solvent extracts were totally evaporated under N_2 at 50 ºC.

1.5.5.2 Derivatization procedure

In this study, THC was derivatized by silylation, reacting with BSTFA and 1% TMCS. BSTFA is the silylation reagent that reacts with Δ^9-THC replacing active hydrogens by a –Si(CH3)^3 (trimethylsilyl) group. TMCS increases the reactivity of BSTFA. Δ^9-THC measurement was performed with the addition of 60 µL of BSTFA +1%TMC. The samples were vortex mixed and heated for 60 min at 70 ºC. After cooling to room temperature, the samples were injected into the GC-MS system.

1.5.6 Gas-chromatography mass spectrometry conditions

Quantitative GC-MS analysis was performed on a Varian CP-3800 gas chromatograph (USA) equipped with an ion-trap Varian GC-MS Saturn 4000 mass detector. Chromatographic separation was achieved using a capillary column VF-5ms (30 m × 0.25 mm i.d. × 0.25 µm) and a high-purity helium C-60 carrier gas. An initial temperature of 100 ºC was maintained for 1 min, increased to 300 at 15 ºC/min, and held for 1 min, giving a total run time of 24.33 min approximately. The flow of the carrier gas was maintained at 1.0 mL/min. The injector port was set at 250 ºC. Analyses were performed in full scan in splitless injection mode.

The obtained full scan chromatogram was reprocessed using the following selected qualifier ions and retention times for each analyte, presented in Table 2. The underlined ions were used for quantification.

Table 2. Retention times and m/z ions of Δ^9-THC and IS by GC-MS.

Analytes	Retention time (minutes)	Fragments (m/z)
Δ^9-THC	12.32	315; 371;386
IS	7.76	105; 182

The integration of the chromatographic peaks for quantitative analysis was performed by monitoring the fullscan chromatogram with specific selected m/z ions allowing more precise peak integration.

1.6 Method validation of Δ^9-THC

Selective and sensitive analytical methods for the quantitative evaluation of psychoactive substances and their metabolites are critical for a successful conduction of analytical studies. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix such as blood, plasma and oral fluid is reliable and reproducible for the intended use. The Food and Drug Administration guidance, that is actually accepted by the biopharmaceutical industries as the gold standard method validation approach, established the fundamental parameters for method validation that include: accuracy, precision, selectivity, sensitivity, reproducibility, and stability (Department of Health and Human Services, 2001).

In this work, the validation methods of Δ^9-THC for blood samples were based on the determination of selectivity, linearity, LOD, LLOQ, precision, accuracy, recovery and reproducibility. In order to obtain these validation data, calibration curves were prepared by spiking blank whole blood with appropriate concentrations of Δ^9-THC standard.

1.6.1 Selectivity

Six blank samples with no analytes or IS added were extracted by LLE as described previously and analyzed by GC-MS to detect possible chromatographic interferences with THC. Chromatographic selectivity was evaluated by the presence or absence of co-eluting peaks at the retention times of the analytes. Three independent experiments were performed.

1.6.2 Linearity

The method linearity was determined by evaluation of the regression curve (ratio of analyte peak area and IS peak area *versus* analyte concentration) and expressed by the determination coefficient (r2) using spiked samples. The calibration curve ($y = mx + b$) was obtained using nine different concentrations (0, 1, 5, 10, 25, 50, 100, 500, 1000 ng/mL). The mean slopes were obtained for calculating the concentration of real samples (unknown concentrations). These concentrations were prepared daily as mentioned before.

1.6.3 Limit of detection and lower limit of quantification

In this work, LOD and LLOQ were obtained based on the standard deviation of the response and the slope of the calibration curve. The LOD and LLOQ are expressed accordingly to the following equations respectively. The σ is the standard deviation of the response and *S* is the slope of the calibration curve.

$$LLOQ = \frac{10\sigma}{S} \qquad LOD = \frac{3.3\sigma}{S}$$

1.6.4 Precision

The precision of an analytical procedure is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (Department of Health and Human Services, 2001). It is expressed as the coefficient of variation (%CV). Intraday precision data was quantified by analyzing the areas of three replicates of three concentrations (low, 10; medium, 100; and high, 1000 ng/mL) and calculating the %CV. The areas of the same three concentrations, injected on three consecutive days, were used to calculate the interday repeatability (%CV). A %CV value of ≤15% for interday and intraday analysis was considered satisfactory.

1.6.5 Accuracy

The accuracy of an analytical method as the closeness of agreement between the conventional true value and the value found. It is expressed as a percentage. In this work, the accuracy of the THC method was determined by spiking blank matrix with three different THC concentrations (low, 10; medium, 100; and high, 1000 ng/mL) and through the calculation of the percentage deviation between the calculated value and the nominal value [accuracy (%) = (experimental concentration/theoretical concentration) × 100]. A deviation percentage of ≤15% was considered satisfactory.

1.6.6 Recovery

The recovery was determined by analyzing two sample groups of the same concentrations (10, 100 and 1000 ng/mL) in triplicate. In the first group, THC and the internal standard were added before the liquid-liquid extraction as following mentioned above. In the second group, THC and internal standard were added after the liquid-liquid extraction, before drying. The recovery was evaluated by the comparison of the mean response of the two groups. The response of the unextracted group represents 100% recovery. A deviation percentage of ≤20% was considered satisfactory.

1.6.7 Reproducibility

The reproducibility of an analytical method is determined by analyzing the same concentration of the sample five times. The objective of reproducibility is to verify that the same method will provide the same result. In this work, the reproducibility was determined by analyzing the same concentration (100ng/mL) five times.

1.7 Analysis of amphetamines

For detection of amphetamines in blood samples, it was used a preliminary test. This method consists in identifying the presence of the most known amphetamines as: MDMA, MDA and methamphetamines in blood samples, using a GC-MS program called NIST (national institute of standards and technology). This program has the majority of the standards of psychoactive substances. Knowing the time retention and the ions characteristics of each amphetamine, is possible to identify those in your sample chromatograms, compared with this data base.

1.7.1 Sample preparation for gas-chromatography mass spectrometry

To each eppendorf 1 mL of blood sample and 500µL of methanol were added. All tubes were vortex mixed and centrifuged at 13000 rpm for 20 min. The organic layer was transferred to new eppendorf and it was reposed for 30min. After the compounds that were in solution have precipitated, the sample was centrifuged again at 13.000 for 30 min. This procedure was repeated 3 times for all compounds presents in blood samples being totally removed. After that, the samples were injected into the GC-MS system.

1.8 Analysis of cocaine

For detection of cocaine in blood samples, it was used a commercial First Check$^{@}$ test. This is a screening test that allow detect cocaine presents in biological samples.

1.8.1 Sample preparation

To each eppendorf 150 mL of blood sample and 50µL of $HClO_4$ (20%) were added. All tubes were vortex mixed and centrifuged at 13000 rpm for 10 min. 100µL of the supernatant was transferred to new eppendorf and 33,3 µL K2CO3 (1.52 M) was added to neutralize the pH. The samples were vortex mixed and centrifuged at 13.000 for 1 min. 100µL of the supernatant was added to the screening test. A positive control sample with (10µg/mL) of cocaine standard was prepared in the same conditions. 100µL of this solution was also added to the screening test. If the blood samples contain cocaine metabolites, will appear one line: the line control.

Chapter III

Results

Surveys

1. Surveys

1.1 Socio-demographic characteristics

In this study participated 78 willingly young adults who were in nightclubs. Most of the contacted persons agreed with data collection, but were fearful to provide a blood sample. Those 78 engaged, 73,08% of participants were male and 26,92% were female (Figure 11), with the range of ages between 18 and 30 years, being the average ages 22,18 years (Table 3).

The majority of the participants were students 75,64%, (Figure 12). In the student population, 16,9% were in high school, 76,3% were graduation students, 3,4% were master students and 3,4% were doing PhD (Table 4). Figure 13 shows the courses frequented by these participants and figure 14 shows the distribution among types of studies.

Live permanently in Coimbra 42,31% of the inquired, while 30,8% live temporarily and 26,92% do not live in Coimbra (Figure 15). The major reason for the participants to live temporary in this city (91,66%), is due to studying in the university of Coimbra and 2 (8,34%) were due to professional reasons. In relation to personal habitation, 69,2% live with family, 16,7% live with friends, 6,4% live alone, 3,8% live with boyfriend/girlfriend and 3,8% live in a student's residential (Figure 16).

Concerning professions 67,9%, were student, following 7.7% were worker/students and 24,4% corresponded to other professions such as: DJ, worker, bartender, housekeeping, and musicians.

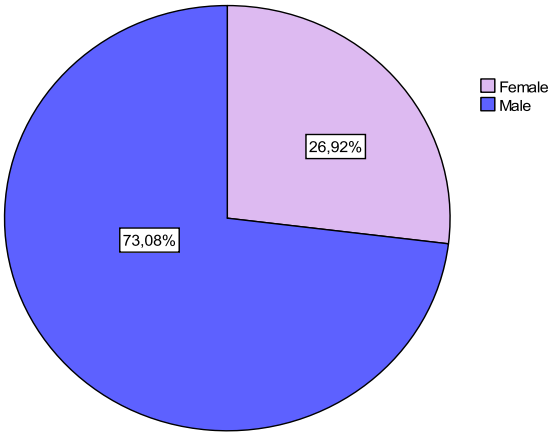

Figure 11. Gender

Table 3. Age

Age (years)									
N	Min	Max	1ºQ	2ºQ	3ºQ	Mean	SD	Median	Mode
78	18	30	20	21	24	22,18	3,66	21,00	20
N: number of answers; **Min:** minimum; **Max:** maximum; **1ºQ/2ºQ/3ºQ:** Quartiles; **SD:** standard deviation									

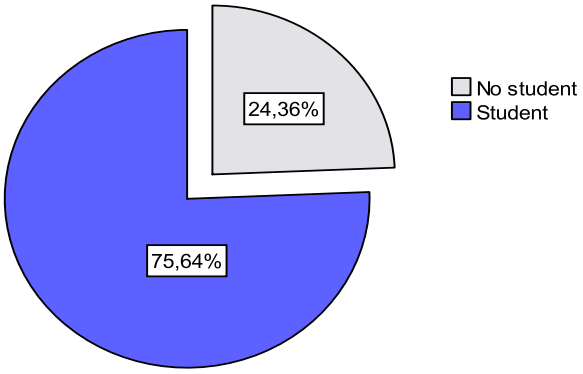

Figure 12. Student vs No student

Table 4. Typology of degree

Students	Academic Degree				Total
	High School	Graduation	Master	PhD	
Count	10	45	2	2	59
% of Total	16,9%	76,3%	3,4%	3,4%	100,0%

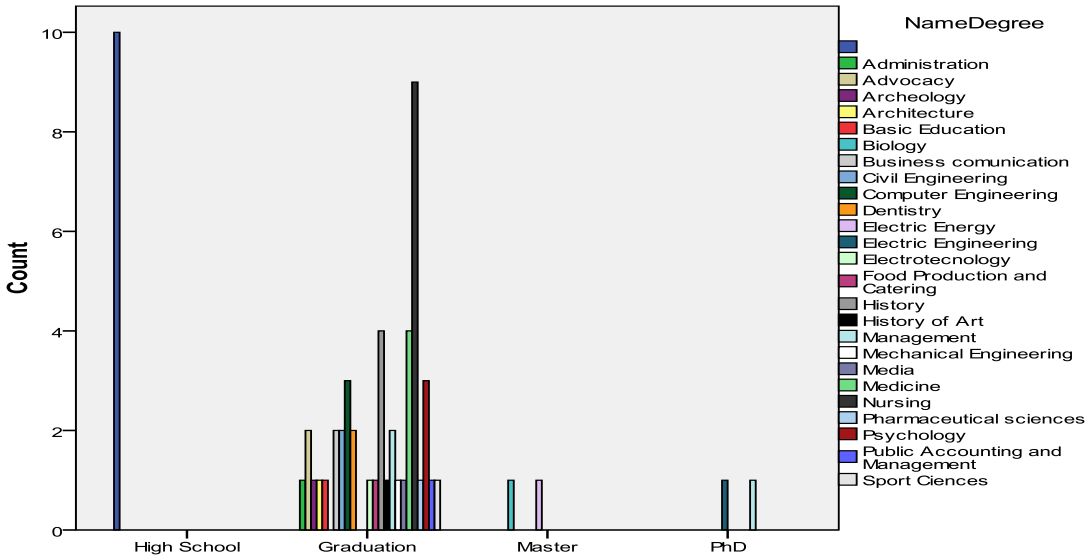

Figure 13. Courses

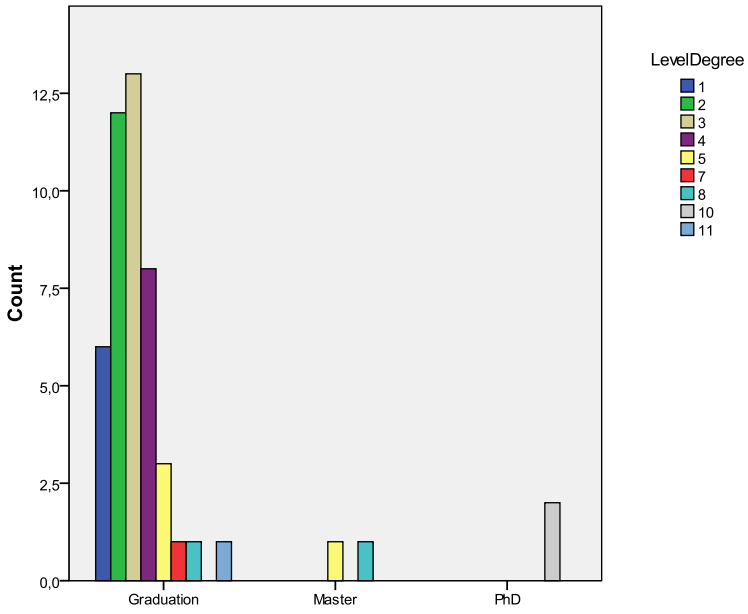

Figure 14. Distribution among types of studies

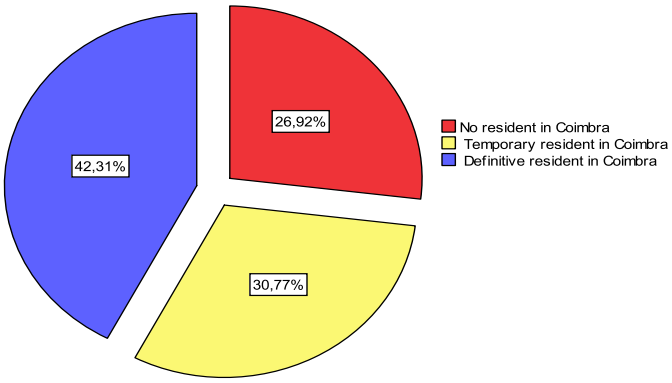

Figure 15. Residence of participants

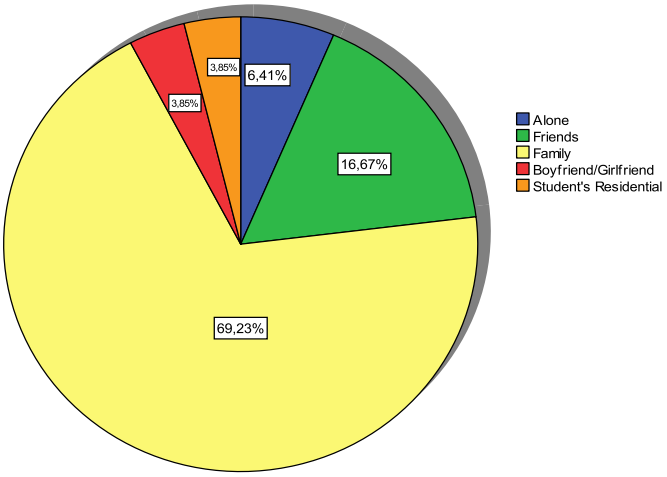

Figure 16. Personal habitation

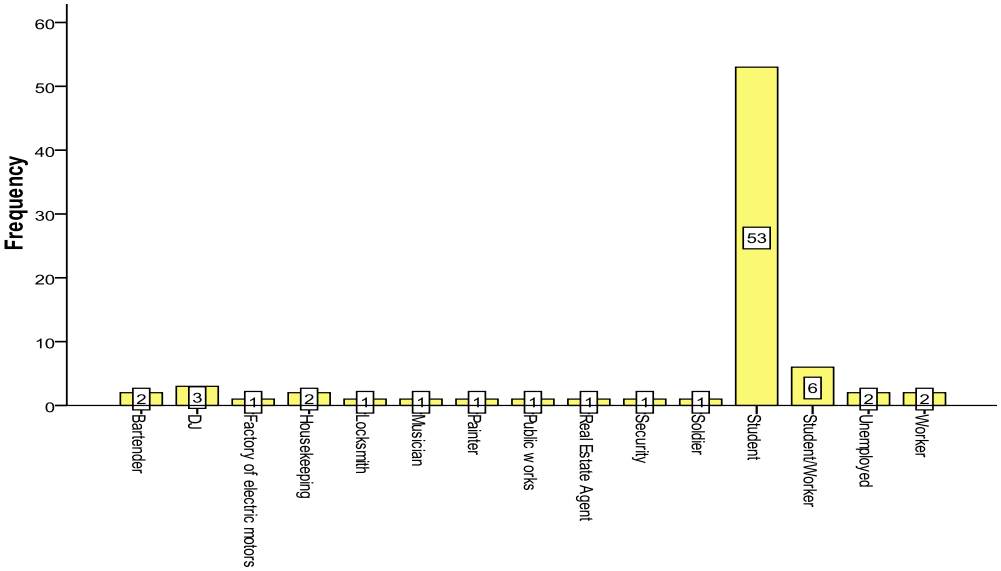

Figure 17. Professional status

1.2 Recreational nightlife habits

Data about recreational nightlife habits revealed an average of 2,09 nights out per week and 8,01 nights out per month. The inquiries visit in average 3,08 recreational spaces in each night out (Table 5).

In relation to the money that the participants spent per night, it varies among less than 5€ and more than 50€. However, the majority of participants spend 10€ per night (Figure 18).

Table 5. Recreational nightlife habits

Recreational nightlife habits							
	N	Min	Max	Mean	SD	Mode	Median
Nights out per week	78	0	7	2,09	1,531	1	2,00
Nights out per month	78	0	30	8,01	5,669	4	8,00
Recreational spaces	78	0	10	3,08	1,618	3	3,00

N: number of answers; **Min:** minimum; **Max:** maximum; **SD:** standard deviation

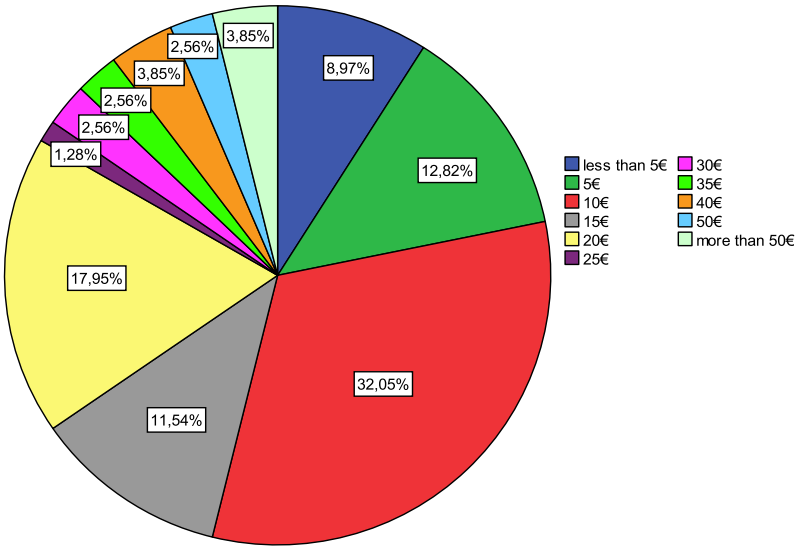

Figure 18. Money spent per night

1.3 Health information

The participants of this study weigh, in average, 69,71 kilograms and height, in average, 1,7372 meters (Table 6). The majority of participants (78,2%) do not have health problems. Among the participants that have health problems (21,8%), about half of them were not taking medication. All participants who were taking medication said that it was prescribed by his doctor (Table 7). The last food intake by participants was, in average, 4,85 per hours ago (Table 6).

Table 6. Health information

Health information							
	N	Min	Max	Mean	SD	Mode	Median
Weight	78	45	115	69,71	13,234	70	70,00
Height	78	1,52	1,93	1,7372	0,08842	1,8	1,7400
Last feed administrated (hours ago)	78	1	9	4,85	2,330	8	5,00

N: number of answers; **Min:** minimum; **Max:** maximum; **SD:** standard deviation

Table 7. Health problems and use of medication

Use of medication		Health Problems			Use Prescription Medication		
		No	Yes	Total	No	Yes	Total
No	Count	61	8	69	0	0	0
	% of Total	78,2%	10,3%	88,5%	0%	0%	0%
Yes	Count	0	9	9	0	9	9
	% of Total	0%	11,5%	11,5%	0%	100%	100%
Total	Count	61	17	78	0	9	9
	% of Total	78,2%	21,8%	100,0%	0%	100%	100%

1.4 Problems due to use of psychoactive substances

Regarding to health problems related to the use of drugs, 19 (6,8%) of the individuals affirmed that already had problems with the use of psychoactive substances. The majority of the participants, 8 (42,11%), were injured and 4 (21,05%) got sick. Only 2 people (10,53%) had a sexual regret, 1 (5,26%) had family or friends problems, 1 (5,26%) had a crisis anxiety or a mental disturbance, 1 (5,26%) had a road accident and 2 (10,53%) have already experienced all of these problems.

In respect to the influence of psychoactive substances in social behavior of the 78 participants, 12,82% answered that the use of drugs interfered to their friendship; 25,6% had already driven under the effect of psychoactive substances and 14,1% had being involved in conflicts with authority due to drug use (Table 8).

In relation to the major risk associated with going out at night, only 7,69% of the inquiries answered “no risk”, 17,95% mentioned the alcohol effects, 16,67% traffic accidents, 14,10% drug effects, 11, 54% alcohol and drug effects and physical violence, 7,69% sexual violence, 5,13% to get unconsciousness, 3,85% stealing or robbery, 2,56% the drug users and 1,28% the police (Figure 19).

Table 8. Problems due to use of psychoactives substances

		Frequency	Percent	Valid Percent	Cumulative Percent
Influence of psychoactive substances in friends relations	No	68	87,2	87,2	87,2
	Yes	10	12,8	12,8	12,8
	Total	78	100,0	100,0	
Drive under psychoactives substances	No	58	74,4	74,4	74,4
	Yes	20	25,6	25,6	25,6
	Total	78	100,0	100,0	
Problems with authority due to use of psychoactive substances	No	67	85,9	85,9	85,9
	Yes	11	14,1	14,1	14,1
	Total	78	100,0	100,0	

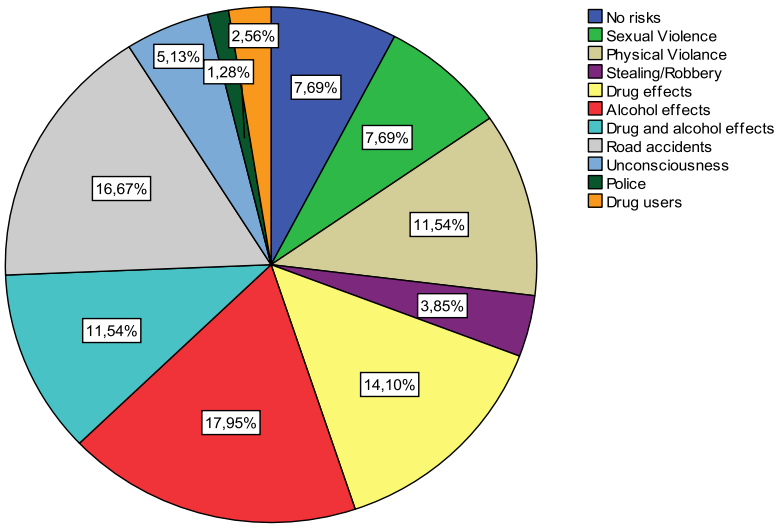

Figure 19. Perception of risk

1.5 Drug abuse patterns

The psychoactive substance more used by young people in recreational settings was alcohol (87,2%), followed by tobacco (64,1%). Regarding to illegal psychoactive substances, cannabis was the most used drug (19,2%), followed by cocaine and amphetamines (2,6%), LSD and smartshop substances (1,3%). In this study, it was not detected the use of other illicit drugs such as: ecstasy, psilocybin, mescaline, heroin, GHB, or flunitrazepam. On the other hand, cathinones or synthetic cannabinoids may fall in the 1,3% use of smartshop substances (Table 9).

The 87,2% of participants who used alcohol, in average, consumed 5,40 fermented drinks and 3,16 distilled drinks (Table 9). All of the 64,1% of participants that used tobacco, consumed smoked tobacco. These young people smoke in average 14,84 cigars when go out at night. The brand of cigarettes more common was Marlboro with 20,51%, following Camel with 12,8%, and 10, 28% used other brands of cigars (Figure 20).

Among the 19,2% individuals who used cannabis, 6,41% do not smoke one complete cannabis cigarette, but only smoke, in average, 4,2 raisins. The rest of them, smoked in average 2,73 cigarettes (Figure 21). The 2,6% of people who used cocaine, in average sniffed 2 lines, and 1,3% shared material. All users of cocaine sniffed this psychoactive substance. About the 2,6% users of amphetamines, 1,3% ingested in average 1 pill, and 1,3% smoked in average 2 raisins. The only one user of LSD (1,3%) used one stamp in that week. The only one user of smartshop substances (1,3%) ingested one pill named creatine (Table 9).

85,9% of the participants do not use substances for relaxing in the end of the night, although 6,41% use cannabis, 5,13% use medicines (2,56% valium, 1,28% guronsan 1,28% alprazolam),1,28% use cocaine and 1,28% use tobacco for sleeping well (Figure 22).

Table 9. Psychoactive substances used by participants during that week

Use of Psychoactive Substances								
		Frequency (Total)	Percent (Total)	Route Administ.	Min	Max	Mean	SD
Tobacco	Cigarettes	50	64,1%	Smoked	2	70	14,84	15,403
Alcohol	Fermented			Ingested	0	20	5,40	5,303
	Distilled	68	87,2%	Ingested	0	13	3,16	3,732
Cocaine	Lines	2	2,6%	Sniffed	1	3	2,00	1,414
Amphetamines	Pills			Ingested	1	1	1,00	0
	Raisins	2	2,6%	Smoked	2	2	2,00	0
Ecstasy		0	0%		0	0	0	0
Cannabis	Cigarettes	15	19,2%	Smoked	0	10	2,73	3,348
	Raisins			Smoked	2	8	4,20	2,280
LSD	Stamps	1	1,3%	Ingested	1	1	__	__
Ketamine		0	0%		0	0	0	0
Psilocybin		0	0%		0	0	0	0
Mescaline		0	0%		0	0	0	0
Heroin		0	0%		0	0	0	0
GHB		0	0%		0	0	0	0
Flunitrazepam		0	0%		0	0	0	0
Cathinones		0	0%		0	0	0	0
Synthetic Cannabinoids		0	0%		0	0	0	0
Smartshops Substances	Pills	1	1,3%	Ingested	1	1	__	__

Administ: Administration **Min:** minimum; **Max:** maximum; **SD:** standard deviation

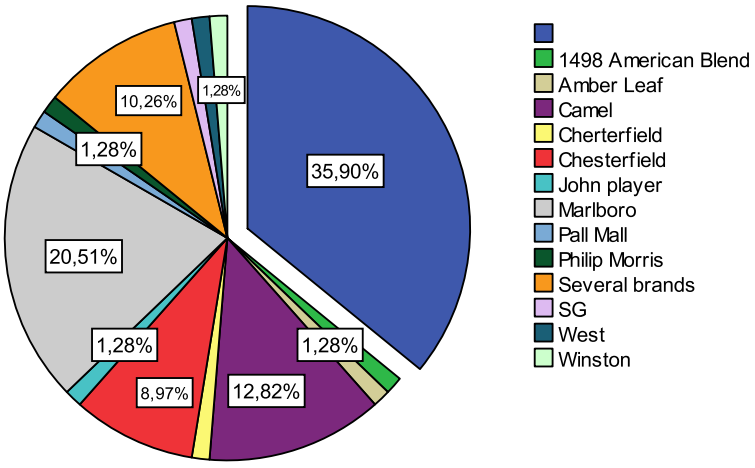

Figure 20. Brands of cigarettes used by participants

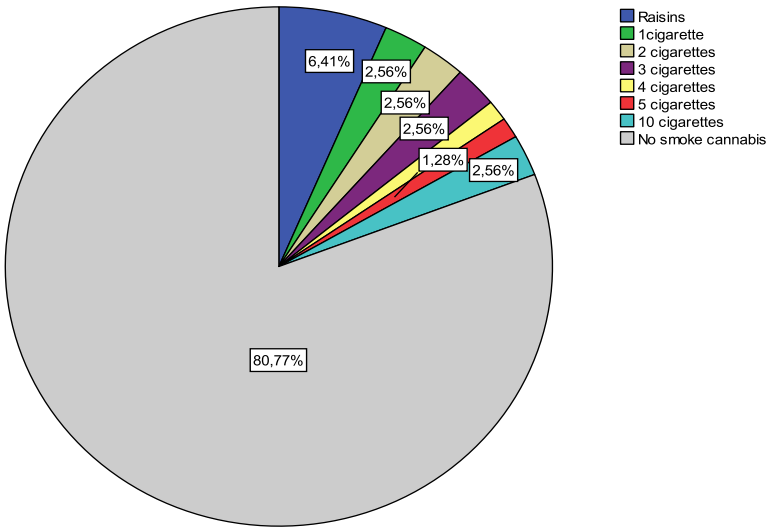

Figure 21. Number of cannabis cigarettes

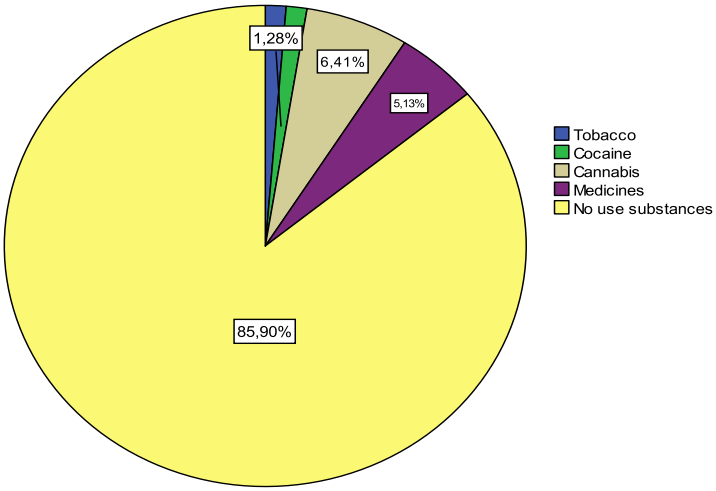

Figure 22. Use of psychoactive substances for sleeping well

1.6 Drug abuse patterns and socio-demographic characteristics

With the objective of analyzing the influence of gender on drug abuse patterns, it was used the non parametric test of Pearson Chi-Square. For the psychoactive substance tobacco, 28(35,9%) are no smokers, being 8 (38,1%) females and 20(35,1%) males. On other hand, 50(64,1%) of the total participants are smokers, being 13(61,9%) females and 37 (64,9%) males. Using the non parametric test of Pearson Chi-Square p=0,806, there are no statistically significant differences between gender and tobacco smoking (Table 10). For alcohol, 10 participants (10,8%) do not consume alcohol, being 3 (14,3%) females and 7(12,3%) males. On the other hand, 68(87,2%) of the total participants

consume alcohol, being 18(87,5%) females and 50 (87,7%) males. Using the non parametric test of Pearson Chi-Square p=0,814 there are no statistically significant differences between gender and consumption of alcohol (Table 11). Concerning cannabis, 15(19,2%) of the total participants used cannabis, being 1(4,8%) females and 14 (24,6%) males. Although it is not possible to determine statistically the influence of gender in cannabis use, it is possible to observe that there is an association between male gender and cannabis use (Table 12). When analyzed the number of tobacco cigarettes, we can observe that males (average 17,22) consume more cigarettes than females (average 8,08). The same occurs with alcohol, the mean of fermented and distilled drinks being higher in males (average 6,43 and 3,76 respectively) than in females (average 2,61 and 1,56 respectively) (Table 13).

Table 10. Gender vs Tobacco

Non parametric test of Pearson Chi-Square p=0,806			Gender		
			Female	Male	Total
Tobacco	No	Count	8	20	28
		% within Gender	38,1%	35,1%	35,9%
	Yes	Count	13	37	50
		% within Gender	61,9%	64,9%	64,1%
Total		Count	21	57	78
		% within Gender	100,0%	100,0%	100,0%

Table 11. Gender vs Alcohol

Non parametric test of Pearson Chi-Square p=0,814			Gender		
			Female	Male	Total
Alcohol	No	Count	3	7	10
		% within Gender	14,3%	12,3%	12,8%
	Yes	Count	18	50	68
		% within Gender	85,7%	87,7%	87,2%
Total		Count	21	57	78
		% within Gender	100,0%	100,0%	100,0%

Table 12. Gender vs Cannabis

			Gender		
			Female	Male	Total
Cannabis	No	Count	20	43	63
		% within Gender	95,2%	75,4%	80,8%
	Yes	Count	1	14	15
		% within Gender	4,8%	24,6%	19,2%
Total		Count	21	57	78
		% within Gender	100,0%	100,0%	100,0%

Table 13. Gender vs number of cigarettes or drinks

	Gender	N	Mean	Std. Deviation	Std. Error Mean
NºCigarettes	Female	13	8,08	4,536	1,258
	Male	37	17,22	17,139	2,818
NºFermented	Female	18	2,61	2,933	,691
	Male	49	6,43	5,624	,803
NºDestilled	Female	18	1,56	2,148	,506
	Male	49	3,76	4,024	,575

Comparing the drug abuse patterns with the professional situation of the participants, we can conclude that, for tobacco and alcohol, the results obtained using the test of Pearson Chi-Square did not provide statistically significant differences between the drug and the professional situation (p=0,478). However, when consumption of cannabis and professional situation were compared, we observed that the majority, 9 (60%), were students and the others are musician 1(10%), DJ 1(10%) and other professions. Applying the same probabilistic test, the results are statistically significant with p=0,029. For cocaine due to the small size of the samples (2 individuals) is not possible to determine the statistic probability (Table 14).

Table 14. Professional Situation vs use of psychoactive substances

			Tobacco		Alcohol		Cocaine		Cannabis		
			No	Yes	No	Yes	No	Yes	no	yes	Total
Professional Situation	Bartender	Count	0	2	0	2	2	0	2	0	2
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,0	,0
	DJ	Count	0	3	1	2	3	0	2	1	3
		% within Tobacco	,0	,1	,1	,0	,0	,0	,0	,1	,0
	Factory of electric motors	Count	1	0	0	1	1	0	1	0	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,0	,0
	Housekeeping	Count	2	0	0	2	2	0	2	0	2
		% within Tobacco	,1	,0	,0	,0	,0	,0	,0	,0	,0
	Locksmith	Count	0	1	0	1	1	0	0	1	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,1	,0
	Musician	Count	1	0	0	1	0	1	0	1	1
		% within Tobacco	,0	,0	,0	,0	,0	,5	,0	,1	,0
	Painter	Count	0	1	0	1	1	0	1	0	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,0	,0
	Public works	Count	0	1	0	1	1	0	1	0	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,0	,0
	Real Estate Agent	Count	0	1	0	1	1	0	0	1	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,1	,0
	Security	Count	1	0	0	1	1	0	1	0	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,0	,0
	Soldier	Count	0	1	0	1	1	0	1	0	1
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,0	,0
	Student	Count	21	32	5	48	52	1	44	9	53
		% within Tobacco	,8	,6	,5	,7	,7	,5	,7	,6	,7
	Student/Worker	Count	2	4	3	3	6	0	6	0	6
		% within Tobacco	,1	,1	,3	,0	,1	,0	,1	,0	,1
	Unemployed	Count	0	2	0	2	2	0	0	2	2
		% within Tobacco	,0	,0	,0	,0	,0	,0	,0	,1	,0
	Worker	Count	0	2	1	1	2	0	2	0	2
		% within Tobacco	,0	,0	,1	,0	,0	,0	,0	,0	,0

Analyzing the household where the participants live with the drug abuse patterns, in relation to tobacco there are no statistically significant differences (p=0,358). However, when we analyse alcohol consumption, 48 (70%) who drink alcohol live with family and 11(20%) live with friends, being the P value 0,73. Therefore, there is not a positive

correlation between the household and use of alcohol. Among the users of cocaine, 1(10%) lives alone and 1(10%) lives with family. Among the users of cannabis, the majority live with family 9(60%) and 4(30%) live with friends, do not having any association between household and use of cannabis (P value = 0,385) (Table 15).

Table 15. Household vs use of psychoactive substances

Household		Tobacco		Alcohol		Cocaine		Cannabis		
		No	Yes	No	Yes	No	Yes	no	yes	Total
Alone	Count	3	2	1	4	4	1	3	2	5
	% within Tobacco	,1	,0	,1	,1	,1	,5	,0	,1	,1
Friends	Count	5	8	2	11	13	0	9	4	13
	% within Tobacco	,2	,2	,2	,2	,2	,0	,1	,3	,2
Family	Count	18	36	6	48	53	1	45	9	54
	% within Tobacco	,6	,7	,6	,7	,7	,5	,7	,6	,7
Boyfriend/ Girlfriend	Count	0	3	1	2	3	0	3	0	3
	% within Tobacco	,0	,1	,1	,0	,0	,0	,0	,0	,0
Student's Residential	Count	2	1	0	3	3	0	3	0	3
	% within Tobacco	,1	,0	,0	,0	,0	,0	,0	,0	,0
Total	Count	28	50	10	68	76	2	63	15	78
	% within Tobacco	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0

1.7 Drug abuse patterns and recreational nightlife habits

The recreational habits among young people are closely related with use of psychoactive substances. Analyzing the graph about frequency of nights out per week and polyusers (Figure 23), we can observe that participants who go out until 4 times in the week, use in general 2 types of psychoactive substances, being that females are entirely in these group. The participants (all males) who frequent recreational spaces more than 4 times a week, in their majority use three types of psychoactive substances, being at least one illegal. The users of four or more types of psychoactive substances, being at least 2 illegal, go out until 3 times per week.

In relation to the gender, females who use only one type of psychoactive substance go out until 2 times at week, while females who use 2 types of psychoactive substances go out more times per week (between1 and 4 times). Males go out more times per week than females, even those who do not use or only use one type of psychoactive substance. (Figure 23).

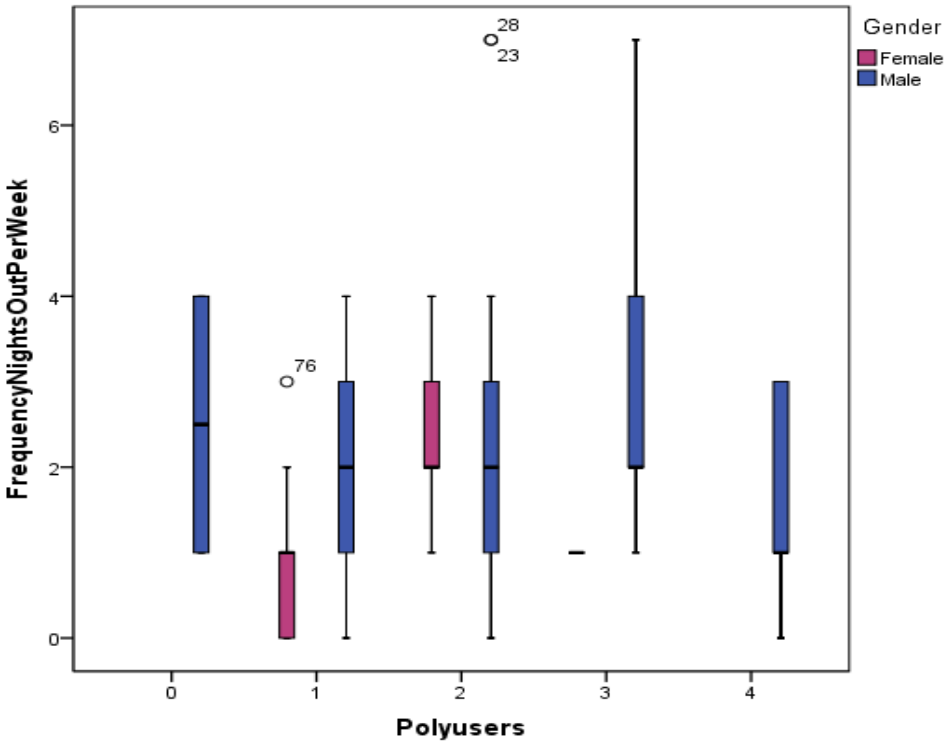

Figure 23. Recreational nightlife habits and polyconsumption

1.8 Drug abuse patterns and health information

Using the non-parametric test of Pearson Chi-Square, there are statistically significant differences between use of medications and polyusers (p=0,002). Among the participants who use medications prescribed by their doctor, only two of them do not use more than one type of psychoactive substances. The majority of them, uses 2 types of psychoactive substances, even when they have taken medications. One individual who had taken medications use 3 types of psychoactive substances, being at least one of them illegal (Table 16).

Table 16. Use of medication and use of psychoactive substances

Use of Medication	Polyusers					Total
	0	1	2	3	4	
No	0	24	30	10	5	69
Yes	2	2	4	1	0	9
Total	2	26	34	11	5	78

1.9 Drug abuse patterns and problems due to use of psychoactive substances

Analysis of the relation between health problems (as injured, get sick, have a sexual regret, anxiety crisis, friends/family problems and traffic accident) and use of substances indicates, in a general, that there is an upper risk for health problems among the users of psychoactive substances. In relation to tobacco, the users have a light increased risk of health problems, with an odds ratio of 1,288. In relation to alcohol, the participants who use alcohol have clearly more problems than those do not drink. In relation to cocaine, the odds ratio is 3,22, which means that the users of cocaine have three times more health risk than no users. The cannabis users have 2,56 times more health risk than no users (Table 17).

Table 17. Health problems due to use of psychoactive substances vs drug abuse patterns

Problems due to use of psychoactive substances		Tobacco		Alcohol		Cocaine		Cannabis		
		No	Yes	No	Yes	No	Yes	no	yes	Total
No	Count	22	37	7	52	58	1	50	9	59
	% within Tobacco	,8	,7	,7	,8	,8	,5	,8	,6	,8
Yes	Count	6	13	3	16	18	1	13	6	19
	% within Tobacco	,2	,3	,3	,2	,2	,5	,2	,4	,2
Total	Count	28	50	10	68	76	2	63	15	78
	% within Tobacco	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0

In relation to other problems, the users of alcohol have a light risk of problems in their personal relations with a odd ratio of 1,373, while cocaine users have an higher risk, with an odds ratio of 7,44. The users of cannabis have a double risk of problems in their personal relations than no users (odds ratio 2,00).

There is a high relation between the use of tobacco and driving problems with a contingency coefficient of 0,923. The risk of driving problems associated with the consumption of alcohol is of 1,5 (odds ratio). The users of cocaine have an increased risk of driving problems 3.00 times more than no cocaine users (odd ratio 3,00). The users of cannabis have 1,6 more risk of driving problems than no users. In relation to problems with authority, the cocaine users have the highest risk concerning this issue with an odds ratio of 6,6, following by cannabis and alcohol consumers with 1,7 and 1,5 odds ratio, respectively (Figure 18).

Table 18. Personal, driving issues, and problems with authorities due to use of psychoactive substances.

Influence of psychoactive substances in:			Tobacco		Alcohol		Cocaine		Cannabis		
			No	Yes	No	Yes	No	Yes	no	Yes	Total
Friend Relation	No	Count	24	44	9	59	67	1	56	12	68
		% within Tobacco	,9	,9	,9	,9	,9	,5	,9	,8	,9
	Yes	Count	4	6	1	9	9	1	7	3	10
		% within Tobacco	,1	,1	,1	,1	,1	,5	,1	,2	,1
Driver	No	Count	21	37	8	50	57	1	48	10	58
		% within Tobacco	,8	,7	,8	,7	,8	,5	,8	,7	,7
	Yes	Count	7	13	2	18	19	1	15	5	20
		% within Tobacco	,3	,3	,2	,3	,3	,5	,2	,3	,3
Authority problems	No	Count	24	43	9	58	66	1	55	12	67
		% within Tobacco	,9	,9	,9	,9	,9	,5	,9	,8	,9
	Yes	Count	4	7	1	10	10	1	8	3	11
		% within Tobacco	,1	,1	,1	,1	,1	,5	,1	,2	,1
Total		Count	28	50	10	68	76	2	63	15	78
		% within Tobacco	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0

Chapter IV:
Implementation of methodologies for the identification and quantification of psychoactive substances in biological samples

1. Implementation of methodologies for the identification and quantification of psychoactive substances in biological samples

1.1 Analysis of ethanol

1.1.1 Pre-treatment of samples

Ethanol is a volatile compound whose detection and quantification in biological matrices can be made with a direct injection, using a capillary column, in the gas chromatography with flame ionization detection (GC–FID). In this work, the analysis of ethanol in blood samples, and in oral fluid samples, were realized according to method previously published method (Pontes et al., 2009). This validated method was chosen because is a rapid and efficient method that requires only the addiction of triton-x100 solution (containing acetonitrile in its composition) for the pre-treatment of the samples. This solution was used to dilute the blood and oral fluid matrices.

1.1.2 Detection by gas-chromatography flame ionization detector

Gas-chromatography flame ionization detector has a high sensitivity, uniform response to hydrocarbons, and a broad linear range that have made the flame ionization detector (FID) one of the most widely used detector in gas chromatography. The FID response of hydrocarbons is proportional to the mass of carbon present in the sample (Jorgensen et al., 1990).

In this work, interferences were ruled out by verifying the absence of peaks in the retention time of ethanol. The retention time for ethanol and IS (1- propanol) were 3.06 min and 5.08 respectively. The acetonitrile, integrant part of triton-x 100 solution has as retention time of 3.71 min (Figure 24).

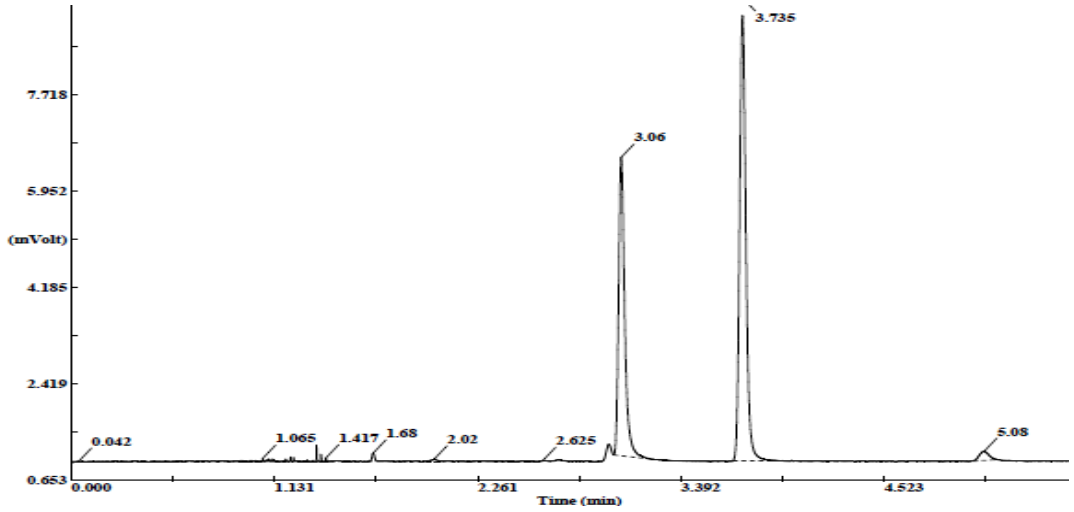

Figure 24. Representative chromatograms obtained from blood sample. The retention time for ethanol, IS (1- propanol) and acetonotrile were 3.06, 5.08 and 3.71 min respectively.

1.1.3 Selectivity of the method

The GC-FID chromatograms of spiked samples were compared with the chromatograms obtained with a blank blood/oral fluid sample. No interference peaks were detected in the retention times of ethanol neither in blood sample (Figure 25) nor in oral fluid sample (Figure 26).

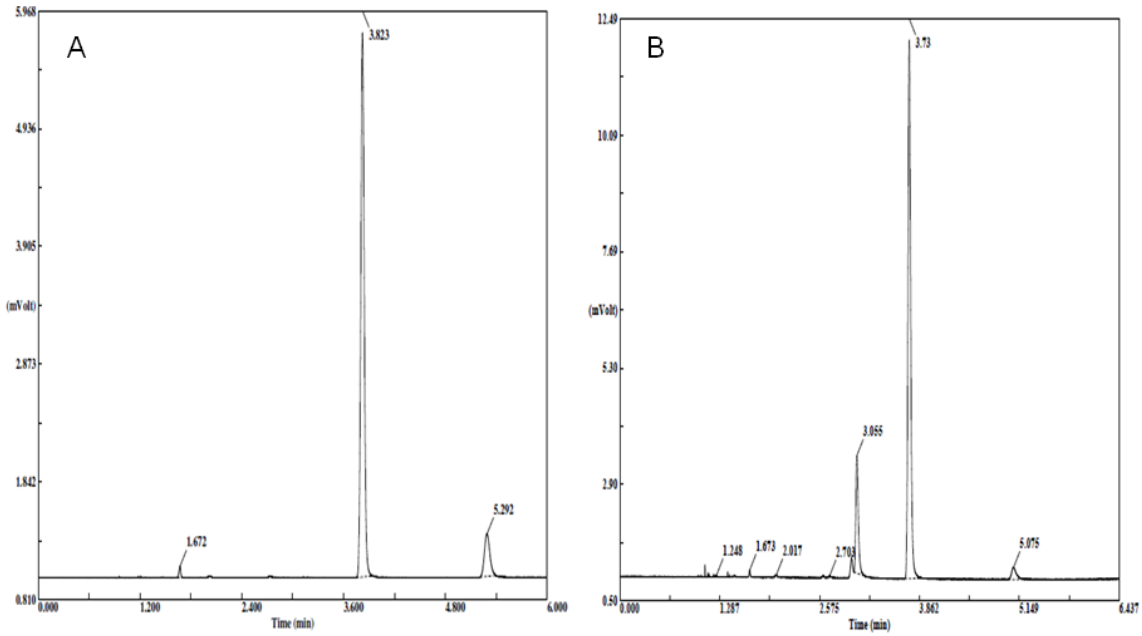

Figure 25. Representative chromatograms obtained from blood sample. The figure A represents a blank blood sample without ethanol. The figure B represents a blood sample containing ethanol.

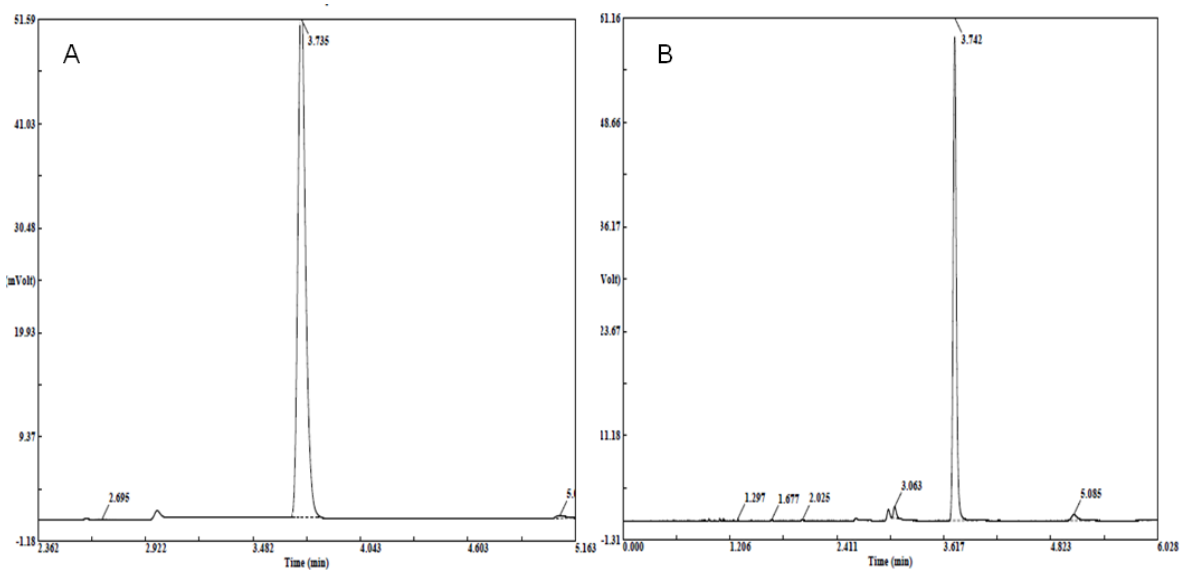

Figure 26. Representative chromatograms obtained from oral fluid sample. The figure A represents a blank oral fluid sample without ethanol. The figure B represents an oral fluid sample containing ethanol.

1.1.4 Calibration curve

In this work, the calibration curve of ethanol for blood samples was evaluated in the range of 0 g/L to 2.4 g/L. This calibration curve was obtained with six concentrations (0; 0.15; 0.30; 0.60; 1.20; 2.40 g/L). For oral fluid samples, the calibration curve was evaluated in the range of 0 mg/L to 0.60 g/L, and the calibration curve was obtained with five concentrations (0; 0.0375; 0.075; 0.30; 0.60 g/L). All blood/oral fluid samples were analyzed according to the procedure previously described (Chapter I) respectively. The weighted least squares regression equations and coefficients of correlation were calculated from these curves. The GC-FID chromatogram peak area ratios of ethanol/IS were determined to establish calibration equation (Figure 27).

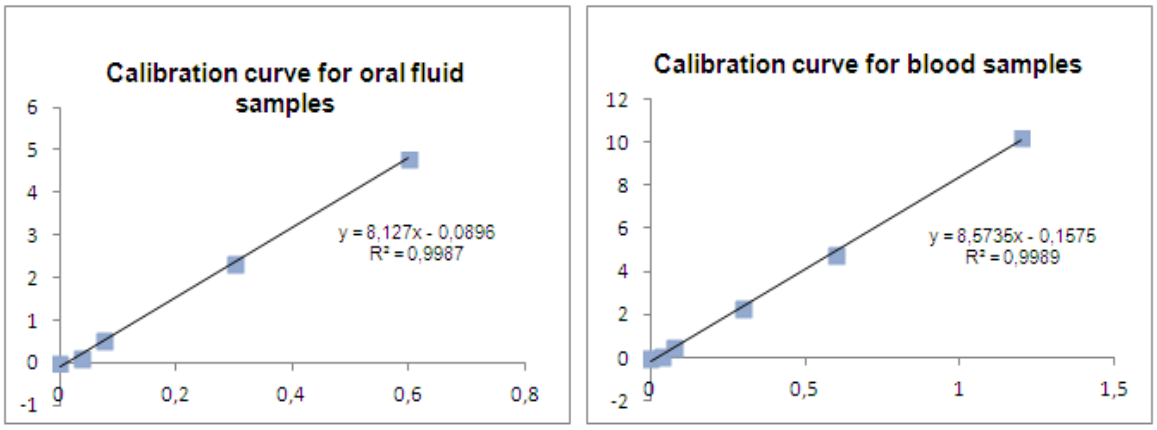

Figure 27. Calibration curves for blood and oral fluid samples. Plotted peak areas of the ethanol/IS peak areas versus concentrations (0; 0.15; 0.30; 0.60; 1.20; 2.40 g/L) for blood samples and (0; 0.0375; 0.075; 0.30; 0.60 g/L) for oral fluid samples

The method was linear at the concentration range established, with determination coefficients (r2) greater than 0.99 for the calibration curves of THC for blood and oral fluid samples (Table 19).

Table 19. Blood and oral fluid linear regressions analysis of ethanol standard solutions.

Type of samples	Y=mx+b	R^2	Concentration range (g/L)
Blood	Y=8.5735x +0.1575	0.9989	0-2.4
Oral Fluid	Y=8.127x +0.0896	0.9987	0-0.6

2.1 Analysis of Δ^9-THC

2.1.1 Sample preparation for gas-chromatography mass spectrometry

2.1.1.1 Extraction of samples

In toxicological analysis, plasma or blood are the ideal samples for the detection and quantification xenobiotics. The concentrations of many xenobiotics, and their metabolites, tend to be higher in blood, thereby facilitating detection (Dinis-Oliveira et al., 2010). However, blood is a complex biological matrix containing many interferents, such as proteins, hormones and blood cells. The separation and elimination of this interferents is an important step for obtain good results. Actually the analyses of cannabinoids in biological samples are continually being developed in forensic toxicology. The mainly methods that are described for the extraction of cannabinoids from blood include solid phase extraction (SPE) (REF) and liquid–liquid extraction (LLE) (Andrews and Paterson, 2012). In this case the liquid-liquid extraction was quick, efficient and more favorable in Δ^9-THC analysis over SPE due to the nature of the sample matrix.

Using SPE method to extract Δ^9-THC in blood samples, it was obtained a bad peak resolution and the compounds were not well separated (Figure 28(1)). However, in LLE the best results were obtained in THC analysis. (Figure 28(2)). The chosen LLE method for this study proved to be simple and rapid in the preparation of samples prior to analysis by GC-MS.

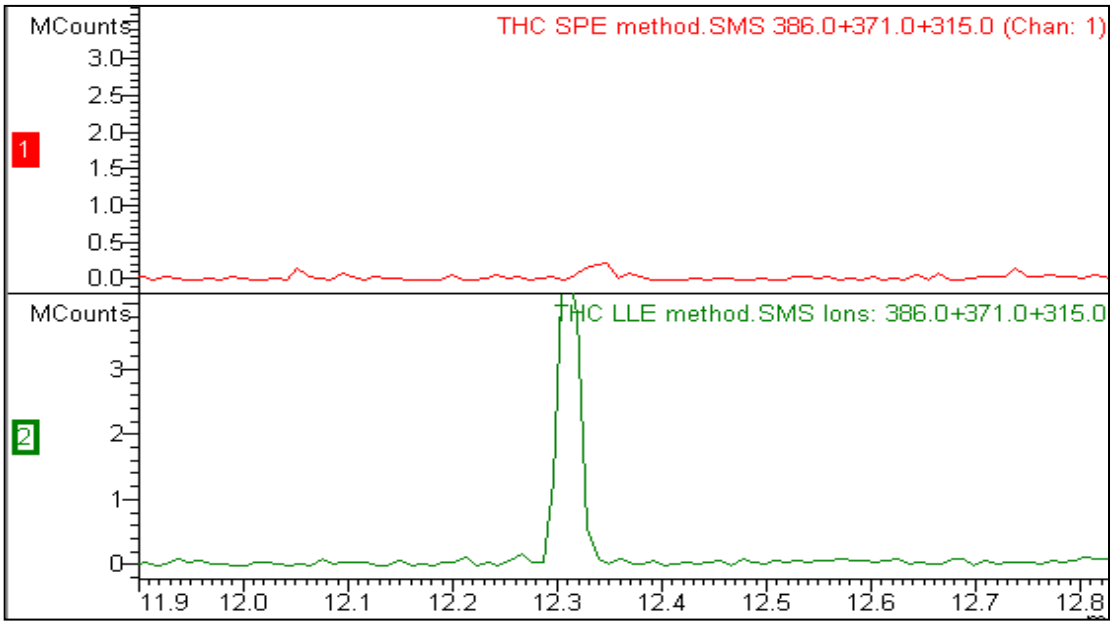

Figure 28. Different methods ofΔ^9-THC extraction in blood samples. 1. Chromatogram of SPE method showing a bad peak resolution. 2. Chromatogram of LLE method showing a good peak resolution and good separation of the compounds.

2.1.1.2 Derivatization

Δ^9-THC has a tri-cyclic 21- carbon structure without nitrogen and with two chiral centers in transconfiguration. It is a volatile viscous oil with high lipid solubility and low aqueous solubility and a pKa of 10.6 (Sharma et al., 2012). Due to the presence of only one hydroxyl group and its long chemical structure, it was necessary to test different compounds, times and temperatures of derivatization for obtaining the best peak resolution.

In this study, Δ^9-THC was derivatized by silylation, reacting with BSTFA and TMCS. BSTFA is the silylation reagent that reacts with Δ^9-THC and replaces active hydrogens with a $-Si(CH3)^3$ (trimethylsilyl) group (Figure 29). TMCS increases the reactivity of BSTFA.

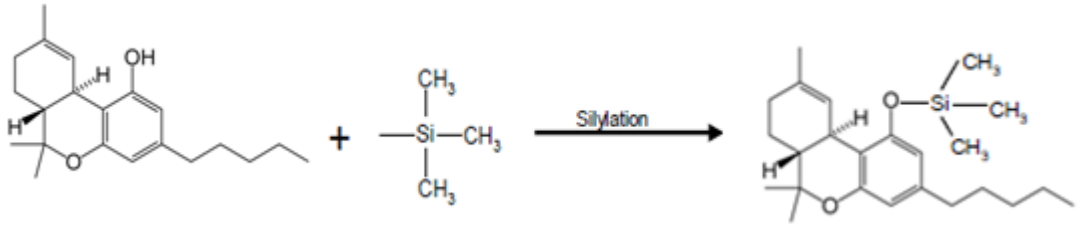

Figure 29. Derivatization reaction of THC

The derivatization process using BSTFA +1%TMC for 1h at 70 ºC, allowed an increase in Δ^9-THC volatility, improving the thermal stability and consequently the detectability of the derivative (Figure 30)

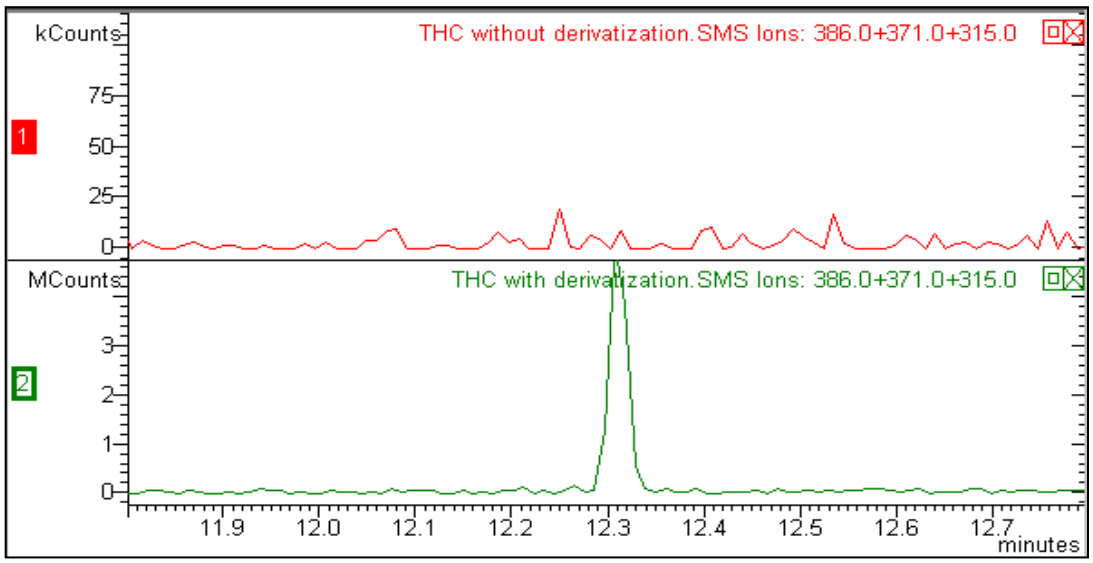

Figure 30. Δ^9-THC with and without the derivatization step. 1. Chromatogram of Δ^9-THC without derivatization step showing a bad peak resolution. 2. Chromatogram of Δ^9-THC with derivatization step showing a good peak resolution.

2.2 Method Validation of Δ^9-THC

2.2.1 Detection by gas-chromatography mass spectrometry

Preliminary tests were performed to determine the best conditions of chromatographic separation and detection in order to obtain the best peak resolution and separation of Δ^9-THC. In the chromatogram, it is possible to identify the peak of Δ^9-THC.

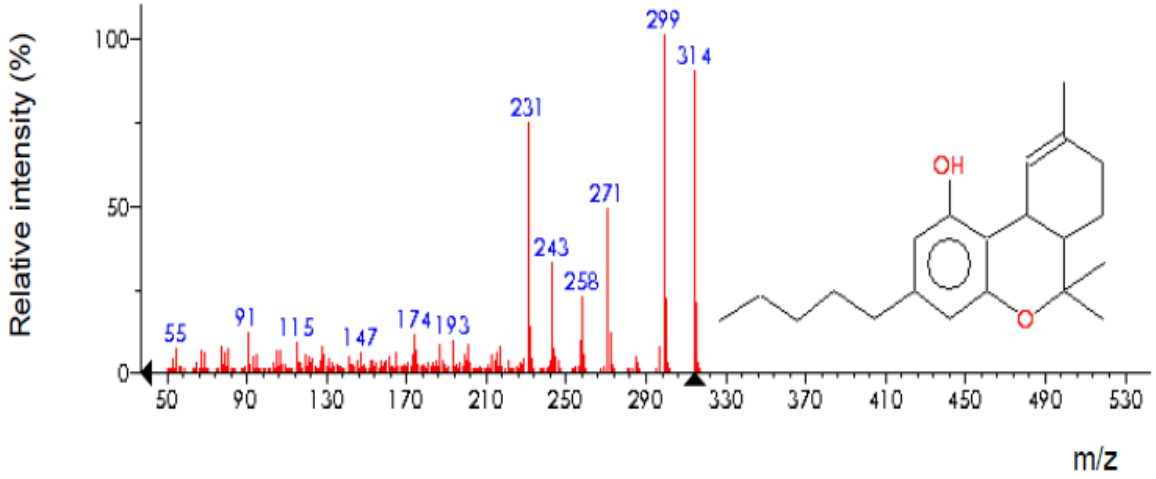

Figure 31. Mass spectrum of Δ^9-THC without derivatization.

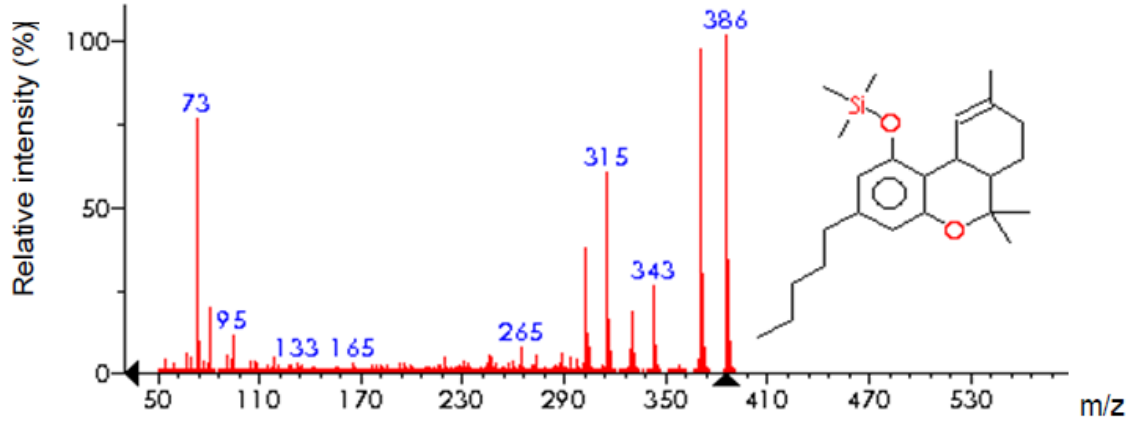

Figure 32. Mass spectrum of Δ^9-THC derivatized.

Based on mass spectrum of each peak, for Δ^9-THC the first peak represents Δ^9-THC without derivatization (Figure 31) the second peak shows Δ^9-THC derivatized (Figure 32). For Δ^9-THC, three ions were used. The most abundant ion m/z 386 was used for quantification and the other ions m/z 371 and 315 were used for the proper identification.

2.2.2 Selectivity

The GC-MS chromatograms of spiked samples were compared with the chromatograms obtained with a blank blood sample. No interference peaks were detected in the retention times of Δ^9-THC (Figure 33) or in the IS and selected ions (Figure 34).

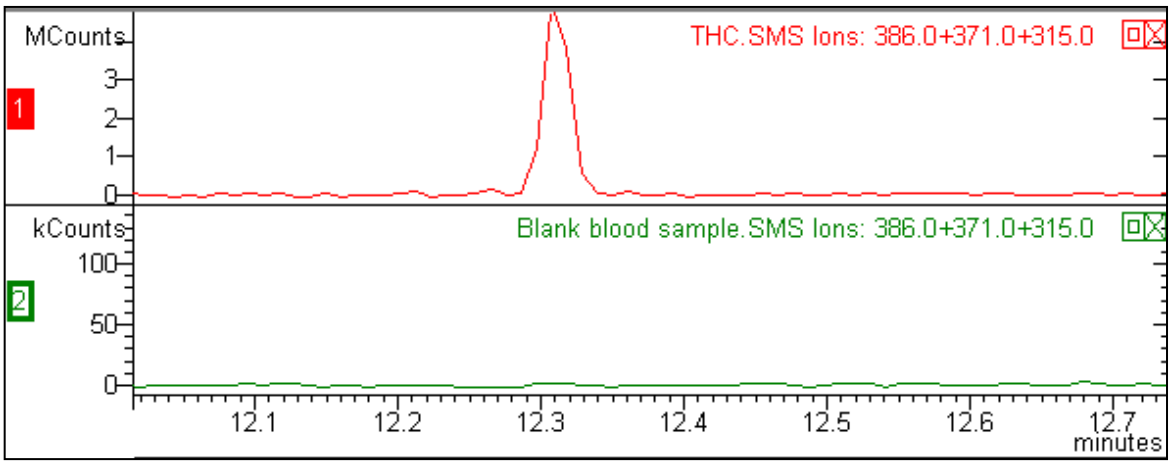

Figure 33. Reconstructed GC-MS (SIM mode) chromatogram of a blank blood sample (1000 ng/mL) and THC (m/z 386+371+315).

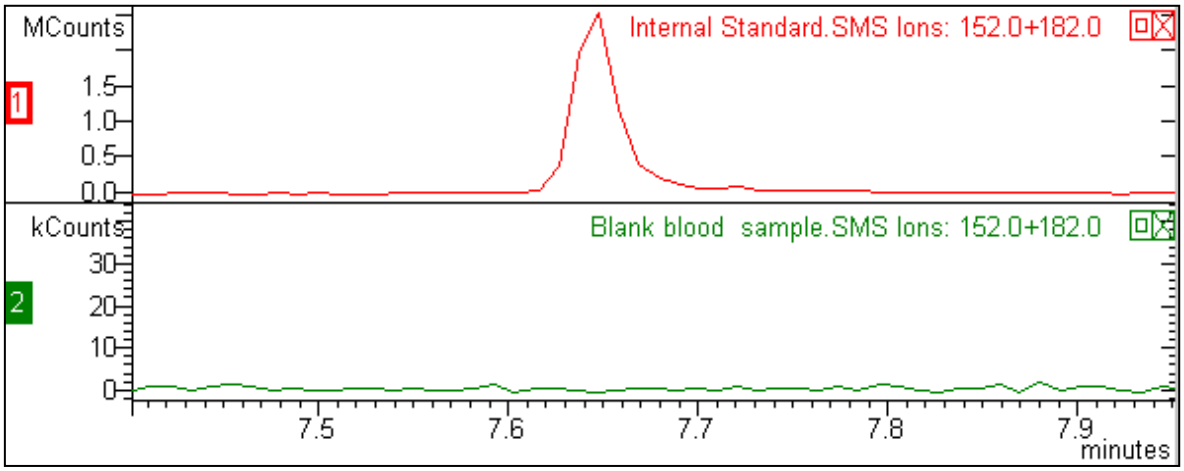

Figure 34. Reconstructed GC-MS (SIM mode) chromatogram of a blank blood sample (1000 ng/mL) and internal standard (m/z 105+182).

2.2.3 Linearity

In this work, the Δ^9-THC linearity studies were evaluated in the range of 0 ng/mL to 1000 ng/mL. This calibration curve was obtained with nine concentrations (0, 1, 5, 10, 25, 50, 100, 500, 1000 ng/mL). The blood samples were analyzed according to a previously described procedure (Chapter I). The weighted least square regression equation and coefficient of correlation were calculated from this curve. The GC-MS chromatogram peak area ratios of Δ^9-THC/IS were determined to establish calibration equation (Figure 35).

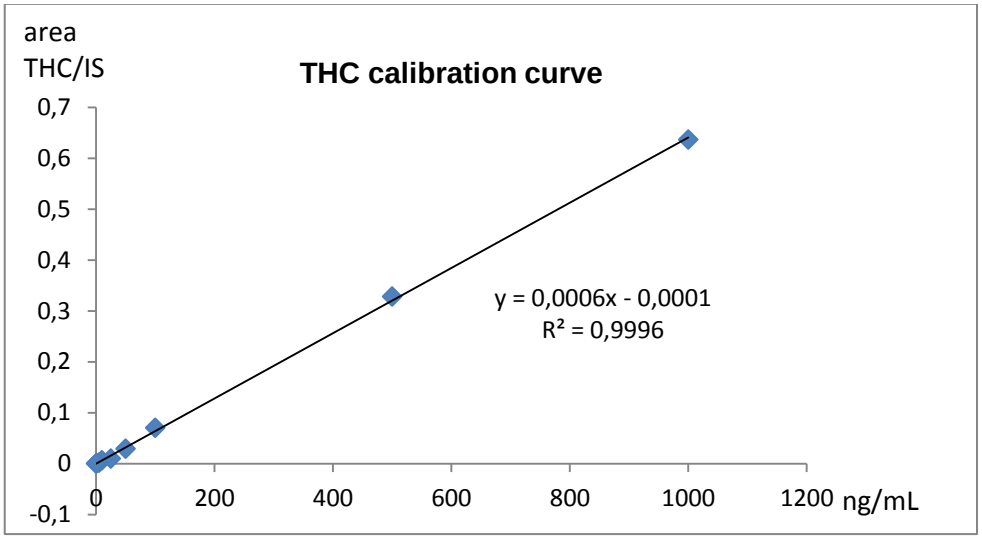

Figure 35. Δ^9-THC calibration curve. Plotted peak area of the Δ^9-THC/IS peak area versus concentrations (0, 1, 5, 10, 25, 50,100, 500, 1000 ng/mL).

The method was linear at the concentration range established, with determination coefficients (r^2) greater than 0.99 for the calibration curve of Δ^9-THC (Table 20).

Table 20. Blood linear regression analysis of Δ^9-THC standard solutions (0-1000 ng/mL).

Psychoactive substance	Y=mx+b	R^2	Concentration range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)
THC	Y=0.0006x -0.0001	0.9996	0-1000	0.23	0.69

LOD, limit of detection; LLOQ, lower limit of quantification

2.2.4 Limit of detection and lower limit of quantification

The LOD is 0.23 ng/mL and LLOQ is 0. 69 ng/mL for Δ^9-THC (Table 20). These results show a good capacity of this method for the quantification of Δ^9-THC analytes, although these analytes were in low concentrations.

2.2.5 Precision

The %CV values calculated for Δ^9-THC intra and inter-day precision studies did not exceed 15%, so that the developed method was considered precise (Table 21).

2.2.6 Accuracy

The accuracies of Δ^9-THC were determined in the range of 98,36-106,59% (Table 21), which are within the proposed acceptance limits for this parameter (100 ± 15%).

2.2.7 Recovery

At three different concentrations of Δ^9-THC (10, 100, 1000 ng/mL), the results obtained indicated an efficient clean-up procedure, with extraction recoveries in the range of 102.95 to 108.68%. The recoveries are within the proposed acceptance limits for this parameter (100 ± 20%) (Table 21).

2.2.8 Reproducibility

At five equal concentrations of Δ^9-THC (100 ng/mL), the results obtained did not exceed 15% of coefficient of variation. The reproducibility of Δ^9-THC developed method was considered reproducible (Table 21).

Table 21. Precision, accuracy, recovery and reproducibility (%) for THC.

Analytes	Concentration (ng/mL)	Intra-day precision (%, n=3)	Inter-day precision (%,n=3)	Accuracy (%, n=3)	Recovery (%)	Reproducibility (%)
THC	10	4.25	7.03	98.35	108.68	6.98
	100	0.34	1.61	101.17	102.95	
	1000	0.64	3.29	106.59	105.09	

2.3 Analysis of amphetamines

The characteristic ions that allow identification of amphetamines are: 44 (amphetamine, MDMA, MDA) and 58 (methamphetamine, MDMA). Comparing the chromatograms obtained from the samples suspected to have amphetamines, with GC-MS program (NIST), no amphetamines were identified.

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2.4 Analysis of cocaine

The screening tests have an absorbent material of each test strip that carries the biological sample across the strip at a specific speed. The biological samples have special molecules that interact with drug-specific antibodies. These antibodies only react to metabolites for the specific drug being test. If the biological sample contains the specific drug metabolites, the special molecules react with antibodies and do not give a color, as happened with the positive cocaine control sample (Figure 36 cocaine). However, if the biological sample does not contain cocaine metabolites, the molecules turn a color, as happened with sample 37 and 38 (Figure 36). The tests have also a control line (C) at the top of the test strip, where a reagent makes the molecules turn a color to indicate that the test is working properly. If the control line does not appear, it means that the test is not working properly. In this study, the control line appears, that means that the test worked well, however another the line T(test) appears. Therefore cocaine levels were below those detectable by this method (Figure 36, sample 37 and 15).

Figure 36. Cocaine screening test

Chapter V

Application of the GC-MS and GC-FID methods to blood and oral fluid samples collected from the volunteers participating in this study

1. Application of GC-FID for detect ethanol in biological samples

The method described by Pontes et al. (2009), with some modifications (shown in chapter II), was applied to the ethanol quantification in blood and in oral fluid samples, collected from young adults frequenting nightclubs and that participated voluntarily in this study. Sixty nine blood samples, 17 oral fluid samples, and 46 exhaled air samples were obtained from these participants. With the objective to compare the results of different matrices, in 9 participants, both types of biological matrices (blood and oral fluid) were collected and analyzed. In some participants samples of exhaled air, to compare this result with the results of biological samples, were also collected. Concerning ethanol quantification, 64 blood samples have had positive results and 5 others gave negative results Eighteen oral fluid samples were positive, and 1 was negative for ethanol. Forty five exhaled air samples were positive and 1 was negative for ethanol.

The analysis of ethanol revealed concentrations ranging from 0 to 2,69 g/L for blood samples, from 0 to 1,18 g/L for oral fluid samples, and from 0 to 3,88 g/L for exhaled air. GC-FID chromatograms of blood and oral fluid samples (from subject number 25) are shown in Figure 37.

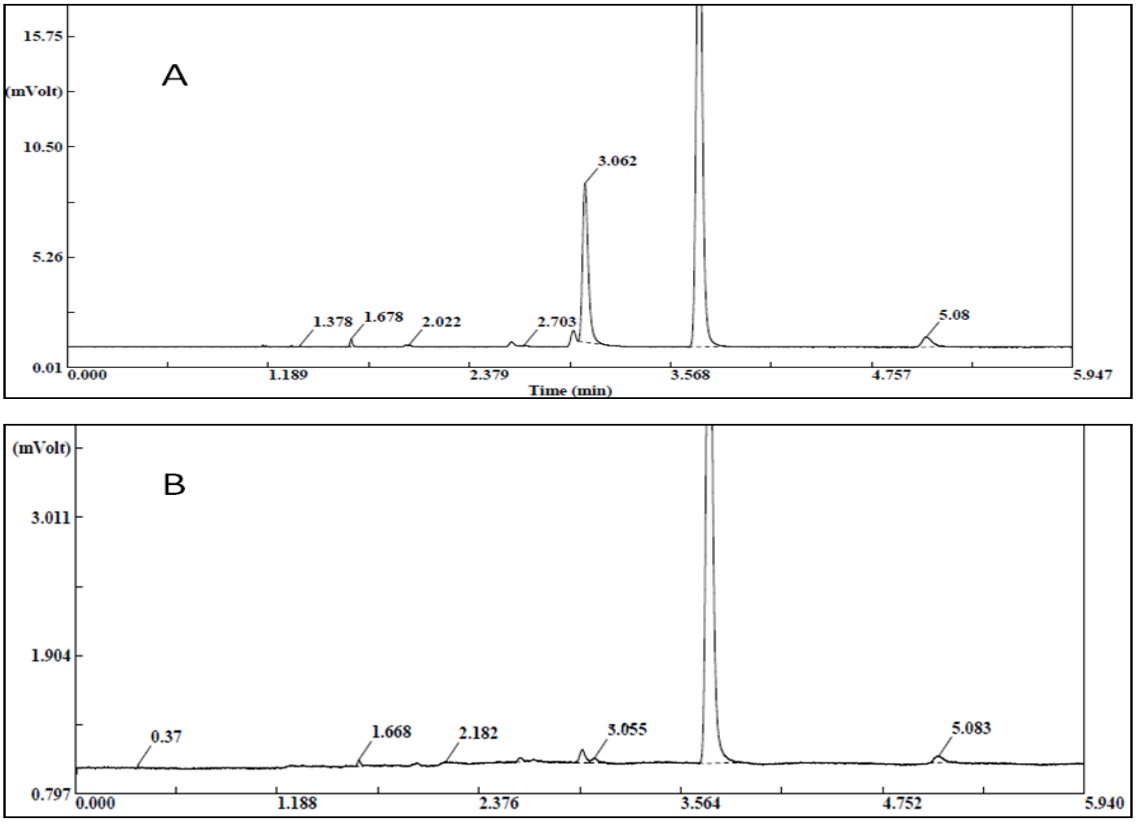

Figure 37. GC-FID chromatograms obtained from a blood sample (A) and an oral fluid sample (B) positive for ethanol (subject number 25). The retention time for ethanol was 3.062 min figure A and 3.055 figure B.

For the correct interpretation of the results, there are some aspects that should be taking into account beyond the concentrations of the ethanol. There are many factors as physiological (age, gender, weight, height), behavioral, genetic, sociocultural and environmental that play a relevant role in determining the huge interindividual variability in the thresholds and lifetime prevalence of ethanol in biological samples (Gemma et al., 2006).

Physiological differences such as bodyweight, height, body surface area and total body water, existing between man and women contribute for toxicokinetic and toxicodynamic differences of psychoactive substances as alcohol, and consequently to differences in the concentration of ethanol in biological samples (Soldin and Mattison, 2009). These anatomical differences between both genders (Table 22) are important reasons to explain the differences found between individuals that consumed the same drinks, exhibiting different concentrations of ethanol in biological samples.

Table 22. Anatomical differences between man and women that influence the concentration of ethanol in biological samples (adapted from Soldin and Mattison, 2009).

Parameter	Reference adult male	Reference adult female	Reference pregnant female
Bodyweight (kg)	78	68	72.5
Height (cm)	176	162	162
Body surface area (cm^2)	18 000	16 000	16 500
Total body water (L)	42.0	29.0	33.0
Extracellular water (L)	18.2	11.6	15.0
Intracellular water (L)	23.8	17.4	18.8

Genetic features involved in ethanol metabolism may also influence the concentration of ethanol in biological samples, at the moment of collection. The hepatic enzymes involved in ethanol metabolism as alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and microsomal P4502E1 (CYP2E1) have shown to be polymorphic, giving rise to altered phenotypes. Recent studies have shown the correlation between metabolic variability and differences in alcohol abuse-related effects (Gemma et al., 2006). Consequently, for the same quantity of ethanol ingested, the concentration found in blood or in oral fluid could be different, due to interindividual variations in alcohol metabolism.

Behavioral features, as mixture of psychoactive substances and ingested alcohol with empty stomach could influence the pharmacokinetics of ethanol. Alcohol is absorbed mainly in stomach and in small intestine. The rate of the absorption depends on the rate of

gastric emptying. When alcohol is ingested with empty stomach, the digestive system is irritated and as there is no food, the absorption of alcohol becomes more rapid. As a result, the blood alcohol concentration (BAC), which is the amount of alcohol in blood stream, increases more rapidly and the effects of alcohol are felt quicker. When alcohol is ingested with food, the gastric emptying is slow, the absorption of alcohol is delayed and peak blood alcohol concentration is reduced. The gastric emptying rate makes an important contribution to interindividual variations in the rate of alcohol absorption and therefore in the magnitude of the acute intoxications (Holt, 1981).

The consumption of alcohol with other drugs, legal (medications) or illegal, causes pharmacokinetic interactions by altering gastric emptying or liver metabolism. Drugs may affect pharmacokinetics of alcohol by inhibiting gastric alcohol dehydrogenase, responsible for converting alcohol to aldehydes, and could cause acute intoxications (Fraser, 1997).

Beyond these interindividual factors there is other variable, the origin of the psychoactive substance, that was not possible to evaluate, but could affect the concentration of ethanol in biological samples.

1.1 Differences between blood, oral fluid and exhaled air samples

Blood samples are the ideal matrix if quantitative measurements are needed (Dinis-Oliveira et al., 2010). However, due to the high volatility of ethanol, special mesures must be taken to prevent ethanol evaporation, during sample handling. For this reason some samples that have low concentrations of ethanol in blood, when analyzed by GC-FID probably become false negatives (subjects number 39 and 64, table 24 chapter VI) For this reason, the method used by exhaled air, at the moment that the surveys are been collected, is the most appropriate. Exhaled air is a non-invasive method used for measuring concentration of volatile xenobiotic as ethanol. Breath testing eliminates the need for taking blood samples, and the results are immediately available (Dinis-Oliveira et al., 2010). Nevertheless, the ideal is to analyze alcohol in both type of matrices for outwit false results.

In this study, oral fluid samples were also used to quantify ethanol. This type of matrix is useful for analyzing quantitatively many xenobiotics, namely drugs of abuse present in saliva. For some xenobiotics as alcohol, there are a good correlation between the concentration found in saliva and the concentration of ethanol found in blood samples. Oral fluid allows detection of psychoactive substances for hours or days and it is a relatively non-invasive method (Dinis-Oliveira et al., 2010). However it has some disadvantages such as: requiring sensitive techniques and collection methods for detect small amounts of the xenobiotic; spitting becomes oral fluid viscous, increasing the

difficulty to handle the sample in the laboratory; it may also be contaminated with food and other debris from the mouth. For these reasons, oral fluid cannot be seen as a substitute for blood or urine drug testing (Drummer, 2006).

According to Jones (1979) (Jones, 1979) there is a relationship between ethanol concentration determined in oral fluid and in blood samples. This relationship is translated by the regression equation y=0.109+1.071x, where y is saliva ethanol concentration (mmol/l) and x is blood ethanol concentration (mmol/l). However, the results obtained in the present work, shows that oral fluid samples have lower concentration of alcohol when compared to blood samples. These results could be explained due to intraindividual reasons. Oral fluid can vary in flow rate, depending on several factors as emotional or hunger. The dry mouth syndrome could be caused by anxiety, lack of proper hydration of the individual and tobacco or THC smoke after drink, can also infuence. Hence, if the volume of oral fluid is less than 1 mL it is required the use of sensitive detection techniques. The stimulated production of oral fluid by citric acid candy, chewing gum or other agents will change the pH and concentration of drug in the oral fluid. This is scientifically proven for lower concentrations (Drummer, 2006).

Other important factor that should be taken into account is the local where the blood was collected. While in study realized by Jones, 1979, he collected capillary blood samples, in the present study venous blood samples were collected. Works from other authors showed that the ethanol present in venous blood is less concentrated than that in capillary blood, until onset of the post-absorptive phase of ethanol metabolism (Jones, 1979). This could explain, in part, the results obtained in the present work.

Finally, the evaporation of ethanol during the sampling procedure, as happens with blood samples could also contribute for the final result.

In conclusion, there is a lot of intra and inter-subject variations due to the technique used, the individual physiology, the characteristic of the compounds and the local of the sample collection, that influence the concentration of alcohol in oral fluid samples.

1.2 Levels of ethanol in biological samples and its effects

Although the concentration of ethanol in biological samples and its respective effects depends on many intra and inter individual factors previously described, the levels greater than 3-4 g/L can be fatal due to respiratory depression. The effects of alcohol ranging from minor impairment of motor coordination and sensation, to amnesia, loss of consciousness and coma when blood levels exceeding 300 mg alcohol/100mL blood (30 mg%) (Marc and Schuckit, 2006)(Table 23).

Table 23. Concentrations of ethanol in blood samples and its effects (adapted from emedicine.medscape.com).

BAC (g/L)	Effects of ethanol
Less than 0.5	No loss coordination and slight euphoria.
0.6 – 0.8	Relaxation, lower inhibition, slight impairment of balance, speech and vision.
0.9 – 1.2	Significant impairment of motor coordination, loss of good judgment and euphoria.
1.3 – 3	Dysphoria, anxiety, nausea, needs of assistance and total mental confusion.
3 – 4	Loss of consciousness.
More than 4	Onset of coma, possible death due to respiratory depression.

Analyzing the ethanol results obtained in biological samples, it is possible to observe that among the 78 individuals, only 6 did not drink alcohol. Among 72 individuals, 17 participants have less than 0.5 g/L in biological samples, 10 individuals have values of ethanol between 0.6 g/L and 0.8 g/L, 9 individuals have values between 0.9 g/L and 1.2 g/L, 27 participants have values between 1.3 g/L and 3 g/L, and 9 individuals have values higher than 3g/L.

With this investigation it was possible to find high levels of alcohol consumption in recreational spaces, by young people. The majority of them had high blood alcohol concentrations that could put them at severe risk. In spite of knowing the bad consequences of alcohol, some of them expected to consume high quantities of alcohol across the course of their night out. This situation is very worrying, mainly when there is an increased risk of social harm associated to alcohol comsumption , particularly violence, intoxication, DST´s, and other risk factors.

Nowadays, some studies evidence the increased effectiveness of strategies to reduce alcohol-related harm in drinking environments, mainly in countries outside Europe. The measures for reducing the availability of alcohol, should focus on the restrictions of alcohol outlet density and increase alcohol price. These measures have significant effect, but are rarely used in practice (Hughes et al., 2011).

2. Application of GC-MS for detecting Δ^9-THC in biological samples

The method validated for Δ^9-THC was applied to analyze THC in blood and oral fluid samples collected from young adults who were in nightclubs and affirmed having used cannabis on that night or on that week. Thirteen blood samples and two oral fluid samples were obtained from these participants. All blood samples were positive and the only two oral fluid samples were negative for Δ^9-THC.

Analysis of blood samples revealed concentrations ranging from 4,69 to 62,40 ng/mL for THC. Reconstructed full scan chromatogram of real blood sample (subject number 14) is shown in Figure 38.

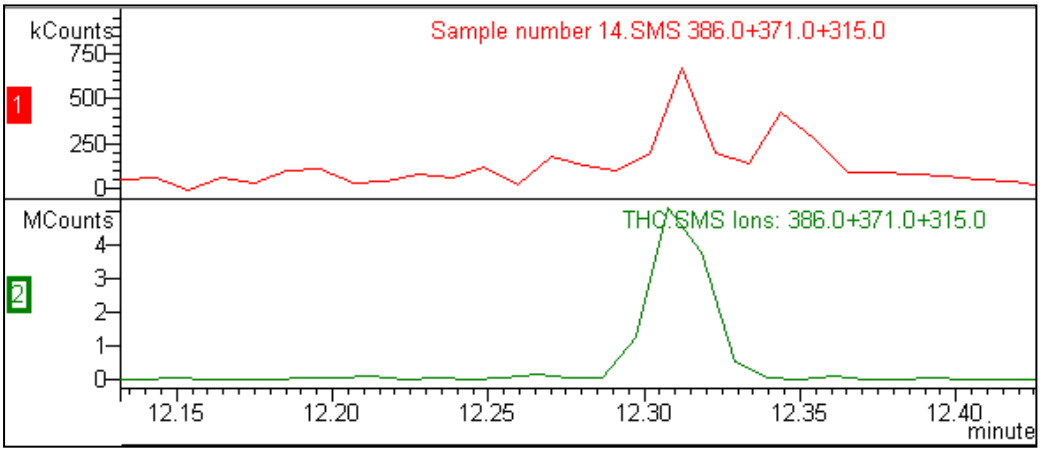

Figure 38. Reconstructed GC-MS Full Scan chromatograms obtained from blood sample (1) positive for THC (subject number 14) and the respective THC standard (2). THC m/z = 386+371+315.

As it happens with alcohol, for the correct interpretation of the THC results is important to take into account some aspects: i) the intra and inter individual physiology of each individual (age, gender, weight, height), ii) their behavioral (mixture of THC with other drugs, route of administration of THC), and iii) genetic features (that caused alterations in toxicokinetic and toxicodinamic of THC). Beyond these interindividual factors, there are also other variables such as: the origin of the THC and the precise quantity that the individuals put in their cigarettes. These factors were not possible to control, but could affect the concentration of Δ^9-THC in biological samples.

Behavioral features as mixture of THC with other psychoactive substances (alcohol or tobacco) could be dangerous, because this mixture becomes stronger and produces more unpredictable effects than if they were used separately. Some of the individuals that participated in this study, had smoked cannabis and ingested alcohol. Cannabinoids and alcohol activate the same reward pathways (dopaminergic system), and CB1 endocannabinoid receptor plays an important role in regulating the positive reinforcing properties of alcohol. When both are used together, its effects become stronger and could cause inhibit vomiting, dizziness and increased paranoia (Mechoulam and Parker, 2003).

The route of administration used by THC consumers may influence the concentration of Δ^9-THC in blood samples. When the drug is administrated orally, over 90

per cent of the dose was absorbed, and the plasma levels of metabolites of Δ^9-THC peaked at three hours. When the drug is inhaled the metabolites peack from 10 to 140 minutes, and the physiologic effects are felt in this moment (Lemberger et al., 1972).

2.1 THC levels in blood samples and its effects

Δ^9-THC has higher lipophilicity, and therefore it is rapidly absorbed and distributed to tissues, passing through the mucosal epithelium into the bloodstream. When the ratio between blood/tissue is in equilibrium, it is possible to determinate a direct correlation of THC blood concentration and effect (Cone and Huestis, 1993; Huestis and Cone, 2004).

The study realized by Schwope et al., 2012 (Schwope et al., 2012) demonstrated the concentrations of THC in whole blood, after some volunteers smoked one single cigarette, that contained 54mg of Δ^9-THC. After 0,25h, they had, in mean, 150µg/L of THC in whole blood (ranging 93 to 250 µg/L), after 4h about 13µg/L (ranging 0-32 µg/L) and after 6h about 8ug/L (ranging 0-27µg/L). The effects that they felt over the time were: "high" (66- 85 µg/L of THC in blood), good drug effects, stoned, stimulated, sedated and anxious (Schwope et al., 2012). The author Mattes *et.al,* 1994 (Huestis and Cone, 2004) reported that 10-15mg of THC resulted in the production of 2,5ng/mL of THC in plasma after 2h following administration. Also, other authors reported that THC plasma levels were about 14 ng/mL, in six volunteers who consumed 20mg of THC (Huestis and Cone, 2004). Other studies, show that an estimated concentration of THC in blood samples, in a range of 7-29 ng/ml, causes 50% of the high effects expected for THC (Cone and Huestis, 1993).

Comparing these studies, for the range of THC from 4,69 to 62,40 ng/mL in whole blood, and knowing that the ratio between blood to plasma of THC concentration is 0,5, it is possible to conclude that the concentration of THC found in the present study, is sufficient for causing 50% of maximal subjective high effects. In fact, among all THC users that participated in this study, only 2 individuals have THC blood concentrations lower than 7 ng/mL. Cannabis is currently one of the most widely used illegal drugs in Coimbra' recreational spaces, an important information for those involved in risk management in Coimbra night life.

Chapter VI

Comparison of participants answers with the identification of psychoactive substances in collected biological samples

1. Alcohol analyzed in biological samples vs answers about use of alcohol

For the question about use of alcohol during that week, 87,18% of the participants refered to have used alcohol while 12,82% indicated absence of alcohol consumption during that week (Figure 39). Following analyses of biological samples, we observed that 92,31% of the participants gave positive results for ethanol and only 7,69% gave negative results (Figure 40). Consulting the table 24, we observe that subject #64 and #39, reported to have used alcohol, but the results where negative for ethanol. This could happen due to individual factors (shown in chapter V) or possible evaporation of ethanol during the sampling procedure. It is important to note that all of these dubious samples were analyzed two times separately in different days. For these reasons is very probable that some individuals have hidden the consumption of alcohol. Although the questionnaire is confidential, several of the participants showed reluctance and were afraid that the study becomes public, or to be shown by media.

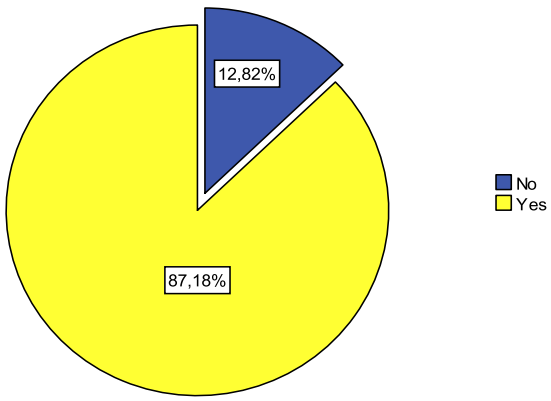

Figure 39. Answers given by participants about alcohol consumption.

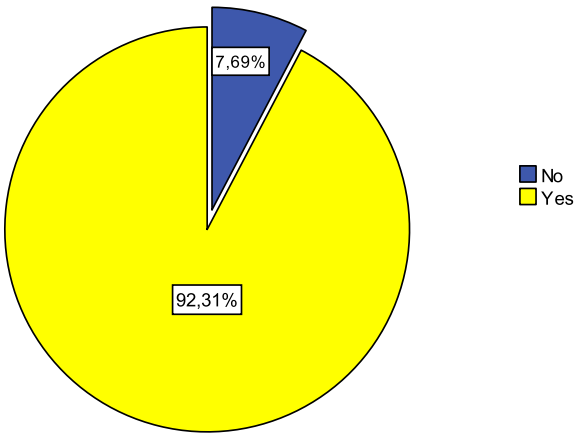

Figure 40. Biological samples analyzed by GC-FID method.

Table 24. Concentration of ethanol in blood, oral fluid and exhaled air samples, compared to responses given by the participants for the question about alcohol.

Nº of samples	Concentration blood samples (g/L)	Concentration oral fluid samples (g/L)	Concentration exhaled air (g/L)	Answer about use of alcohol (Yes/No)	Results obtained by GC-FID
1	1,56	Nc	1.74	Yes	Yes
2	1,19	Nc	1.27	Yes	Yes
3	0,139	Nc	Nc	No	Yes
4	Nd	Nc	Nc	No	No
5	0,604	Nc	Nc	Yes	Yes
6	1,84	Nc	Nc	Yes	Yes
7	1,34	Nc	Nc	Yes	Yes
8	1,75	Nc	Nc	Yes	Yes
9	2,15	Nc	Nc	Yes	Yes
10	0,956	Nc	1.14	Yes	Yes
11	0,770	Nc	0.93	Yes	Yes
12	1,55	Nc	1.64	Yes	Yes
13	1,19	Nc	1.63	Yes	Yes
14	0,843	Nc	Nc	Yes	Yes
15	Nc	0,016	Nc	Yes	Yes
16	0,053	Nc	Nc	Yes	Yes
17*	0,369	0,049	1.14	Yes	Yes
18	Nc	0,116	Nc	Yes	Yes
19*	1,01	0,013	Nc	No	Yes
20*	0,141	0,011	Nc	No	Yes
21	Nc	Nd	Nc	No	No
22	0,136	Nc	Nc	Yes	Yes
23	Nc	0,022	Nc	No	Yes
24	Nc	0,019	Nc	Yes	Yes
25*	1,02	0,020	1.14	Yes	Yes
26*	0,145	0,012	Nc	Yes	Yes
27*	1,48	0,014	Nc	Yes	Yes
28*	0,165	0,012	Nc	Yes	Yes
29*	0,049	0,013	Nc	Yes	Yes
30*	0,154	0,011	Nc	Yes	Yes
31	0,903	Nc	Nc	Yes	Yes
32	0,572	Nc	1.72	Yes	Yes
33	1,83	Nc	Nc	Yes	Yes
34	0,105	Nc	0.70	No	Yes
35	2,07	Nc	Nc	Yes	Yes

36	0,696	Nc	2.33	Yes	Yes
37	1,59	Nc	Nc	Yes	Yes
38	1,11	Nc	Nc	Yes	Yes
39	Nd	Nc	Nc	Yes	No
40	0,782	Nc	Nc	Yes	Yes
41	0,890	Nc	1.52	Yes	Yes
42	1,70	Nc	2.17	Yes	Yes
43	0,181	Nc	0,69	Yes	Yes
44	0,284	Nc	1.52	Yes	Yes
45	0,136	Nc	0.63	Yes	Yes
46	2,05	Nc	3.27	Yes	Yes
47	0,041	Nc	0.66	Yes	Yes
48	1,70	Nc	3.48	Yes	Yes
49	0,059	Nc	0.39	Yes	Yes
50	0,750	Nc	3.12	Yes	Yes
51	1,46	Nc	3.88	Yes	Yes
52	0,808	Nc	3.57	Yes	Yes
53	0,389	Nc	1.12	Yes	Yes
54	0,639	Nc	1.66	Yes	Yes
55	0,611	Nc	1.59	Yes	Yes
56	0,686	Nc	1.88	Yes	Yes
57	2,07	Nc	3.12	Yes	Yes
58	0,678	Nc	1.49	Yes	Yes
59	1,33	Nc	3.27	Yes	Yes
60	1,87	Nc	Nc	Yes	Yes
61	0,122	Nc	1.20	Yes	Yes
62	0,512	Nc	0.9	Yes	Yes
63	0,856	Nc	1.71	Yes	Yes
64	Nd	Nc	Nc	Yes	No
65	Nd	Nc	Nd	No	No
66	Nd	Nc	Nd	No	No
67	0,043	Nc	0,17	Yes	Yes
68	0,620	Nc	2,05	Yes	Yes
69	Nc	1,18	3.30	Yes	Yes
70	1,27	Nc	2,07	Yes	Yes
71	1,16	Nc	Nc	Yes	Yes
72	1,19	Nc	2,72	Yes	Yes
73	Nc	0,026	0,18	Yes	Yes
74	Nc	0,055	Nc	No	Yes
75	Nc	Nc	0,19	Yes	Yes

76	2,69	Nc	3,38	Yes	Yes
77	0,861	Nc	1,49	Yes	Yes
78	1,72	Nc	2,33	Yes	Yes

Legend: * represents that ethanol was analyzed in both matrices (blood and oral fluid); red color represents false negatives; green color represents false positives; Nd – no detectable (below detection limit); Nc-sample no ceded by individual.

2. THC analyzed in biological samples vs answers about use of cannabis

For the question about use of cannabis during that week 19,23% of the participants reported to have used cannabis (Figure 41). Following analysis of the biological samples, we observed that 16,67 % of the participants were positive results for Δ^9-THC (Figure 42). Consulting the table 25, we observe that the subjects *21 and *23, reported to have used cannabis, but the results were negative for Δ^9-THC. This could happen because Δ^9-THC levels in oral fluid samples were below the detection limit of the method. In this case, the hypothesis of the subjects have lied is not likely. However, is possible they may think that the substance which they are using is cannabis, but may be another substance.

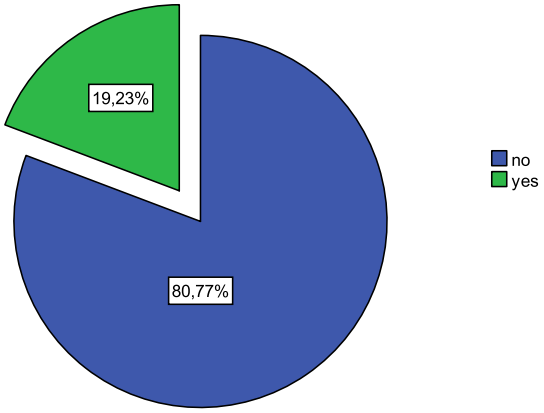

Figure 41. Answers given by participants about use of cannabis

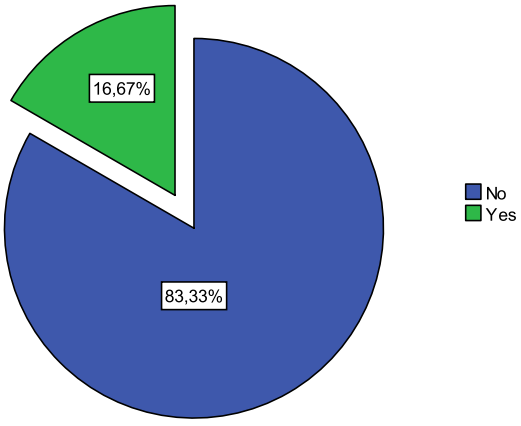

Figure 42. Biological samples analyzed by GC-MS method

Table 25. Concentration of THC in blood samples and oral fluid samples. Answer given by the participants for the question about cannabis, and the respective results obtained by analyze of biological sample (GC-MS).

THC samples	Concentration (ng/mL)	Answers about use of THC (Yes/No)	Results obtained by GC-MS
1	9,02	Yes	Yes
4	7,58	Yes	Yes
8	21,64	Yes	Yes
10	4,69	Yes	Yes
14	62,40	Yes	Yes
21*	**Nd**	Yes	No
22	14,68	Yes	Yes
23*	**Nd**	Yes	No
37	9,90	Yes	Yes
41	32,83	Yes	Yes
43	13,79	Yes	Yes
46	12,95	Yes	Yes
51	5,315	Yes	Yes
52	9,67	Yes	Yes
59	7,437	Yes	Yes

Legend: * The 21 and 23 represent the oral fluid samples; Nd – no detectable (below detection limit).

3. Amphetamines analyzed in biological samples vs answers about use of amphetamines

For the question about use of amphetamines during that week, only two participants reported to have use amphetamines. However, following analysis of the biological samples, it was not possible to detect any amphetamine. The amphetamines that participants used, may be different from those screened in the the data base (NIST), or the quantity found in blood samples were below the detection limit of the method.

4. Cocaine analyzed in biological samples *vs* answers about use of cocaine

For the question about use of cocaine during that week, only two participants reported to have used cocaine. However following analysis of the biological samples, it was not possible detect cocaine by using screening tests. Again, cocaine levels could be below the limit of the detection of the screening tests. Screening tests give us preliminary results. If cocaine had done positive in blood samples, highly sensitive testing methods as GM-MS, should have been performed.

Part IV
Discussion

1. Discussion

This study intended to determine the patterns of drug abuse in recreational nightlife, as well as the associated sociodemographic characteristics, the recreational habits, the health risk and risk behaviors. A better knowledge on this subject may contribute to the prevention of harm associated with the excessive consumption of this type of substances.

In this study, the psychoactive substance more used among young people in recreational settings, in Coimbra, was alcohol (87,2%), followed by tobacco (64,1%). Regarding to illegal psychoactive substances, cannabis was the most used drug (19,2%), followed by cocaine and amphetamines (2,6%), LSD and smartshop substances (1,3%). Comparing this study with a similar one realized by Lomba, 2008 (Lomba et al., 2008) in the same city, it is possible to detect many similarities. In respect of alcohol, this author observed a higher consumption (95,10%) compared to our study. However, it is important to take into account that, although the reported consumption of alcohol was 87,2%, following analysis of the biological samples, 92,3% of the participants were positive for ethanol in blood. The second substance used by the participants in Lomba's study was tobacco, with (79,12%) followed by cannabis with (65,73%). Compared to our study, the consumption of cannabis was significantly higher. Additionally, although the consumption of this drug was 19,2%, it was only possible to detect ∆9-THC in 16,6% of the blood samples by the GC-MS method. The lower number of samples evaluated in our study could explain, in part, these differences.

In relation to the gender of participants, we observed a variance between gender and use of psychoactive substances. The males have tendency of use more psychoactive substances, as alcohol and cannabis, in higher quantities than females. This difference becomes more evident in relation to the use of illegal drugs as cannabis, cocaine and others. These results are agreement to general drug patterns in European countries. In fact, in these countries, one of the most used drug among young people is cannabis, males being those who generally use it more frequently. These differences are very significant in Portugal. The use of cannabis by male and female, in Europe have a ratio from 1.8 in Norway to 5.9 in Portugal (EMCDDA, 2005) which was confirmed in part our study, in which we found a ratio of 14.

In respect of recreational habits, we concluded that the majority of participants, who frequent recreational spaces with assiduity, use more psychoactive substances than the participants who go out occasionally. A study realized by Calafat, 2008, has the same finding. In his study, it was observed that as the participation in recreational nightlife increase, the percentage of people who did not get drunk decreases. Additionally, the

frequency of drunkenness increases (Calafat et al., 2008). Also in our study, was possible to observe a discrepancy among genders in relation to recreational habits. Males have more tendencies for going out more times, using more than one type of psychoactive substance in the same night, and use more illegal drugs than females. Studies realized by EMCDDA, 2005 corroborate that the number of females in relation to males is generally lower for illegal drugs and have lower incidence of recent or frequent patterns of drug use. These discrepancies between genders tend to be lower over the years (EMCDDA, 2005). Another study realized in two different Spanish islands (Ibiza and Mallorca), reported that cannabis and cocaine were more commonly used by males in both locations (Hughes et al., 2009).

Relatively to the frequency of nights out per week, Lomba, 2008, affirmed that inquired youngsters go out about seven days per month, mainly on weekends. This is concordant with our study. However, we observed a light increased in nights out per month, with an average of 8,01 (Lomba et al., 2008).

In respect of problems due to use of psychoactive substances, this study clearly showed that the participants who used more harmful substances, had more problems in all sectors. Relatively to health problems due to use of psychoactive substances, our study revealed higher incidence in cocaine users (odds ratio of 3,22), followed by cannabis (2,56). The use of alcohol was also a positive relation. In fact, a study realized by Hughes, 2009 in two different locations (Malorca and Ibiza) reported that illness, discussions, physical fighting, unintentional injuring and needing to go to hospital, were more commonly reported by users of illicit drugs, especially in Ibiza. This fact could occur due to drug patterns in Ibiza have revealed to be relatively high, when compared with studies in other places, as in Portugal or whole Spain. In contrast, the drug patterns in Malorca had more concordance to our study. The number of cocaine users was 7,5% and ecstasy was 4% (Hughes et al., 2009).

Another subject that is associated with the consumption of psychoactive substances is the risky sexual behavior. Dowing, 2010 reported a comparative cross-sectional between Germany, British and Spanish users. She concluded that the main factor for 34,1% of the participants contributing for sex on holiday, was high levels of drunkenness (Dowing et al., 2010). However, in your study this data does not have statistic relevance. Only one user reported to have had a sexual regret due to use of psychoactive substances. This could happen due Coimbra is not a characteristic touristic city. In fact, the number of touristic people in our study was much reduced.

Driving under influence of psychoactive substances and related driving-related problems, are also relevant subjects in our study. It is known that alcohol and cannabis users have an increased risk of 1,5x and 1,6x of having driving-related problems,

respectively, while cocaine users have a 3x increased risk. In our study, we verified that 25,6% of the participants have already driven under influence of psychoactive substances.. These results are in partial agreement with the study published by Calafat, 2008, in which he observed a higher number of participants that had already driven drunk, or drugged (65,2%) (Calafat et al., 2008).

Nowadays, several authors have addressed some interventions that could be implemented in recreational spaces, with objective to reduce harm associated with recreational nightlife venues. Calafat, 2012, is one of these authors that highlight the importance of regulating standards that should be implemented in European recreational settings. For this author, the use of preventive interventions including venue management, underage checkouts, staff training and collaboration with the police, are some of prevention standards that could exist in nightlife industry (Calafat et al., 2012). In fact, all of these measures could implemented; however for many countries, including Portugal, it means more costs. Nevertheless, to ensure promotion of health and safety in nightlife spaces, risk assessment and risk control are lifesaving issues that should become an incentive to each and every one of us.

Part V

Conclusion and future perspectives

1. Conclusion

I. Drug abuse patterns in Coimbra recreational nightlife was monitored in this work through the use of a survey and collection/analysis of biological samples.

II. Although this is a pilot study with only 78 samples, it was possible to obtain significantly different data.

III. In general males go out with more frequency, using more psychoactive substances, both in quantity as in diversity, than females.

IV. Alcohol was the most used psychoactive substance (87,2%), followed by tobacco (64,1%), cannabis (19,2%), cocaine and amphetamines (2,6%) and finally LSD and smartshop substances (1,3%).

V. Males have more tendencies to be polyusers.

VI. Cocaine users have the highest risk for health problems perceptive to the users, followed by cannabis, alcohol and finally tobacco.

VII. Relatively to biological samples, ethanol was the substance that has more discordance, comparing to the surveys. 92,31% of the participants gave positive results for ethanol while only 87,18% of them answered positively for the consumption of alcohol.

VIII. The THC results show more concordance, when compared to the answers of the surveys. All samples were positive for THC, except two oral fluid samples.

IX. A sensitive, reproducible, precise, accurate and inexpensive GC-MS method was developed and validated to identify and quantify THC in whole blood samples.

X. The proposed GC-MS method was successfully applied in the quantification of THC in real blood samples and shown to be appropriate for routine analysis. Blood samples represent the most used matrix with relevant importance in toxicological analysis.

XI. Liquid-liquid extraction revealed to be the best extraction, allowing to obtain the best peak resolution and separation of the compounds.

XII. The use of exhaled air to analyze the concentration of ethanol revealed to be the best method. However, the use of more than one matrix to quantify and qualify ethanol is essential for having a precise and better result.

2. Future Perspectives

I. Using screening methods, it was not possible to detect amphetamines and cocaine. The next step will be analyzing these samples with high sensitive methods.

II. More samples should be collected for have a better perspective of drug abuse patterns in Coimbra.

III. Some biological samples should be collected in accidental or injured individuals that recurred to the hospitals due to use of psychoactive substances.

IV. Other psychoactive substances, including the new psychoactive drugs, should be identified and quantified in biological samples.

Part VI

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