UNIVERSIDADE Ð
COIMBRA

Diogo Oliveira da Costa

DEVELOPMENT AND VALIDATION OF A GC-MS METHOD FOR IDENTIFICATION AND QUANTIFICATION OF LSD IN STREET SEIZED SAMPLES

Dezembro de 2020

Agradecimentos

Este trabalho não seria possível sem a colaboração e apoio de diversas pessoas, portanto, seria injusto não dar o devido crédito a todos aqueles que estiveram ao meu lado.

À Doutora Ana Isabel Matias, pelo exemplo de resiliência e superação; pelos ensinamentos, pelo incentivo constante e por toda a disponibilidade oferecida.

À Professora Doutora Ermelinda Eusébio, pela compreenção, atenção e dedicação sem reservas.

Às minhas colegas de curso, que apoiaram-me desde os primeiros momentos em Coimbra.

Aos funcionários do Laboratório de Polícia Científica da Polícia Judiciária, pelo acolhimento cordial, apoio e pelos momentos de descontração.

Aos meus amigos Victor Gonzalez, Eduardo Braz e Scheilla Azevedo pelo apoio incondicional durante os tempos mais cinzentos.

Ao meu padrinho Leonardo Mathias e à minha mãe de coração Christiane Costa pelo amor e carinho de sempre, mesmo à distância.

À minha avó Isaura Oliveira, por ter me criado como um filho e por sempre acreditar em mim, mesmo quando eu próprio não o faço.

E claro, à Coimbra! Porque "uma vez Coimbra, para sempre saudade".

A todos vós, um muito obrigado!

Abstract

Lysergic acid diethylamide (LSD) is one of the most potent known psychoactive substances. It was first synthesized in 1938, but its hallucinogenic properties were only accidentally discovered five years later. From the 1950s through the 1960s, the recreational use of LSD become popular among academics and young people, associated with 60´s counter-culture movement. Due to the spread of illegitimate use and the increasing number of people who had developed negative side effects under treatment, LSD was put under international control in 1971.

Although the use as a recreational drug peaked during the 1960s, LSD is still one of the most consumed hallucinogenic substances worldwide. It is commonly found in blotter papers. The Portuguese law establishes the limits quantities of illegal substances defined as possession for personal use based on estimations of the average required for 10 days' consumption. Therefore, LPC must provide reliable quantitative results suitable for criminal justice.

The aim of this work was to develop and validate a GC-MS method to quantify LSD in blotter papers seized. An extraction protocol by soaking the blotter into methanol was established, and the trimethylsilyl derivative was successfully formed by reaction with MSTFA/PYR.

The linearity between 2 to 20 µg/mL was observed, with satisfactory repeatability (lower than 7 %RSD) and intermediate precision (lower than 10 %RSD), and accuracy (between -6.5 to 11.5 %RE, n = 3). The limits of detection and quantification were calculated as 1.08 µg/mL and 3.23 µg/mL, respectively.

The method was applied in six real samples, which concentration varied from 10.3 (± 4.0) to 109.1 (± 5.7) µg/blotter. The method was validated and showed to be useful to analyze LSD in blotter papers.

Resumo

A dietilamida do ácido lisérgico (LSD) é uma das mais potentes substâncias psicoativas conhecidas. Foi sintetizada pela primeira vez em 1938, mas as suas propriedades alucinogénias só foram descobertas cinco anos depois. Durante os anos 1950 e 1960, o uso recreacional do LSD tornou-se popular entre académicos e jovens, associado ao movimento de contracultura dos anos 1960. Devido ao aumento do uso ilegal e ao crescente número de pacientes que desenvolveram efeitos adversos, o LSD foi colocado sob controlo internacional em 1971.

Embora o uso recreacional tenha atingido os níveis mais altos durante a década de 1960, o LSD continua a ser uma das substâncias alucinogénias mais consumidas em todo o mundo. É comumente encontrado sob a forma de selos de papel. A legislação Portuguesa estabele os limites para que a posse de substâncias ilegais seja considerada para uso pessoal, de acordo com estimativas da quantidade média necessária para dez dias de consumo. Por isto, o LPC deve fornecer resultados quantitativos confiáveis para fins de judiciais.

O objetivo deste trabalho foi desenvolver e validar um método de GC-MS para quantificar LSD em amostras de selos apreendidos em Portugal. Foi estabelecido um protocolo de extração por metanol, e o derivado trimetilsilil foi formado através de reação com MSTFA/PYR.

O método apresentou resposta linear na faixa entre 2 a 20 μg/mL, com valores satisfatórios de repetibilidade (inferior à 7 %RSD) e precisão intermédia (inferior à 10 %RSD), assim como satisfatórios valores de acurácia (entre -6.5 a 11.5 7 %RE , n = 3). Os limites de deteção e quantificação estimados foram, respetivamente, 1.08 μg/mL e 3.23 μg/mL.

O método foi aplicado em seis amostras reais, cujas concentrações variaram entre 10.3 (± 4.0) e 109.1 (± 5.7) μg/selo. O método foi validado e foi demonstrada a sua aplicabilidade para a análise de LSD em selos de papel.

Abbreviations

AMU	Atomic mass unit
25C–NBF	[2-(4-chloro-2,5-dimethoxyphenyl)ethyl][(2-fluorophenyl)methyl]amine
25IP–NBOMe	{2-[2,5-dimethoxy-4-(propan-2-yl)phenyl]ethyl}[(2-methoxyphenyl)methyl]amine
5–APB–NBOMe	1-(benzofuran-5-yl)-*N*-(2-methoxybenzyl)propan-2-amine
25I–NBOH	2-({[2-(4-iodo-2,5-dimethoxyphenyl)ethyl]amino}methyl)phenol
5-HT	5–hydroxytryptamine
ASD	Average single dose
CHCl₃	Chloroform
DMT	Dimethyltryptamine
DLO	Drug law offence
SDT-LPC	Drugs and Toxicology Sector
EI	Electron ionization
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ENFSI	European Network of Forensic Science Institute
EU	European union
GC-MS	Gas chromatography– mass spectroscopy
GC	Gas-chromatography
SICAD	General–Directorate for Intervention on Addictive Behaviours and Dependencies
GPCR	G–protein–coupled receptor
IS	Internal standard
PJ	Judicial police
LOD	Limit of detection
LOQ	Limit of quantification
LC	Liquid chromatograph
LSD	Lysergic acid diethylamide
LEO	Lysergic acid ethyl–2– hydroxyethylamide
LAE	Lysergic acid ethylamide
MS	Mass spectrometry
m/z	Mass–to–charge ratio
Cmax	Max concentration observed
MeOH	Methanol

MDMA	Methylenedioxymethamphetamine
MAOI	Monoamine oxidase inhibitor
25I–NBF	*N*-(2-fluorobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine, monohydrochloride
1P–LSD	*N,N*-diethyl-7-methyl-4-propanoyl-6,6a,8,9-tetrahydroindolo[4,3-fg]quinoline-9-carboxamide
BSTFA	*N,O*–bis–trimethylsilyl–trifluoroacetamide
GNR	National republican guard
UNCTE	National Unit to Combat Drug Trafficking
NPS	New psychoactive substance
NADH	Nicotinamide adenine dinucleotide
MSTFA	*N*–Methyl–*N*–trimethylsilyl–trifluoroacetamide
OLS	Ordinary-least squares
OCG	Organized crime group
KOH	Potassium hydroxide
PSP	Public security police
PYR	Pyridine
RSD	Relative standard deviation
RE	Relative standard error
RT	Retention time
LPC	Scientific police laboratory
SWGDRUG	Scientific Working Group for the Analysis of Seized Drugs
SIM	Selected ion monitoring
SSRIs	Selective serotonin reuptake inhibitors
SNFL	Slovenia national forensic laboratory
s	Standard deviation
SOP	Standard operation procedure
TV	Test value
TLC	Thin–layer chromatography
Tmax	Time of Maximum concentration observed
TIC	Total ion current
TMS-LSD	Trimethylsilyl-Lysergic acid diethylamide
UNDCP	United nations drug control program
UNODC	United Nations Office on Drugs and Crime
S^2	Variance
WLS	Weighted least squares

List of Figures

List of Tables

Table of Contents

1 Introduction

1.1 The Discovery of LSD

Lysergic acid diethylamide (LSD) is a prototypical human hallucinogen and one of the most potent known psychoactive drug.[1] It is a semi–synthetic product derived from Ergot alkaloids, the metabolic products of the fungus *Claviceps purpurea,* which naturally grows on rye and other cereals. It was first synthesized in 1938 by the Swiss natural products chemist Dr. Albert Hofmann working for Sandoz Laboratories in Basle, Switzerland.[2–4]

Hofmann was an enthusiast in plant and animal chemistry. In his doctoral work at the University of Zurich, he conducted important research on animal substances, for which he received his doctorate with distinction in 1929. At the same year, he joined the pharmaceutical–chemical department of Sandoz, under the leadership of Dr. Arthur Stoll. The department research goal was to obtain pure compounds by isolating the active principles of known medicinal plants e.g., substances from foxglove (*Digitalis*), Mediterranean squill (*Scilla maritima*), and ergot of rye (*Claviceps purpurea*). In their natural form, most of these substances are easily decomposed and their concentrations are subject to great variation, which makes pharmaceutical manufacturing difficult.[4]

The first years of Hofmann in the department were devoted almost exclusively to studding the cardioactive glycosides obtained from the squill, in which he contributed to elucidate its chemical structure. When these studies were concluded in 1935, he found himself looking for a new field of research and asked to resume the investigations on ergot, which were abandoned after Stoll succeed in isolating the Ergotamine in 1918, brought to market in 1921 as a medicament to stopping postpartum haemorrhage and in the treatment of migraine.[2,3,5]

Meanwhile, English and American laboratories had begun investigations on ergot alkaloids and had succeeded in isolating and characterizing the nucleus common to all those substances. They named it lysergic acid. Soon later, four institutions, including Sandoz, published simultaneously and quite independently the isolation of a relatively simple structure alkaloid, the ergobasine, also used in obstetrics. Since the ergobasine is present in ergot only in insignificant quantities, Hofmann set as his first goal to obtain it

synthetically. Despite the chemical instability of the lysergic acid, and its rebounding with basic radicals, in 1935 he achieved the synthesis of a compound identical to the natural alkaloid, by combining the two products of ergobasine chemical cleavage: lysergic acid and propanolamine. After this first success, the researches on ergot alkaloids went forward on two fronts: first, the attempt to improve the pharmacological properties of ergobasine by variations of its amino alcohol radical;[4]

Secondly, the production of new lysergic acid compounds from which, on basis of their chemical structure, other pharmacological properties could be expected. In 1938, Hofmann synthesized the lysergic acid diethylamide, abbreviated to LSD–25, as it was the twenty–fifth substance in this series of lysergic acid derivatives. He planned the synthesis of LSD expecting to obtain a circulatory and respiratory stimulant, considering its chemical structural similarity to nicotinic acid diethylamide (Coramine), an analeptic already known at that time. During the pharmacological tests of LSD, it was observed that LSD was 70% as effective as ergobasine in controlling blood flow in the uterus. It was also noted that the animals became restless after LSD administration. Nevertheless, the compound was declared to be of no pharmacological interest.[4–6]

Five years later, Hofmann decided to re–synthetize the LSD and submit it to pharmacological department for further tests, a quite unusual procedure. He repeated the synthesis and obtained a few centigrams of the substance. At the final step of the synthesis, he experienced atypical sensations which obligated him interrupting his work and go back home.[5] At that time, he was not sure whether the symptoms were due to some exogenous source. He concluded that perhaps the LSD he had been working with somehow could have been responsible. To test that hypothesis, he decided to conduct a self–experiment with 25 µg of LSD tartrate diluted in water. Forty minutes after orally ingesting the drug, he described had been feeling the first psychoactive symptoms, e.g., slight dizziness, unrest, difficulty in concentration and visual disturbances. This led to his infamous bike ride home heavily impaired, known as "Bicycle Day". [2–4,6]

This self–experiment revealed the powerful hallucinogenic properties of LSD i.e., its ability to alter thought, perception and mood without affecting memory.[7] Hofmann stated he had been aware for the entire duration of the experiment. He wrote a report on his experience to Dr. Stoll, also sending a copy to the director of pharmacology department, Dr. Ernst Rothlin, both of whom asked whether he was sure about the stated

dose. At that time no other substance was known by provoking psychoactive effects at micrograms dose. Subsequent experiments showed that oral doses of 30 µg of LSD tartrate was enough to promote psychoactive effects in humans.[3–6]

The first scientific study on the effects of LSD was published in 1947.[8] From the early 1950s, through the 1960s, Sandoz made LSD available to research institutes and psychiatrist as an experimental drug, under the trade name Delysid (*D–Lysergsäure–Diethylamide*). As a result, a considerable number of clinical studies with LSD had been published, amounting to about 1,000 clinical case reports discussing treatment of approximately 40,000 subjects. Despite such high enthusiasm, the relatively rudimentary clinical instruments, lack of controls, and poor follow–up used in those early clinical studies often led to inconclusive results, and it was difficult to assess whether LSD had any real therapeutic value.[5,6,9]

The popularization of LSD associated with the raising of the "60s counter–culture movement", led to unqualified people setting up LSD therapy practices, which whereas often extremely lucrative. The recreational use started primarily among academics and medicinal professionals who became acquainted with LSD in their work. They began using it themselves, sharing with associates, and holding LSD "parties".

The spread of illegitimate use and an increasing number of patients who had had LSD treatment and developed negative side effects, forced the countries to assess their laws involving "Investigational Drugs". Sandoz stopped the production and distribution of LSD altogether in 1965. In 1971, LSD was put on international control, classified in the schedule I of the United Nations Convention of Psychotropic Substances. Schedule I is the most restrictive category, with drugs in it defined as having no medical use and as being unsafe to use, even under medical supervision, with a high potential for abuse.[1,7]

1.2 Properties and Chemical Synthesis

LSD belongs to the family of indolealkylamines, substances which possess an indole nucleus structurally similar to 5–hydroxytryptamine (5–HT or serotonin), a monoamine neurotransmitter that modulates human mood and behaviors.[10] LSD is a crystalline substance, colourless, odourless and tasteless; with a melting point at 82.5 °C; slightly soluble in water (67.02 mg/L at 25 °C) and neutral organic solvents; extremely

sensitivity to both light and heat.[11] The substance exists in four isomeric forms. The (R) stereoisomer is more potent than the (S) form (**Figure 1**). LSD is normally produced as tartrate salt, which dissolves in water and ethanol to give clear and odourless solutions.[7]
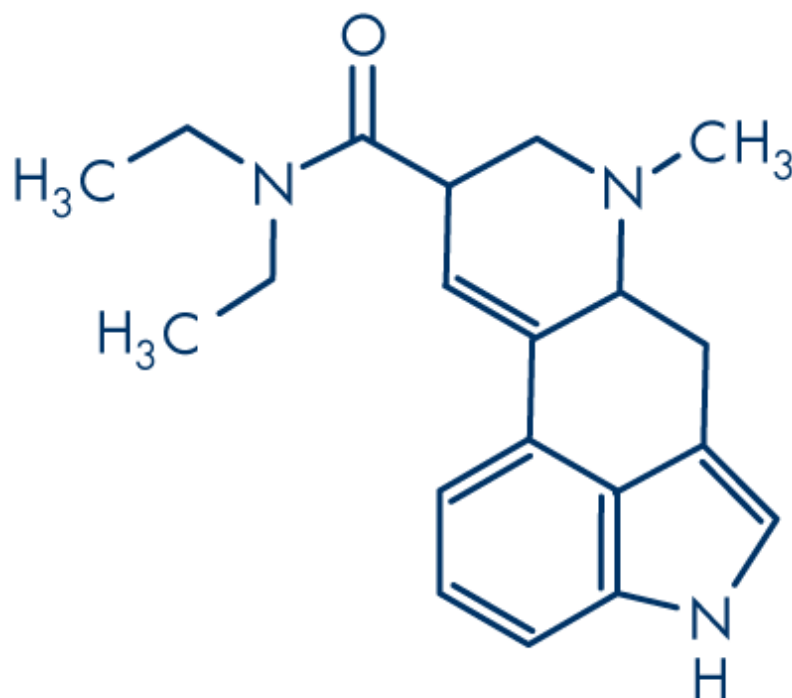
Figure 1 – Chemical structure of LSD.

The first synthesis of LSD reported by Stoll and Hofmann is illustrated in **Figure 2**. The ergotamine was heated with anhydrous hydrazine to produce racemic isolysergic acid hydrazide. This process racemizes the lysergic acid nucleus at C(5) and also epimerizes the carboxylic acid moiety at C(8). The *d*–isolysergic acid hydrazide was separated from the racemic mixture using *d*–di(*p*–toluyl)–tartaric acid. The resolved *d*–isolysergic acid hydrazide was treated with ethanolic KOH to epimerize the C(8) position and afford d–lysergic acid hydrazide. This hydrazide was then treated with nitrous acid to afford the corresponding lysergic acid azide, which was layered over with a cold diethyl ether solution of diethylamine, whereupon the diethyl group attacked and replaced the azide. The *d*–lysergic acid *N,N*–diethylamide thus obtained was then crystallized as the natural tartrate salt.[6,12]
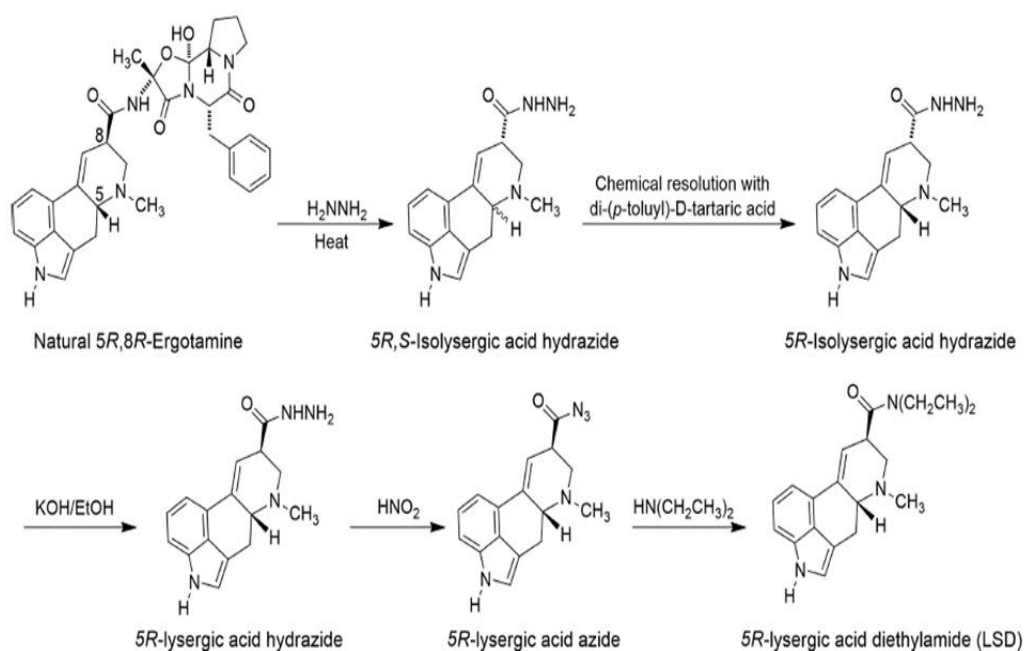
Figure 2 – The first LSD synthesis.
Adapted from Nichols, 2018.[6]

Sandoz held the patent on LSD until 1963, afterward other methods were developed either by legal or clandestine laboratories.[13–15] Unlike some of the illegal substances e.g., methamphetamine, the manufacturing of LSD requires a solid knowledge of organic chemistry, sophisticated laboratory setup (e.g., almost all procedures typically require a final purification by chromatography), and several chemicals and precursors that are under control.[6,16]

The common street dose forms are *blotters*, sheets of absorbent printed paper usually with coloured pictures featuring cartoon characters, geometric and abstract motifs (**Figure 3**). The paper sheets are usually perforated into 0.5x0.5–1x1 cm squares, being each considered a single dose. The blotters are prepared by dipping the paper in an aqueous alcoholic solution of LSD tartrate, or by dropping the solution onto individual squares. Over the years, LSD have been found in different dosage forms such as small tablets "microdots", sugar cubes, thin gelatine squares "windowpanes", capsules, and water or alcohol solutions.[7,17–19]

Figure 3 – LSD seized blotter.

1.3 **Toxicological Data**

1.3.1 *Toxicokinetics*

Following oral administration LSD is rapidly absorbed from the gastrointestinal tract. However the ingestion of food, as well as the pH of the stomach and duodenum, influence its absorption.[20] The duration and intensity of effects are dose–dependent. In a recent study, Dolder and colleagues measured the plasma concentration after two different doses of LSD via oral administration. In both cases, the $t_{1/2}$ of LSD was reported as 2.6 h, with a max concentration observed (C_{max})of 1.3 and 3.1 ng/mL, and a time of maximum concentration observed (t_{max})of 1.4 and 1.5 h, respectively, for the 100 and 200 µg doses. The acute subjective and sympathomimetic responses to LSD lasted up to 12 h and were closely associated with the concentrations in plasma over time.[21,22]

LSD is rapidly metabolized into some structurally similar metabolites. Canezin and colleagues found the following LSD metabolites in human urine: nor–LSD, lysergic acid ethylamide (LAE), 2–oxo–LSD, 2–oxy–3–hydroxy–LSD, 13– and 14–hydroxy–LSD as glucuronides, lysergic acid ethyl–2– hydroxyethylamide (LEO), and "trioxylated LSD".[23] The inactive 2–oxo–LSD and 2–oxo–3–hydroxy LSD originates from NADH–dependent microsomal liver enzymes. LAE is formed from the enzymatic *N*–dealkylation of the diethylamide moiety, and nor–LSD from the *N*(6)–demethylation of LSD. Aromatic

hydroxylation leads to 13– and 14–hydroxy LSD, which are excreted either as free compound or as conjugated with glucuronic acid.[6,7,20–22]
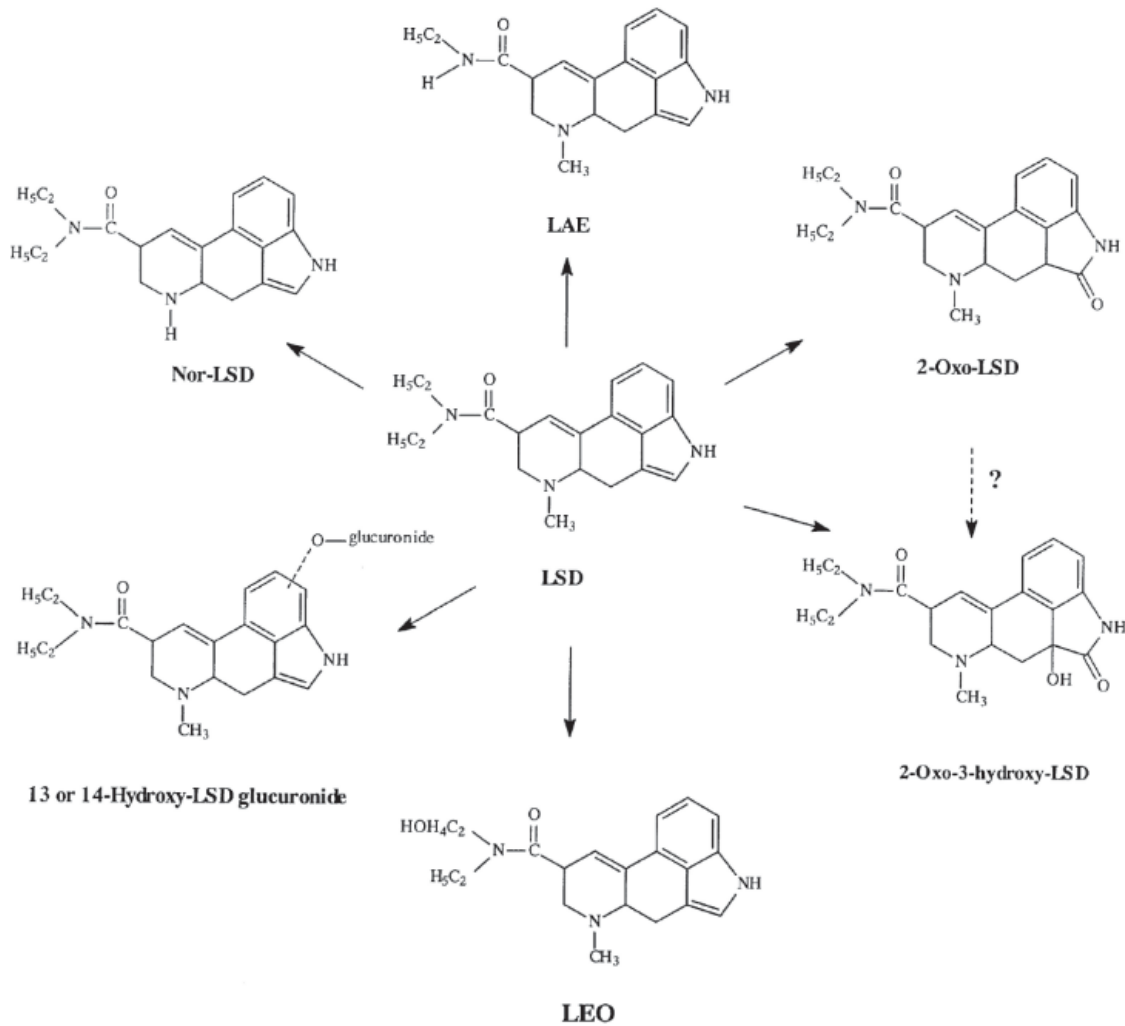
Figure 4 – The main human urine LSD metabolites.
Adapted from Canezin and colleagues, 2001.[23]

The major metabolite in urine is 2–oxy–3–OH–LSD, which may be present at 16−43 times higher than urinary LSD. The rate of excretion peaks about 4–6 h after administration, and LSD and its metabolites are detectable in urine for as long as 4 days after ingestion.[10]

1.3.2 *Toxicodynamics*

LSD exhibits complex interactions with essentially all aminergic G–protein– coupled receptors (GPCRs). It acts as a 5–HT receptor agonist on 5–HT$_{1A}$ receptors in

the *locus coeruleus* (LC), the raphe nuclei (RN), and the cortex, inhibiting the firing and serotonin release. The hallucinogenic effect of LSD is linked to its affinity for the $5–HT_{2A}$ receptor, where it acts as a partial agonist, mediated by cortico–cortical neural circuits rather than by thalamo–cortical circuits. Other phenethylamine and indolamine hallucinogens share this property. There is a strong correlation between the LSD dose and the potency at $5–HT_{2A}$ receptors.[1,5,6,20]

Moreover, there is evidence that LSD interacts with dopaminergic systems. Behavioural studies in rats demonstrated a time–dependent change in pharmacology, from initial (15–30 min) $5–HT_{2A}$ receptors activation, while after 90 min D_2–receptors may mediate major parts of LSD reactions, which might explain the enormous range of effects LSD engenders in humans.[1,5,6,20]

Tolerance to autonomic and psychological effects of LSD occurs after 2–3 days with moderate daily doses. Sedative–hypnotics like diazepam are often used in the emergency room for acute presentations of LSD intoxication to help reduce panic and anxiety. The chronic administration of selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitor (MAOI) antidepressants decrease $5–HT_2$–receptors expression in several brain regions, thereby reduce LSD effects. Lithium and some tricyclic antidepressants have also been reported to increase the effects of LSD.[6,20]

Following the LSD administration, the most common psychiatric complication is episode of anxiety or panic (with severe, terrifying thoughts and feelings, fear of losing control, fear of insanity or death, and despair), the "bad trip". These complications are not lethal; however, they increase the risk of suicidal reactions. Other complicated reactions may include temporary paranoid ideation and, as after–effects in the days following a LSD experience, temporary depressive mood swings and/or increase of psychic instability.[7,20]

After moderate dose (75–150 µg p.o.), the phycological effects observed are euphoria; enhanced capacity for introspection; hypnagogic experience and dreams; illusions and pseudo–hallucinations; alterations of thinking and time experience; "bad trips"; flashback phenomena; psychosis–like symptoms; distorted perception of the size and shape of objects, movements, colour, sounds and touch, as the user´s own body-image as well. Although deaths causally linked to LSD, such as overdose, are extremely rare, its acute effects may promote irrational acts which lead to suicide or accidental death

incidents. At level of neurocognition, the acute effects are decrease of attention and concentration, thinking processes can be also affected, intellectual functions are impaired, psychomotor functions (coordination and reaction time) are frequently impaired memory was also affected. The somatic effects including mild autonomic changes of mydriasis, tachycardia, tachypnea, hyperthermia, hypertonia, and hyperglycemia; rarely, some increase in body temperature; respiration remains generally unchanged; parasympathetic stimulation: diaphoresis and salivation are frequent, nausea may occur, emesis is exceptional, and flushing of the face is more frequent than paleness; temporary headache and near–syncope; slight unsteadiness of gait to full ataxia, positive Romberg's sign, and mild tremor.[7]

1.4 Portuguese Drug Policy

The main drug law in Portugal is Decree–Law no. 15/93, of 22 January, which defines the legal regime applicable to the trafficking and consumption of narcotic drugs and psychoactive substances. It was passed by the national government in accordance with the United Nations Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances, 1988, which Portugal has signed. The Decree–Law annexes six tables listing the plants, substances, and preparation under control.[24,25]

During the 1990s, Portugal faced one of the highest prevalence rates for overdose deaths and several others drug–related issues in European Union (EU). In order to respond to the public health crisis, the Portuguese government gathered a committee of multidisciplinary experts – including physicians, lawyers, psychologists, and social activists.[26] The committee recommended the drug use discriminalization i.e., the elimination of criminal penalties for drug use and possession for personal use, regardless the kind of drug. In despite of its conservative values, the government adopted a ground–breaking response and followed most of the committee's recommendations thus passed Law no. 30/2000, of 29 November.[27]

On basis of average single dose (ASD), Law no. 30/2000 (Section 2) stipulates the threshold quantities of illegal substances defined as possession for personal use, these quantities derived from estimations of the average required for 10 days' consumption. The ordinance no. 94/96, of 26 March (Section 9), pursuant to Decree–Law no. 15/93, of

22 January (Section 71.a), stipulates the ASD for the most frequently consumed plants, substances or preparations listed in tables I to IV.[24,27,28]

In cases of possession of amounts below the threshold, where there is no suspicion of involvement in drug trafficking, the suspected drug users are referred to the local Commission for Dissuasion of Drug Addiction, composed of three members, one being a legal expert named by the Ministry of Justice, and two being medical doctors, psychologists, sociologists, social workers, or other qualified professionals on drug addiction. The two last are named by the Ministry of Healthy and by the Governor representant. Punitive administrative sanctions can be applied, but the main objectives are to explore the need for treatment and to promote healthy recovery.[27]

Drug trafficking in Portugal can incur a sentence of 1–12 years' imprisonment, depending on the specific criteria, such as the type of substance and the quantity. The Portuguese law also stablishes a more lenient maximum sentence (three years) for presumed offenders are selling drugs to finance their own addiction (supply–user). On the other hand, aggravating circumstances, which include trafficking as part of a criminal organization and if the offense causes death or serious bodily harm, drug trafficking sentences can increase to 25 years.[24]

1.5 Criminal Investigation

The criminal investigation comprises a set of legal steps, in light of the criminal procedure law, aiming at the assessment of whether a criminal offence has been committed, at identifying its perpetrator(s) and detecting his/their responsibility and at finding and collecting evidence.[29]

In Portugal, the criminal investigation is regulated by Law no. 49/08, of 27 August (Criminal Investigation Law). In the Section 2 is established that both the Public Prosecutor (at inquiry stage) and the Examining Judge (at the preliminary judicial stage) are assisted by criminal police bodies.[29] The Portuguese criminal police bodies are the Judicial Police (PJ), National Republican Guard (GNR), and the Public Security Police (PSP).[29]

The Portuguese Judicial Police is the higher criminal police body, organized under the aegis of the Ministry of Justice.[30] The Criminal Investigation Law at the Section 8

determines the PJ´s reserved competence, i.e., matters which cannot be assigned to other criminal police bodies, including the investigation on illicit drug trafficking.[29]

According to the Decree–Law no. 137/19, of 13 September (Judicial Police organizational structure), the Scientific Police Laboratory (LPC) is a central unit of technical and scientific support to criminal investigation. The LPC is composed by sectors concerning to a wide range of the forensic sciences specialties (**Figure** 1).[30]
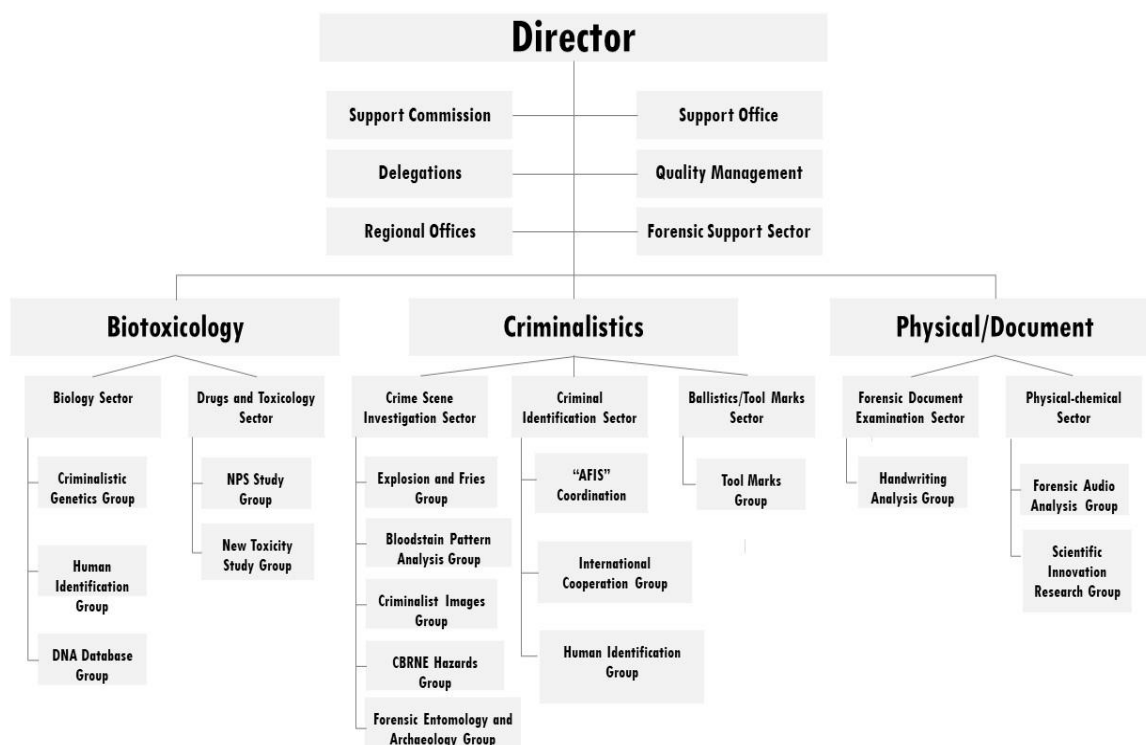
Figure 5 – LPC organizational chart.

The Drugs and Toxicology Sector (SDT–LPC) provides analytical identification of the illicit substances listed on tables I–VI of DL– 15/93, of 22 January, and performs the quantification of the substances listed on the ordinance no. 94/96, of 26 March as well. The SDT–LPC is also responsible to analyse and report the results of the new psychoactive substances (NPSs).[31]

1.6 **Illicit LSD Market in European Union**

The illicit drug market is a major global industry, encompassing production, trafficking, and retailing. It represents the major source of income for organized crime

groups (OCGs). The total value of the EU retail market for illicit drugs in 2017 was conservatively estimated at EUR 30 billion.[32]

The illicit drug market inflicts both direct and indirect impacts on society, extended beyond its financial value. In addition to the harms caused by the use of drugs themselves, links with wider criminal activities and terrorism, the negative impact on the legal economy; violence in communities; damage to the environment; and the increasingly important issue of how the drug market can fuel corruption and undermine governance.[32]

This section presents information from both national and international organizations, civil society, and open sources databases within the Europe, with emphasis on LSD. This type of information helps law enforcement and government officials to make more informed decisions.

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) gathers data related to drug reported by the 28 EU Member States, Norway and Turkey, and provides them with a factual overview of European drug issues and a solid evidence base to support the drug debate. EMCDDA collects Portuguese data from the General–Directorate for Intervention on Addictive Behaviours and Dependencies (SICAD) and from the National Unit to Combat Drug Trafficking (UNCTE) of Judicial Police.

The majority of the reporting countries reports data on seizures in terms of number of seizures and in terms of the quantity seized. These data are an important indicator of drug market, once they are related to all seizures made in each country by their law enforcement agencies. The majority of the reported seizures involve small quantities of drugs confiscated from users.[33]

Although the use as a recreational drug became popular during the 1960s, and returned in the 1980s, LSD is still one of the most often used hallucinogenic substances in Europe. The analysis of the entire EMCDDA´s database on reported seizures of hallucinogens substances (2002–2018), could lead to a misleading interpretation since there was no reported data on hallucinogens seizures except LSD until 2010. From 2010 the law enforcement agencies were encouraged to report other hallucinogens substances, thence prior data is purposely omitted in this section.
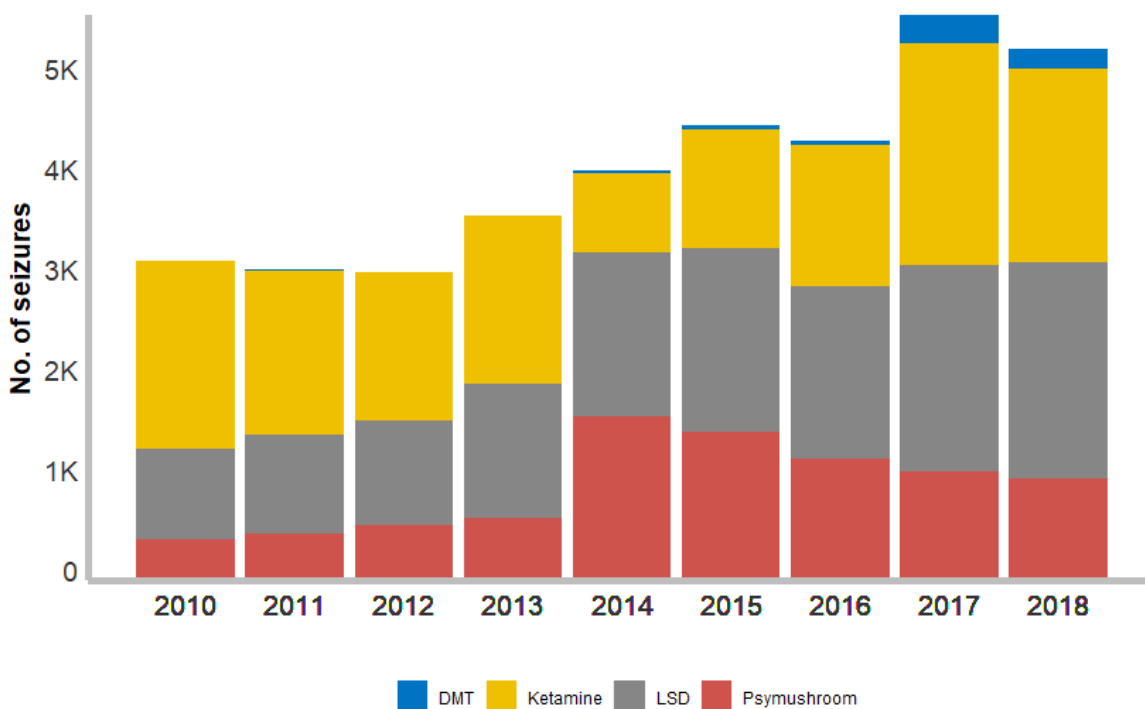
Figure 6 – Number of reported seizures of Ketamine, DMT and hallucinogens in EU. Source: EMCDDA.[34]

Between 2010 and 2018 there were more than 36,000 seizures of hallucinogens substances reported across the Europe (**Figure 6**). Ketamine is the most frequent substance reported (38.5%), followed closely by LSD (36.9%). The total seizures rate has slightly increased and peaked in 2017, when there were reported 5,626 seizures.[34]

The number of seizures of LSD reported (**Figure 7 (A)**) has slightly increased between 2002 and 2009, and it has more than doubled between 2010 and 2015. It represents an increase of more than 167% compared to the first year reported. In 2018 there were 2,153 seizures reported, the highest level so far.[34]
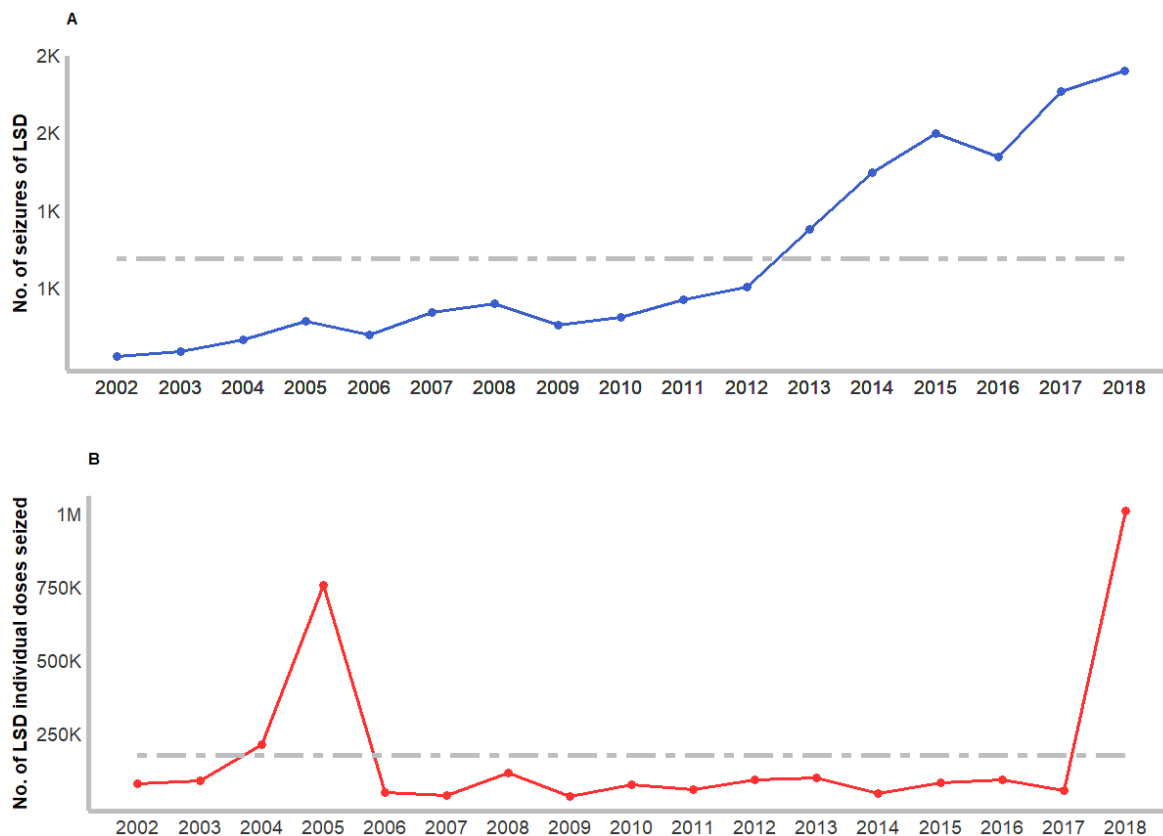
Figure 7 – (A) Number of seizures of LSD in European Union. (B) Quantity of individual doses of LSD seized in European Union.
Source: EMCDDA.[34]

The quantity of individual doses of LSD seized (**Figure 7 (B)**) looks levelled off, with the exception of 2005 and 2018.[34] However, quantities seized may fluctuate from year to another, due to a small number of large seizures. For instance, most of the quantity seized in 2018 (93 %) is due to the seizure of 788,606 LSD´s doses by the Spanish Civil Guard and the Austrian Federal Police, supported by Europol.[35]

In Portugal, the highest number of illicit drug seizures involves cannabis resin, cocaine, herbal cannabis, and heroin.[36] The number of seizures of LSD (**Figure 8 (A)**) has been generally low and stable for a number of years. Although, Portugal is the seventh country ranked in quantity of LSD individual doses seized **(Figure 8 (B))**.[34] Besides, the LSD´ blotters, there were two seizures of LSD under gum unit dose (2017 and 2018) and one seizure of LSD alcohol solution.[34,36,37]
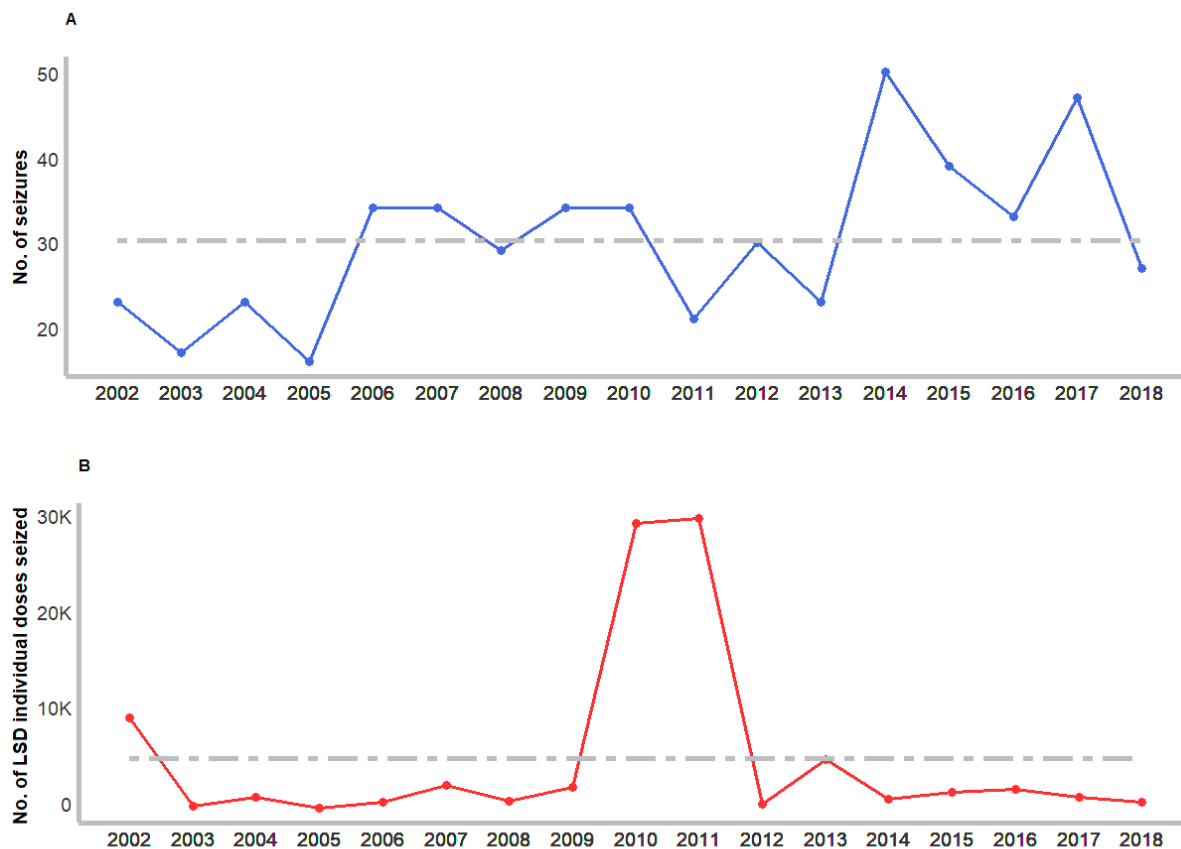
Figure 8 – (A) Number of seizures of LSD in Portugal. (B) Quantity of individual doses of LSD seized in Portugal.
Source: EMCDDA.[34]

Nonetheless, LSD appears to become more available. Special attention must be paid to the increase of electronic music events in Portugal in the last two decades. In 2019, 278 music festivals occurred in Portugal, amounting more than 2,1 millions of people attended.[38] It is well known the pattern use of certain drugs among the attenders of electronic dance music, including substances alleged to be LSD.[39] Some of these events provide drug–checking services (sometimes called pill testing), which enables individual drug users to have their synthetic drugs chemically analyzed. In 2016, a drug–checking service implemented at one of the biggest electronic music festival analyzed 745 samples, in which 42 % were believed to be methylenedioxymethamphetamine (MDMA), and 28% were believed to be LSD.[39]

During the last study conducted by EMCDDA on illicit drug web market, LSD was the most common hallucinogen offered and there was a large variation in the price of retail doses (250 µg or less). The average retail prices were lowest on the Italian IDC 2.0 market EUR 11.00 (± 4.7) and highest on the Russian Hydra market EUR 23.4 (± 4.5).[40]

The overall prevalence levels of LSD use in Europe is low, compared to other illicit drugs, such as cocaine, cannabis and MDMA. The average estimative of lifetime experience within the adult population (15–64) is 5.24 % (±3.06), the highest level are observed for population aged 25–34, 4.48% (±3.15).[34]
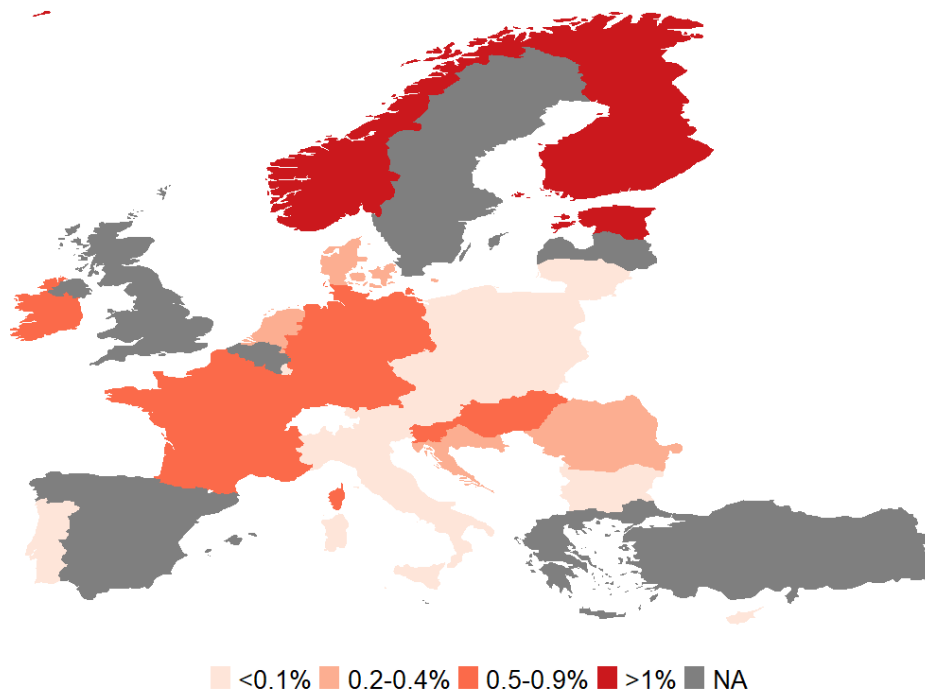
Figure 9 – Last year prevalence use of LSD among young adults (15–34).
Source: EMCDDA.[34]

Among young adults (15–34), national surveys report last year prevalence estimates average of 0.53 % (±0.49) in 2018 or most recent survey year, with the exception of Finland (2.0 %) , Estonia (1.7 %), and Norway (1.3 %).[34] In meanwhile, Portugal shows one of the lowest last year prevalence among young adults levels of Europe (0.1 %), and a lifetime prevalence use in the general population relatively low (0.4 %).[34,36]

Hospital emergency data is useful for assessing both the acute drug harms and the public heath impact related to drug use, although only a small number of countries reported these data. Most of the available data is reported by sentinel station system without national coverage.[33] In 2018, there were reported (25 countries) 1,633 acute emergencies related to hallucinogen substances, in which 729 were due to ketamine and 184 due to LSD.[34] Moreover, data from 27 sentinel hospitals in 19 European countries (n

= 9,134) shows that ketamine and LSD are, respectively, the 16th and 18th most frequent drugs recorded in emergency presentation. Due to under–reporting, these are likely to be minimum figures.[33]
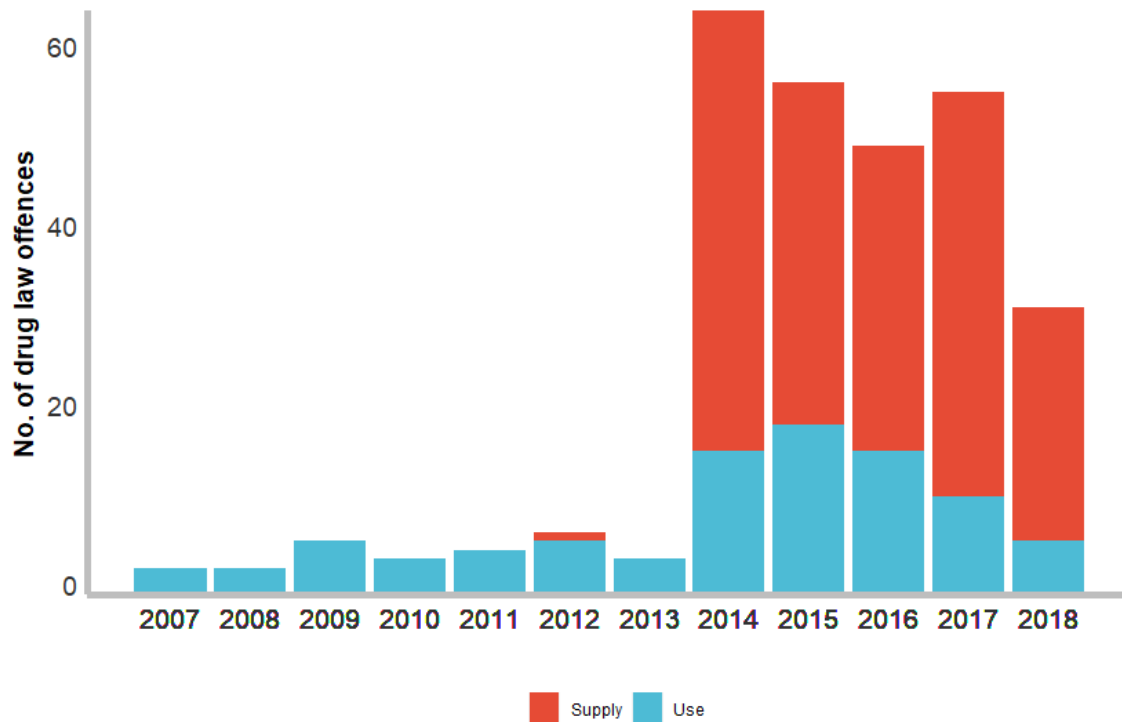
Figure 10 – The number of drug law offences related to LSD, by their nature.
Source: EMCDDA.[34]

The overall number of drug law offences (DLOs) related to LSD is relatively low (n = 292). Mainly, until 2013 there were only use–related offences reported. After 2014, the number of supply–related offences (including both "supply" and "use–supply" categories) reported have been significantly higher than the use–related offences.[34]

The pattern distribution of the nature of the DLOs linked to LSD in Portugal (66.1% were supply–related offences) differs both from the Portuguese general picture viewed in other illicit drugs cases (79.9 % were use–related offences), and the LSD´ DLOs cases in Europe, in which 69.2 % were use–related offences.[34]

1.7 The Aim of This Thesis

The LPC is responsible for analyzing all illicit drugs seized in Portugal. The aim of this work is to develop and validate a GC-MS method for analyzing LSD, suitable to be implemented in the LPC routine.

2 *Analytical Methods and Method Validation*

2.1 **Analytical Methods**

In this work, Thin–layer chromatography (TLC) and gas chromatography– mass spectroscopy (GC–MS) were used for the analysis of seized materials suspected to contain LSD, therefore they fundamentals and operating will be briefly described.

Techniques for the analysis of drug samples may be classified into three categories based on their discriminating power (Table 1). International regulatory agencies such as the European Network of Forensic Science Institute (ENFSI) and the United Nations Office on Drugs and Crime (UNODC), recommend the combination of more than one analytical method to ensure more reliable results.

For example, when a Category A technique is incorporated into an analytical scheme, then at least one other technique (from either Category A, B or C) must be used. On the other hand, when a Category A technique is not available, then at least three different validated methods must be employed. In cases where hyphenated techniques are used e.g. gas chromatography coupled with mass spectrometry, will be considered as separate techniques provided that the results from each are used.[41–43]

In the routine of most forensic chemistry laboratories, screening tests (e.g., colour test, TLC, and immunoassay) are prior conduced to provide a rapid but non–confirmatory result. Nevertheless, positive results to these tests are only presumptive indications of the possible presence of the suspected drug. Likewise, negative results do not ensure the absence of the substance. It is therefore mandatory to confirm such results by use of complementary techniques.

Table 1: Categories of Analytical Techniques.

Category A	Category B	Category C
Infrared spectroscopy	Capillary electrophoresis	Colour tests
Mass spectrometry	Gas chromatography	Fluorescence spectroscopy
Nuclear magnetic resonance spectroscopy	Ion mobility spectrometry	Immunoassay
Raman spectroscopy	Liquid chromatography	Melting point
	Pharmaceutical identifiers	Ultraviolet spectroscopy
	Thin layer chromatography	

Adapted from Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG), 2013.[43]

2.1.1 *Colour test*

LSD can be identified by a colorimetric method. The LSD methanolic extract is spotted onto filter paper, dried, and examined under ultraviolet light (360 nm). LSD gives a strong blue fluorescence. However, most of ergot alkaloids, whether or not under legal control, will give similar results.[44]

2.1.2 *Thin–layer Chromatography*

Thin Layer Chromatography is a technique used to isolate and identify non–volatile mixtures. TLC is one of the fastest, least expensive, simplest, and easiest chromatography technique. Like other chromatographic techniques, TLC depends on the separation principle. The separation relies on the relative affinity of compounds towards both the phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

LSD can be analyzed by TLC. The methanolic extract is spotted to the silica plate and placed into the TLC chamber, previously saturated with the solvent system (acetone). Once the spots are developed, the Ehrlich's reagent (*p*–dimethyl–aminobenzaldehyde) is

applied. The dried plate is examined under ultraviolet light (360 nm) and a blue/purple stain indicates the presence of LSD.[17,44]

2.1.3 *Gas Chromatography–Mass Spectroscopy*

Gas chromatography coupled with mass spectroscopy (GC–MS) is considered the gold standard in chemistry and toxicological forensic analysis. It overcomes limitations inherent to their individual applications e.g., Gas-Chromatography (GC) can separate many volatile and semi–volatile compounds but not always selectively detect them whereas Mass Spectrometry (MS) can selectively detect many compounds but is uncapable to separate them. GC–MS is an ideal technique for both qualitative and quantitative determination of volatile and semi–volatile compounds in a wide variety of samples.[45]

2.1.3.1 Basic Principles and Instrumentation

The effluent (individual compounds) elutes from the GC capillary column, which is inserted directly into the MS ion source. There is a variety of ionizations techniques used for mass spectrometry. Some ionization techniques are very energetic and cause extensive fragmentation e.g., electron ionization (EI), whereas other techniques are softer and only produce ions of the molecular species e.g., chemical ionization.[46] EI source, formerly called electron impact, is the most widely used in organic mass spectrometry. The major libraries and databases of mass spectral data are of EI mass spectra.[47]

In the EI source, ions are generated by bombarding the gaseous sample molecules with a beam of high–energetic electrons (generally 70 eV). EI produces a mixture of positive and negative ions, as well as neutral species. Positive ion EI mass spectra are more commonly recorded because these ions form more readily. Since approximately 10 eV is enough to ionize most organic molecules, the excess energy leads to extensive fragmentation by breaking covalent bonds. The fragmentation process is predictable and is the source of powerful structure elucidation potential of mass spectrometry.[46,47]
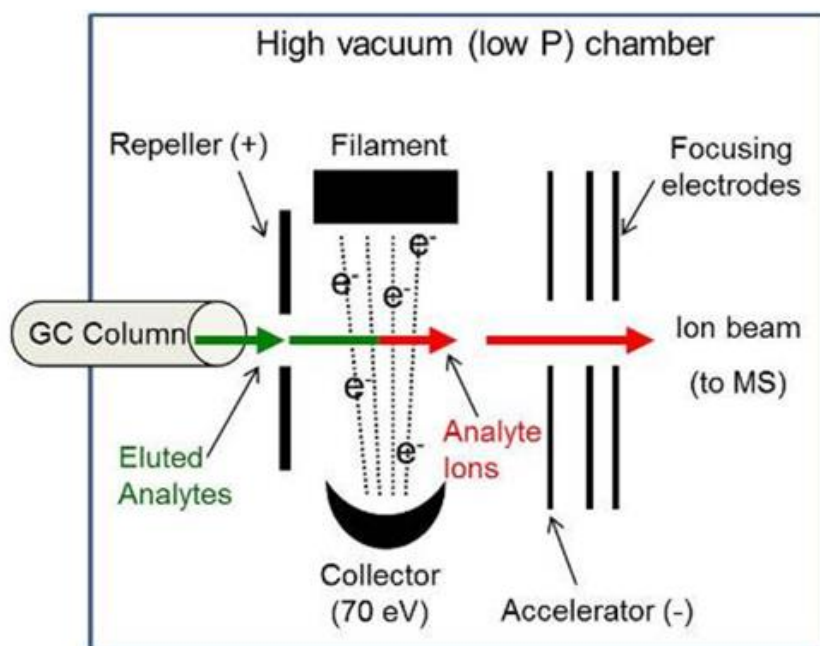
Figure 11 – Schematic of an electron ionization source.
Adapted from Schug, 2014.[48]

This ionization technique works well for many gas–phase molecules but induces extensive fragmentation so that the molecular ions are not always observed. Reduction of the ionization voltage is a commonly successful used strategy to obtain a discernible molecular ion. On the other hand, the fragmentation reduction yields different mass spectrum that cannot be compared to spectra library.[46]

Once the gas–phase ions have been produced, they need to be separated according to their masses, which must be determined. The physical property of ions that is measured by a mass analyzer is their mass–to–charge ratio (*m/z*) rather than their mass alone. Several mass analysers have been developed, and each of them has its advantages and limitations.[46]

Quadrupoles are mass analysers (or mass filters) which consist of four rods with DC and RF voltages applied. An ion of a specific ratio *m/z* will be stable and can pass through the quadrupole only when a specific DC/RF voltage combination is applied.[46,49]
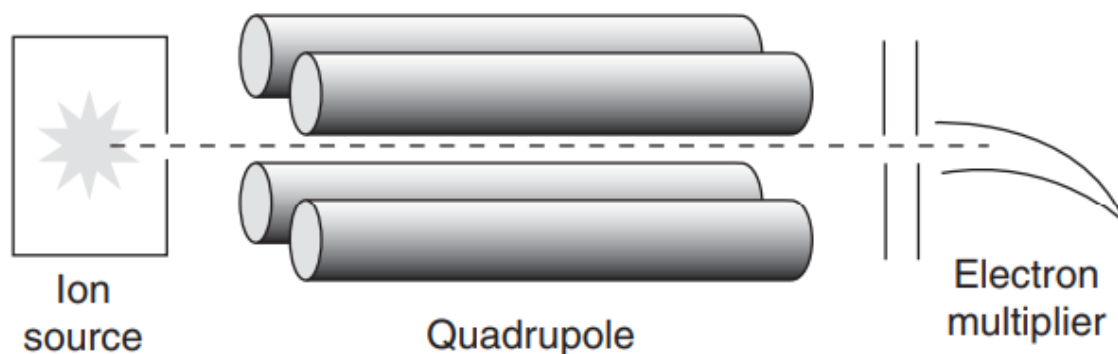
Figure 12 – Schematic diagram of quadrupole mass analyzers.
Adapted from Sleeman, 2005.[49]

2.1.3.2 Data Acquisition System

Single quadrupole can be operated in two modes. The most selective mode to a single quadrupole mass analyzer is the selected ion monitoring (SIM). Hereby, a fixed set of DC and RF voltages is applied to the quadrupole and thus only a single *m/z* can pass. Ions with different *m/z* are filtered out. It increases sensitivity for the target analytes through the selective detection of ions most indicative of the compounds of interest. In total ion current (TIC), the equipment acquires a continuous range of *m/z* data to detect all possible compounds within the sample, however, it has less sensitivity and specificity compared with SIM mode.[46,49]

Moreover, it is also possible to obtain scan information and SIM information quite simultaneously throughout the chromatographic run. In synchronous SIM–Scan, the equipment rapidly and sequentially switches between a TIC acquisition and a SIM acquisition. It is useful for unknown sample analysis when both targeted and non–targeted analytes may be present. Nonetheless, optimal quantitative approaches are achieved at lowest speed scan acquisitions, which are obtained by singular SIM methods.[50,51]

In SIM mode, it is common practice to monitor three ions per compound: the signal of one ion is used for quantitating, and the other are used for qualitative information. Usually, only two ions are monitored for internal standard (IS): one for quantitating and one qualifier. When using SIM ion area to quantitate the peak, it is recommended to set

15 to 20 scan data points over each peak. This parameter must be evaluated by examining the standard solution at the lowest concentration.[47,52]

2.1.3.3 Internal Standard Method

The internal standard method is used to improve quantitative analysis. This method is based on a comparison of the intensities of the signal corresponding to the target analyte to be quantified with one of a reference compound called the internal standard. A known amount of internal standard is added to all the samples, standards, and blanks. The calibration curve is prepared where the y–axis is the ratio of responses and the x–axis is the analyte concentration in the standards.[46,53]

The internal standard method can compensate for certain types of errors when they influence both the analyte and the reference species to the same proportional extent. Thereunto the chosen reference species should show physical and chemical properties that are as close as possible to those of the target compound. Moreover, the internal standard must be absent from the sample, and its added amount should be in an appropriate ratio to the analyte after consideration of the dynamic range.[46]

2.1.3.4 Derivatization

GC–MS techniques are proved to analyse numerous volatile and thermostable analytes, demanding little or none sample preparation prior to analysis. However, several drugs are molecules with polar functional groups, which in their natural form are poorly vaporized and barely enter into the capillary column in order to be eventually detected. A chemical derivatization step is required to create sufficiently volatile and thermostable forms of those compounds.[55]

Although derivatization is regarded as one of the most critical step of sample preparation due to its time consuming and the demand of careful handling, there are several advantaged associated with the GC analysis of derivatized compounds e.g., increased selectivity, sensitivity and the possible identification and quantification of numerous species on a single column, simultaneously.[56]

Analytes with active hydrogens from the functional groups such as –COOH, –NH, and –OH can be derivatized by alkylation, acylation, or silylation. Silylation is the most widely used derivatization procedure for GC analysis, in which an active hydrogen atom

is replaced by a silyl group. The most common silylation procedure is the trimethylsilylation. A number of silylation reagents are used, each of which has its own advantages and disadvantages.[57] Further discussion is presented in Section 4.1.

The MSTFA (*N*–Methyl–*N*–trimethylsilyl–trifluoroacetamide) is an effective trimethylsilyl donor like BSA (*N,O*–Bis–trimethylsilyl–acetamide) and BSTFA (*N,O*– bis–trimethylsilyl–trifluoroacetamide). The LSD trimethylsilyl derivative (TMS–LSD) is formed by the displacement of the active amine proton at the indole moiety. TMS–LSD is more volatile, less polar, and more thermally stable; hence it is possible to obtain a spectra with more structural information.[58,59]
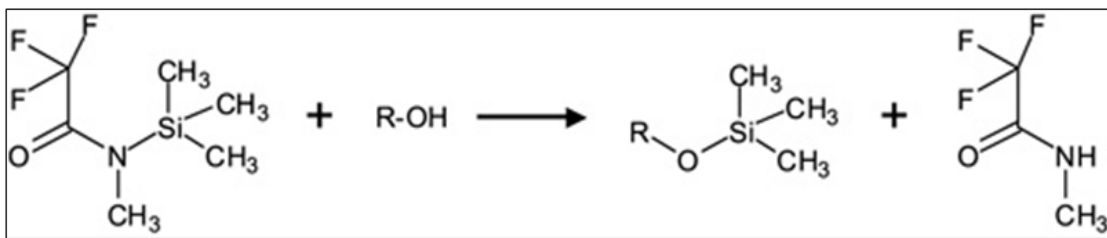
Figure 13 – Silylation of hydroxyl groups with MSTFA and the formation of the byproduct *N*–methyltrifluoroacetamide.
Alcohol (R–OH) shown, but reaction also applies to R–COOH, R=NH, R–NH2, R–SH.
Adapted from Knapp, 1990.[60]

All TMS derivatives are more sensitive to moisture, therefore must be handled under dry conditions. The addition of desiccant is highly recommended for later reuse. Also, the TMS derivatives are more susceptible to hydrolysis than their parent compounds.[58,59]

2.2 Sampling

According to ISO/IEC 10725, sampling is defined as a procedure whereby a part of a substance, material or product is taken to provide for testing or calibration a representative sample of the whole.

Once seized materials are not common samples but unique prosecution's evidence, the sampling strategies must meet the needs for the prosecution and courts in their specific situation, thereby considering the costs and laboratory management aspects.

A representative sampling can be performed on a population of units in which similar external characteristics are observed. If more than one group of homogeneous material are presented in a seizure, they must be separated into as many groups as dissimilarities. Each group will be considered as a whole population and will be sampled and analyzed individually.[65,66]

Sampling includes both the sampling to obtain the laboratory sample and the subsampling in the laboratory to obtain the test sample from which the test portion will be drawn.[61]

The arbitrary sampling is often used by scientific police laboratories worldwide. However, they have no statistical foundation and may lead to an exceptionally large sample in case of large seizures. The United Nations Drug Control Program (UNDCP) recommended method is bellow demonstrated:[66]

for N<x ∴n=N ;

for x≤N≤y ∴n=z ;

for N>y ∴n=√N

where, x=10, y=100 e z=10.

2.3 Validation of Analytical Methods

The objective of analytical forensic methods is to obtain results with a measurement quality relevant for the criminal justice purpose i.e., get consistent, reliable, and accurate results. Thus, before being implemented in laboratory routine these methods must be evaluated and tested to prove in an objective way that they are suitable for their intended application. This previous step is called validation.[41,42]

The validation of a method follows standardized set of experimental tests. ISO/IEC 17025 is the most relevant standard for chemical laboratories. It stablishes that all method developed in–house should be validated, and the validation must be as extensive as

necessary to meet the requirements. It is used to assess laboratories that seek accreditation status.

The data produced throughout the validation process is used to assess the quality of results. In this work were adopted the three related performance characteristics *trueness*, *precision,* and *uncertainty* to describe the method performance. It is important to note that different guidelines may use other concepts, such as types of error (random, systematic and gross errors), accuracy (trueness and precision) and uncertainty.[41,42,61]

2.3.1 *Selectivity*

Selectivity is a parameter concerned with the extent to which the method can be applied to identify and/or quantify the analytes in the presence of other similar substances, in a sample matrix under the stated conditions of the method.[42,61]

In many types of analysis, selectivity is essentially a qualitative assessment. However, for quantitative purposes, it is important to stablish that the measured property is only due to the analyte.[61,62]

Interferences may cause bias by either increasing or decreasing the signal attributed to de measurand. There are two kinds of interference effects: i) the proportional effect, in which the magnitude of the effect for a given matrix is proportional to the signal. It affects the slope of the calibration function; ii) the fixed effect, in which the effect is independent of the concentration of the analyte. It is often referred to as a background or baseline interference and affects the intercept of a calibration function.[61]

The selectivity of a method is usually investigated by studying its ability to measure the analyte of interest in samples to which specific interference have been deliberately introduced.[42]

2.3.2 *Limit of Detection and Limit of Quantification*

The limit of detection LOD is the lowest concentration of the analyte that can be detected by the method at a specified level of confidence. Likewise, the lower limit of quantification LOQ is the lowest level at which the analyte can be quantified by the method at a specific level of confidence.[61]

It is important to distinguish between the instrument detection limit and the method detection limit. The first, can be assessed by presenting a blank sample directly to the instrument without any sample preparation or by based on signal–to–noise measures e.g., chromatographic analysis. To assess the method, the samples must have been submitted through the whole measurement procedure.

There are several approaches for assessing LOD and LOQ. For methods where a measurable signal is obtained for a blank (e.g., spectrophotometry), the analysis of blank samples (i.e., matrices containing no detectable analyte), work well. However, for chromatographic methods, samples with concentration levels close to or above the LOD are required, once these techniques depending on detecting a peak above the noise.[61]

In practice, LOD and LOQ are calculated to be the analyte concentration corresponding to the standard deviation (s) at low levels multiplied by a factor K.

2.3.3 *Linearity and Model Choice*

The relationship between an analytical method response and the concentration of an analyte is mathematically demonstrated throughout a calibration model. The most often calibration model is the simple linear regression using the least squares method, although in some cases the calibration data are best treated by weighed regression.[43]

In accordance with ISO 8466 rules (ISO 8466–1 and 8466–2) the linearity of the analytical response must be studied by means of statistical tests, that allow to decide the best fit model.[67]

2.3.3.1 Homoscedasticity

Whether the chosen linear model is adequate can be investigated informally by plotting the residuals over the x values. A random distribution of the residuals about zero (homoscedasticity) suggests the model is suitable.[63] On the other hand, systematic trends indicate nonlinearity (heteroscedasticity) or a change in variance with level, it means that the calibration data are best treated by weighted-least squares (WLS) regression.[64]

The test of homoscedasticity can be carried out by means of the Hartley–Test (based on Fischer distribution). The null hypothesis H_0 assumes that the lower calibration point variance s_{min}^2 is equivalent to those the higher point s_{max}^2 i.e., homoscedasticity.[64,65]

$$TV(f) = \frac{s_{max}^2}{s_{min}^2} \qquad \qquad \textbf{2--1}$$

The $TV(f)$ must be compared to the critical value $f_{0.01(v_n;v_1)}^b$. If the calculated test value exceeds the critical value, the null hypothesis must be rejected and a WLS approach is suggested.

2.3.3.2 Linearity

The Mandel´s fitting test is an improved version of the f–test, in which the effects of the degrees of freedom are considered. The null hypothesis H_0 assumes the polynomial model does not lead to a significantly better adjustment compared to the linear model. The test value $TV(f_M)$ is obtained by subtracting the sum of squares $ss_{resid(1)}$ and $ss_{resid(2)}$ divided by the difference of their respective number of degrees of freedom $(n-2)$ and $(n-3)$:[63,66]

$$TV(f_M) = \frac{\left(ss_{resid(1)} - ss_{resid(2)}\right)}{(n-2)-(n-3)} \times \frac{1}{s_{resid(2)}^2} \qquad \qquad \textbf{2--2}$$

The $TV(f_M)$ calculated must be compared to the critical value. If the calculated $TV(f_M)$ is greater than the critical value $f_{0.01(1;n-3)}^u$, the null hypothesis H_0 must be rejected in favor to the alternative hypothesis H_A. Thus, an indication of non–linearity of the calibration function is obtained.[63,67,68]

2.3.4 *Working Range*

The working range is the interval over which the method provides results with an acceptable uncertainty.[61] The lower limit of the working range is often considered the limit of quantification, LOQ. The upper end of the working range is defined by concentration at which significant anomalies in analytical sensitivity are observed.[41,42]

The method working range relates to the concentration in the laboratory samples i.e., the concentrations in the samples coming into the laboratory. The instrument working

range refers to the analyte concentrations in the test samples i.e., samples submitted through the whole measurement procedure (the solution injected into GC).[41] During validation both the instrument working range and the method working range should be assessed.[42,61]

Linearity is an important parameter used to investigate the relationship between the signal response (e.g., peak area ratio of the analyte and internal standard) and analyte concentration, within the lower and upper ends of the working range. It aims to determine the kind of this relationship (e.g., linear, quadratic, exponential etc.), demonstrate that the working range is suitable for the purpose and verify whether the proposed instrument calibration procedure is adequate.[61]

Linearity is firstly assessed by visual inspection. However, this is not sufficient to prove that the method is fitted for purpose. The full assessment is supported by data from precision and trueness studies and significance statistical tests.[42,61]

2.3.5 *Precision*

Precision is a measure of the closeness agreement between independent test results obtained under stipulated conditions.[62] It is usually expressed by statistical parameters which describe the spread of the results e.g., the standard deviation (S) or relative standard deviation (RSD), calculated from a from a series of replicate measurements under specified conditions.[64]

 The two most common precision measures in a single laboratory validation are repeatability and within–laboratory reproducibility (also called intermediate precision).[41] Repeatability is a measure of the variability in results when a measurement is performed by a single analyst using the same equipment over a short timescale. Within–laboratory reproducibility is a measure of the variability with different analysts and over extended timescales, within a single laboratory.[41,64]

Precision is generally concentration dependent and should be measured at different concentrations within the working range, typically at the lower, mid, and upper parts. If relevant, the relationship between the precision and the analyte concentration should be established e.g., verify whether the standard deviation is proportional to, or linearly dependent on, analyte level.[41,61,62]

2.3.6 *Trueness (Bias)*

Trueness is an expression of how close the mean of a set of measurements (produced by the method) is to a reference value. In consideration of the finitude of the number of results measurements, trueness cannot be measured but assessed. This assessment is expressed quantitatively in terms of *bias*. [41,61]

Bias of analytical method is usually determined by the study of reference materials RMs, recovery experiments using spiking samples or by comparison with results obtained with another method.[69] Significance testing is highly recommended.

In the absence of suitable reference materials, recovery studies (spiking experiments) may be used to give an indication of the likely level of bias. This approach consists in analyzing samples by the method under validation both in its original state and after the addition of a known quantities of analyte to the test portion. The result (bias) may be expressed in absolute terms or, more often, as a relative spike recovery (also denoted apparent recovery).[62,69]

Although a good recovery is not a guarantee of trueness, a failure to determine part of or all the analyte present certainly reflect an inherent problem with the method. Hence, it is necessary to assess the efficiency of the method for detecting all of the analyte presence.[62,69]

The acceptability of bias should be decided on basis of overall bias measured (method bias and laboratory bias) against the reference value. Bias should be shown to be negligible or corrected for, but in either case the uncertainty associated with the determination of the bias remains an essential component of overall uncertainty.[69]

2.3.7 *Measurement Uncertainty*

Uncertainty is an interval associated with a measurement result which express the range of values that can reasonably be attribute to the quantity being measured.[69] Although the word *uncertainty* relates to the general concept of doubt, the knowledge of the uncertainty of measurement provides assurance that results and conclusions from method are fit for purpose.[42]

The uncertainty on the result may arise from many possible sources e.g., sampling, environmental conditions, uncertainties related to laboratory equipment, random effects, etc. In estimating the overall uncertainty, all these effects should be taken in account.

2.3.8 *Processed Sample Stability*

It is important to demonstrate the extent to which the analyte is stable during the whole analytical procedure.[42] It includes the circumstances in which samples that have undergone the method preparation cannot be immediately analyzed.[43]

2.3.9 *Outliers on Calibration Fit*

The ordinary-least squares (OLS) method is strongly sensitive to the presence of deviating points in the dataset. Once it is based on minimizing the sum of squares of the y–residuals, a suspect point with a large y–residual can have a significant effect on the estimated slope and intercept of the regression line, and thus on the analytical information obtained. The Mandel´s test measures the influence of a suspect calibration point on whether increasing the total variation of the adjust s_{resid}^2 with n and $n - q$ experimental values.[70]

The null hypothesis H_0 states that the model with the suspected value removed does not decrease the random error $H_0: s_{resid(\text{n}-\text{p})}^2 \leq s_{resid(\text{n}-\text{p}-\text{q})}^2$.

$$TV(f_M) = \frac{\left(ss_{resid(\text{n})} - ss_{resid(\text{n}-\text{q})}\right) \Big/ \left((n - p) - (n - p - q)\right)}{s_{resid(\text{n}-\text{p}-\text{q})}^2} \qquad \textbf{2–3}$$

$ss_{resid(\text{n})}$ is the sum of squares of the model with all n calibration points and $ss_{resid(\text{n}-\text{q})}$ is the sum of squares of the model without q suspected points. It is highly recommended to remove one suspected calibration point at once. If the test value $TV(f_M)$ is lower than or equal to the critical value $f_{0.01(q;n-q-p)}^b$ the null hypothesis H_0 is accepted i.e., the removed value does not contribute for weakening the fit and, therefore must be retained. On the other hand, if the null hypothesis H_0 is rejected in favor to the alternative hypothesis H_a the suspected value can be safely removed.

2.3.10 *Standard Deviation of the Adjust*

The standard deviation of the adjust demonstrates s_{fit} mathematically the quality of the model increases therefore the sensitivity. In the case of OLS adjust the sensitivity is constant (b_1) over the entire working range. The s_{fit_1} for OLS model is giving by 2–4):[65]

$$s_{fit_1} = \frac{s_{resid(1)}}{b_1} \qquad \qquad \textbf{2–4}$$

In the event of a WLS model, the sensitivity is giving by the first derivative of the calibration function. The s_{fit_2} is derived from the residual standard error and the sensitivity E in the centre $\bar{x}_i$ of the working range.

$$s_{fit_2} = \frac{s_{resid(2)}}{E} \qquad \qquad \textbf{2–5}$$

Where E is the slope (tangent) of the calibration function at the centre $\bar{x}_i$ of the working range:

$$E = b_1 + 2 \cdot b_2 \cdot \bar{x}_i \qquad \qquad \textbf{2–6}$$

2.3.11 *Calibration Curve Limits Values*

There are several approaches for assessing the detection and quantification limits e.g., determination on blank, signal/noise or by assessing the linearity study. The method for estimating the analytical limits by using data from the linearity study are demonstrated below. The first step is estimating the dependent variable limits (y_{LOD} and y_{LOQ}):

$$y_{LOD} = 2 \times t^u_{0.05(n-p)} \times s_{fit} \qquad \qquad \textbf{2–7}$$

$$y_{LOQ} = 6 \times t^u_{0.05(n-p)} \times s_{fit} \qquad\qquad 2\text{--}8$$

in which $t^u_{0.05(n-p)}$ is the quantile of t–distribution and s_{fit} is the standard deviation of the adjust.

For the correct estimate of both y_{LOD} and y_{LOQ}, they must be converted into the sample domain by the calibration model:

$$x_{LOD} = \frac{y_{LOD} - b_0}{b_1} \qquad\qquad 2\text{--}9$$

$$x_{LOQ} = \frac{y_{LOQ} - b_0}{b_1} \qquad\qquad 2\text{--}10$$

3 Materials and Methods

3.1.1 *Glassware and Laboratory Equipment*

The glassware and laboratory equipment used in this work are listed below:

- Adam® analytical balance

- Binder® heating chamber ED 400

- Hirschmann® volumetric lasks 1, 2, 5 and 10 mL

- Vortex mixer

- Normax® Pasteur pipette

- Gilson® pipettes P20, P100 and P1000

- Gilson® tips 2 and 200 µL

- Agilent® vial 2 mL, PTFE septa

- Agilent® insert polypropylene 250 µL

3.1.2 *Standards and Reagents*

The standards and reagents used in this work are presented in **Table 2**:

Table 2: Standards and reagents.

Reference	Description	Molar weight (g/mol)	Supplier	Purity (%)	Lot
Lysergic acid diethylamide (LSD)	Primary standard	323.44	Lipomed	>98.5	LSD397FB50
Tetracosane	Internal standard	338.65	Sigma–Aldrich	99	MKBL7013V
Methanol (MeOH)	Solvent	32.042	Merk	≥99.9	1917250

Chloroform (CHCl_3)	Solvent	119.38	Merk	≥99.9	K51085045908
n–Hexane	Solvent	86.18	Merk	≥99.9	H1043741000
N,O–Bis–trimethylsilyl–trifluoroacetamide (BSTFA)	Derivatization reagent	257.40	Sigma–Aldrich	–	BTRF850
N–Methyl–N–trimethylsilyl–trifluoroacetamide (MSTFA)	Derivatization reagent	199.25	Sigma–Aldrich	–	BCBN4262V
Pyridine	Reagent	79.1	Merk	–	1097280500

The NPSs used in interference studies are listed in **Table 3**. These substances were provided by Slovenia National Forensic Laboratory (SNFL) in the context of the RESPONSE PROJECT.[71]

Table 3: Potential interfering substances.

Common names	Systematic name	Molar weight (g/mol)	Structural formula
1P–LSD	N,N-diethyl-7-methyl-4-propanoyl-6,6a,8,9-tetrahydroindolo[4,3-fg]quinoline-9-carboxamide	379.22	
5–APB–NBOMe	1-(benzofuran-5-yl)-N-(2-methoxybenzyl)propan-2-amine	295.38	
25C–NBF	[2-(4-chloro-2,5-dimethoxyphenyl)ethyl][(2-fluorophenyl)methyl]amine	323.79	
25I–NBF	N-(2-fluorobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine, monohydrochloride	415.24	
25I–NBOH	2-({[2-(4-iodo-2,5-dimethoxyphenyl)ethyl]amino}methyl)phenol	413.26	

| 25IP–NBOMe | {2-[2,5-dimethoxy-4-(propan-2-yl)phenyl]ethyl}[(2-methoxyphenyl)methyl]amine | 343.47 | |
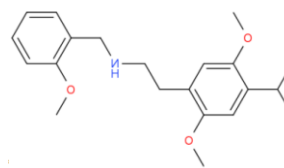
3.1.3 *Preparation of Standard Working Solutions*

For the preparation of standard working solutions, 1.1 mg of LSD was weighed out and dissolved in methanol (MeOH) to give a 1.1 mg/mL, which was diluted again to give a 100 µg/mL stock solution. The highest concentration standard working solutions were then prepared by serial dilution of the stock solutions (Table 4), while the lowest concentrations working solutions were prepared by diluting the 100 µg/mL standard solution (Table 5). This procedure was adopted with the intention of reducing the risk of pipetting error. All standard solutions were stored in the laboratory refrigerator prior to analysis.

Table 4: Preparation of LSD highest concentration level standard solutions.

Solution	Added volume µL	Concentration µg/mL	Final volume mL
ss_1	600	60	1
ss_2	500	50	1
ss_3	400	40	1
ss_4	300	50	1
ss_5	200	20	1
ss_6	150	15	1
ss_7	100	10	1

Table 5: Preparation of LSD lowest concentration level standard solutions.

Solution	Added volume µL	Concentration µg/mL	Final volume mL
ss_8	500	5	1
ss_9	200	2	1
ss_{10}	100	1	1
ss_{11}	50	0.5	1

3.1.4 *Preparation of Internal Standard Solution*

The internal standard solution was prepared by dissolving 250 mg of tetracosane in 5 mL MeOH:CHCl_3 1:1 (v/v) to give a 500 µg/mL stock solution (this solution is used in several laboratory routines analysis). The stock solution was then diluted again with MeOH:CHCl_3 1:1 (v/v) to give a 100 µg/mL solution. The internal standard solution was stored in the laboratory refrigerator.

3.1.5 *Preparation of Interference Substances Solution*

A mixture of six NPS (1P–LSD, 5–APB–NBOMe, 25C–NBF, 25I–NBF,25I–NBOH, 25IP–NBOMe) was prepared from individual stock solutions. The mixture solution was diluted at three concentration levels (high, middle, and low) to be used in the interference study. The mixture solutions were stored in the laboratory refrigerator prior analysis.

3.1.6 *Samples*

All samples suspected to contain LSD were seized in Portugal from 2019 to 2020. The sampling procedure demonstrated in (2.2) was followed. The total amount of unitary doses was record and both the whole material mass and the sample mass weighted for later.

3.1.7 _Extraction_

To each blotter 1 mL of MeOH was added in a test tube than sonicated for 30 minutes at 30º C. The blotter was removed from test tube and the extract was split into two equal parts: 500 µL was reserved for GC–MS investigation on NPSs (without derivatization).

Into the last 500 µL of sample 100 µL was added of the internal standard solution, vortex mixed for 30 s and then evaporated for dryness under a gently steam of N_2.

3.1.8 _Silylation_

The residues were resuspended with 50 µL of MSTFA/PYR 1:1, and vortex mixed for 60 s. The solution was then transferred to a 250 µL insert and heating the capped vial at 60º C for 30 min. The vials were allowed to cool in a desiccator for 30 minutes at least prior GC–MS analysis.

3.1.9 _Instrumental Analysis_

The analysis was performed in a 7890B Gas Chromatograph equipped with 7693 autosampler, coupled to a 5977B mass spectrometer detector (Agilent, Santa Clara, CA, USA). The GC–column was a HP5–MS UI (Agilent Technologies, 30 m, 0.250 mm, 0.25 mm) with helium as the carrier gas, at a flow of 1.4 mL/min. An initial oven temperature of 150º C was set with a 0.5 min isothermal period followed by heating up to 250º C at a rate of 15º C/min and held for 2 min, then heating up to 290º C at a 10º C/min and held for 6.5 min. The total run time was 19.7 min. The injection volume was 1 µL in split mode (10:1), and the injector temperature was set at 280º C. Transfer line temperature was set at 300º C. The mass detector was operated in electron impact ionization at 70 eV. For screening analysis and method optimization studies, the mass analyzer was set to operate in scan mode, ranging from m/z 40 to m/z 450. The spectral identification was referenced to the 2020 SWGDRUG Mass Spectral Library. The validation studies and the real sample analysis were carried out in SIM mode, with the solvent delay time was set to 8.5 min. The data were acquired and analysed using Agilent MassHunter Workstation Software - Qualitative Analysis.

4 Results and Discussion

4.1 Optimizations

4.1.1 *Optimization of Derivatization Procedure*

LSD is low–volatile, thermal unstable compound and tends to undergo adsorptive losses during the GC analysis, thereby its quantitative analysis by GC–MS is preferred after suitable derivatization. In the majority of published works, the use of BSTFA (*N,O*–bis(trimethylsilyl)trifluoroacetamide) was preferred, whence the first derivatization reagent studied in this work.[72,73]

In a first moment, derivatization with BSTFA was extensively investigated either alone or combined to pyridine. After qualitatively settling the derivatization conditions settled the derivatization conditions (e.g., reagent volume, time and temperature of derivatization), the results were statistical analysed by f–test (Table 6).

Table 6: f–test table that indicated the statistically significant difference between the BSTFA and the BSTFA/PYR, given a 95% confidence level.

Factor	MS	f–test	p[H_0]
BSTFA	$1.73 \: x \: 10^{-3}$	42.66	0.0061
BSTFA/PYR	$4.06 \: x \: 10^{-5}$		

As demonstrated above, statistically significant differences were observed in using the BSTFA alone or associated with pyridine, in which the combined reagents produced better results. Nevertheless, as showed in

Figure **14**, it was not possible to detect the TMS–LSD throughout GC–MS analysis unless at high concentrations. It is possible to observe the chromatographic peaks of the test samples fortified at 100 µg/mL (black line) and at 80 µg/mL (red line), despite their low intensity ($< 10^2$), but there were no detectable signal for the test sample at 50 µg/mL (blue line). The analyses were carried out in both TIC and SIM modes; however, the TIC chromatogram allowed a better visualization as shown below.
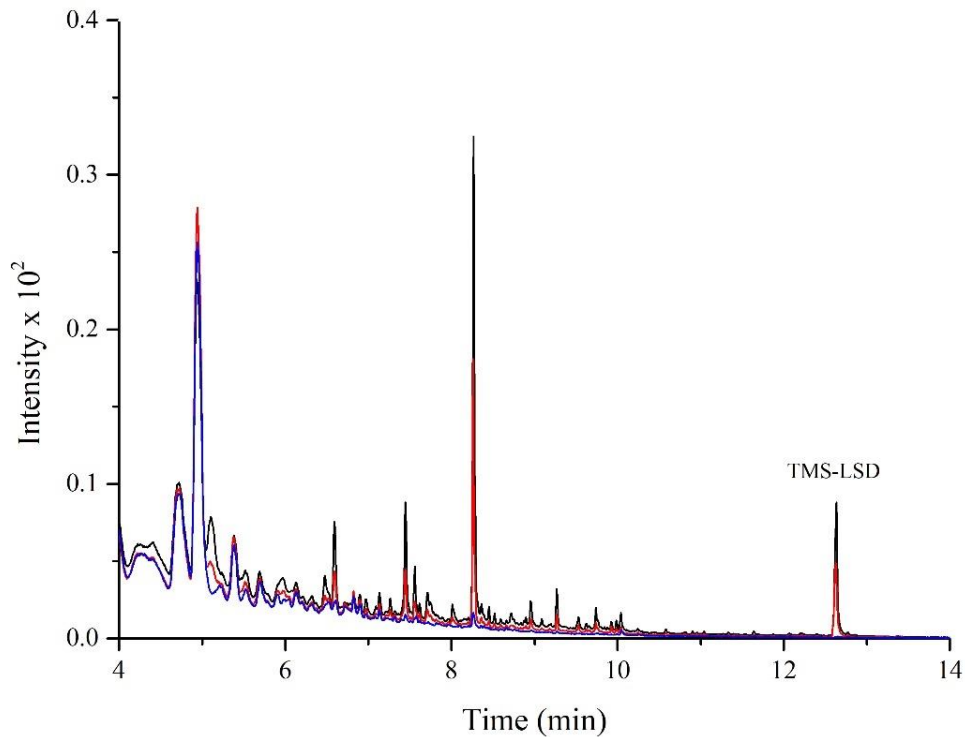
Figure 14 – Representative chromatogram of test samples fortified at three different levels of LSD, 100 µg/mL (black line), 80 µg/mL (red line), and 50 µg/mL (blue line). All test samples were derivatized with BSTFA/PYR (1:1), and analyzed by GC–MS in TIC mode.

Given the unsatisfactory results obtained with BSTFA, MSTFA derivatization procedures were investigated. Contrariwise to BSTFA, MSTFA on its own was not able to react with LSD and produce the TMS derivative. Therefore, a MSTFA/PYR (1:1) association was elected. The effect of derivatizing temperature (60 º C and 70 ºC) was evaluated with f–test (Table 7).

Table 7: f–test table that indicated there is no statistically significant difference between the temperatures studied, given a 95% confidence level.

Temperature (ºC)	MS	f–test	p[H_0]
60	$7.60 \ x \ 10^{-4}$	3.84	0.08
70	$7.41 \ x \ 10^{-4}$		

Given there was not statistical difference observed between studied temperature levels, and taking into consideration savings in energy, the lowest temperature, i.e. 60°C, was selected to be used in all further experiments.

The amounts of MSTFA/PYR (1:1) were varied from 40 to 100 µL. The areas of TMS–LSD chromatographic peak are plotted against each amount of reagent (Figure 15). The greatest chromatographic peak areas ratio was observed by using 50 µL of the reagent, which indicates that is the most favourable reagent volume.
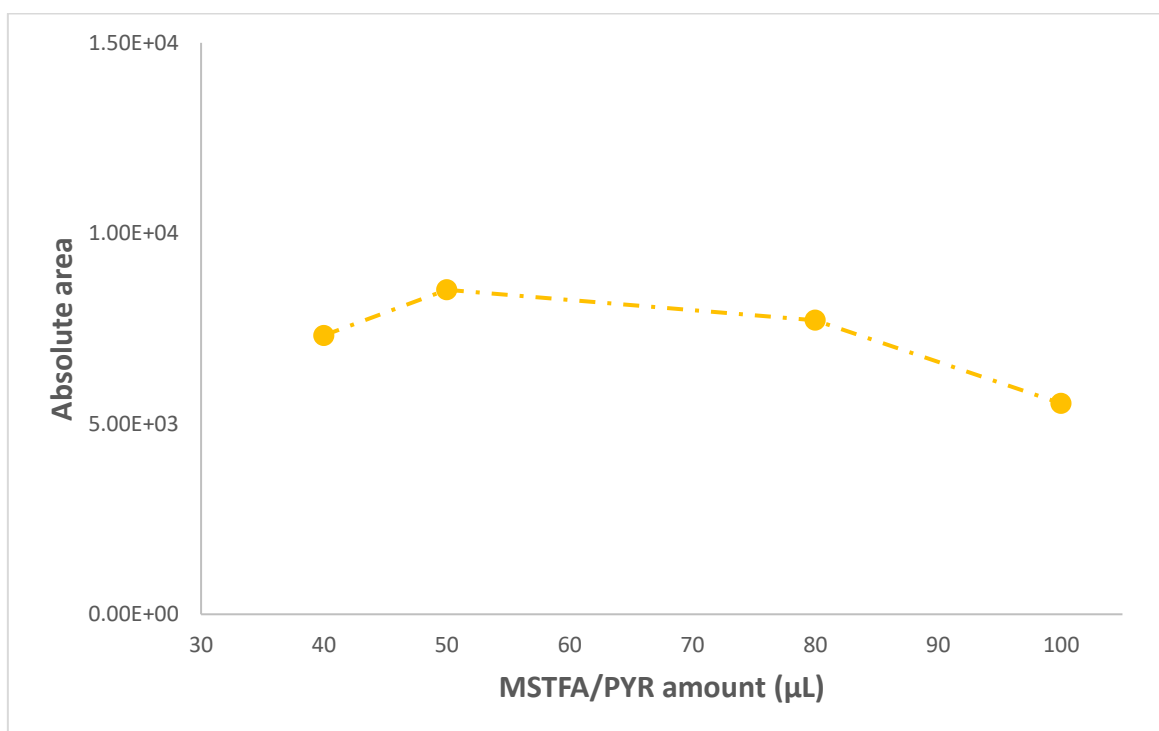
Figure 15 – LSD–TMS chromatographic peak areas ratio obtained by GC–MS with different amounts of reagent.

4.1.2 *Optimization of Extraction Method*

Extensive research on literature was carried out prior to experimental studies. However, most of the published protocols for analysing blotter samples suspected to contain LSD were developed aiming Liquid Chromatograph (LC) rather GC techniques, in which aqueous solvents are not an analytical issue.

In LPC, LSD blotters used to be analysed by LC as well, therefore its extraction standardized protocol included an aqueous solvent extraction followed by a liquid–liquid extraction (LLE) with dichloromethane. The last step would undesirably co–extract the

drawing pigments, increasing the matrix complexity and therefore jeopardize the GC–MS method.

From the above, a new extraction approach was needed to better fit to GC–MS technique. The selected method consists in soaking a single blotter into 1 mL of MeOH, and sonicating for 30 min at 30 ºC under UV–light protection. In a first moment, seventeen samples known to contain LSD were submitted to the proposed procedure and then analysed by TLC (Figure 16). The results are summarized in Table 8:

Table 8: TLC results from MeOH soaking extraction study.

ID	TLC result	ID	TLC result
Sample A	–	Sample I	+
Sample B	+	Sample J	–
Sample C	+	Sample K	+
Sample D	+	Sample L	–
Sample E	+	Sample M	+
Sample F	–	Sample N	+
Sample G	+	Sample O	+
Sample H	+		

+ positive for LSD, – negative for LSD.

For interpretation purposes, the samples stains were compared to the LSD standard stain. Among the 17 analyzed samples, 11 were positive for LSD (> 65 %). However, it is not possible to assert whether the negative results are due to a lack of efficiency of the proposed extraction protocol, or the samples undergone decomposition during the storage time. The extraction procedure was later evaluated again by GC–MS (4.2.9), in which all tested samples were positive for LSD.
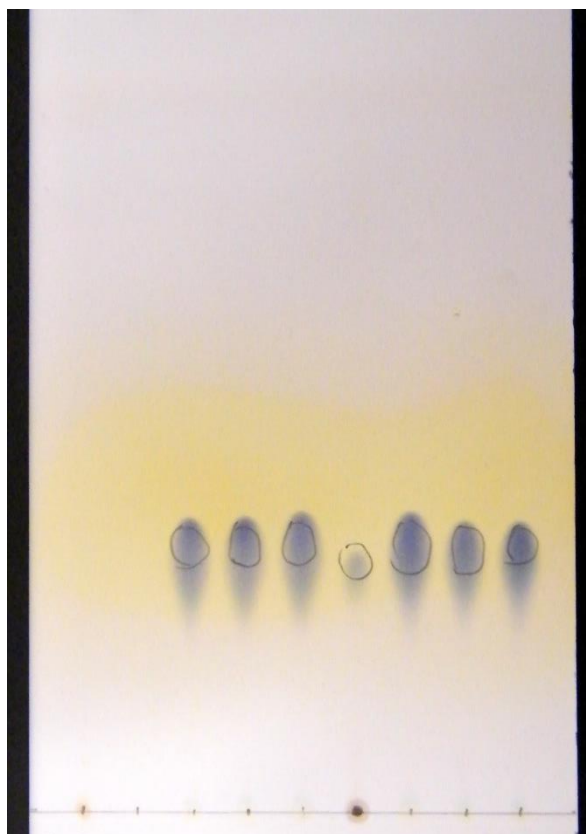
Figure 16 – TLC plate with seven LSD positive samples and two negative samples.

4.1.3 *Optimization of Chromatographic Parameters*

The optimization of the chromatographic parameters was carried out by analyzing test samples fortified at several levels of the analyte. In the first moment, test samples were analyzed by the methods already implemented in laboratory routine for analyzing the monitored compounds e.g., amphetaminic compounds, cocaine, opioids, and tetrahydrocannabinol. Once it was not possible to detect the target analyte by those means, several approaches based on published protocol were tested. All analysis were carried out in both scan and SIM modes. The methods and their parameters are resumed in Table 9.

Table 9: Chromatographic methods tested.

Method	Injection	Oven programming	Source
1	1 µL in split (40:1) at 280º C	150º C for 1 min, raised until 270º C at a 12º C/min rate, and kept for 8 min	LPC
2	1 µL in split (40:1)	150º C for 1 min, raised until 270º C at a 12º	LPC

	at 280° C	C/min rate, and kept for 8 min	
3	1 µL in split (40:1) at 250° C	220° C raised until 320° C at a 20 °C/min rate, and kept for 6 min	74
4	1 µL in split (40:1) at 285° C	160° C for 1 min, raised until 220° C at a 30° C/min rate, and kept for 3 min 220° C raised up to 300° C at a 2° C/min rate, and kept for 4.8 min	75
5	1 µL in split (40:1) at 250° C	200° C for 1,5 min, raised until 300° C at a 30° C/min rate, and kept for 3 min	76

By the exhaustive analysis obtained from the several approaches tested some key factor could be highlighted. The TMS–LSD was only detected when the chromatographic run was performed until high temperatures ≥ 290º C. However, when the oven programming was set to begin at relative high temperatures ≥ 200º C, two undesirable outcomes were verified: the IS was not detected; and the baselines were extremely high, which suppressed the analyte signal (IS signal as well). It happened probably due to the elution of several interferents components (e.g., TMS derivative by–products, columns components, matrix interferences) throughout the chromatographic run at the same time.

The way found to overcome that issue was by setting the temperature injection to a high enough temperature which allowed getting most of the sample components at vapor phase immediately (avoiding condensation), while the oven programming was set to start at soft temperatures and low increasing rates. It allowed getting MSTFA/PYR excess and its by–products to eluate before the solvent delay time end. One advantage of MSTFA over other silylating reagents is the volatility of its by–products, which usually elute with the solvent front. In addition, the combination of moderate temperatures and soft increasing rates improved the separation ability. It was clearly visualized as both the IS and TMS–LSD peak shapes was getting better as the softer rates were tested. The oven temperature programming and the other relevant method are displayed in Table 10 and **Table 11**, respectively.

Table 10: Optimized oven temperature programming.

	Temperature ºC	Rate ºC/min	Holding time min
Setpoint	150	15	0.5
Rate 2	250	10	2.0
Final	290		6.5

Another relevant factor observed was setting an appropriate transfer line temperature to preventing sample from condensing on eluting from GC column. For this purpose, the transfer line was set to 10 ºC above the highest point at chromatographic oven programming.

Table 11: Optimized injection, column and outlet parameters.

Injection volume µL	Split ratio	Injection dispense speed µL/min	Injection temperature ºC	Split flow mL/min	Solvent delay time min	Septum purge flow mL/min	Column flow mL/min	Transfer line temperature ºC
1	10:1	6000	300	14.0	8.5	3	1.4	300

4.1.4 *Optimization of Mass Analyzer Parameters*

A test sample was prepared with 60 µg/mL of the internal standard and 20 µg/mL of LSD and, then analyzed in scan mode under the conditions described in 3.1.9. The TIC chromatogram and both IS and TMS–LSD background subtracted mass spectra are showed below.
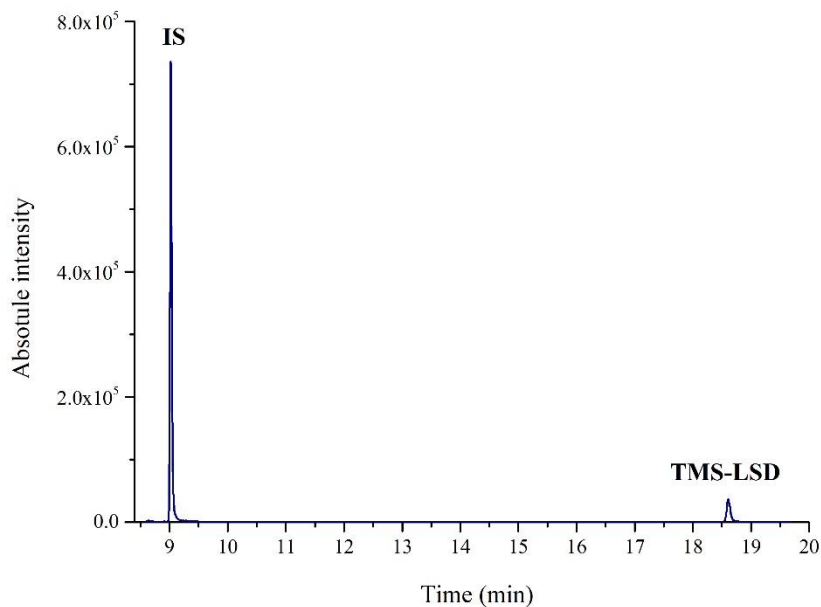
Figure 17: TIC chromatogram obtained from a test sample. Internal standard RT = 9.02 min and LSD-TMS RT = 18.61 min.
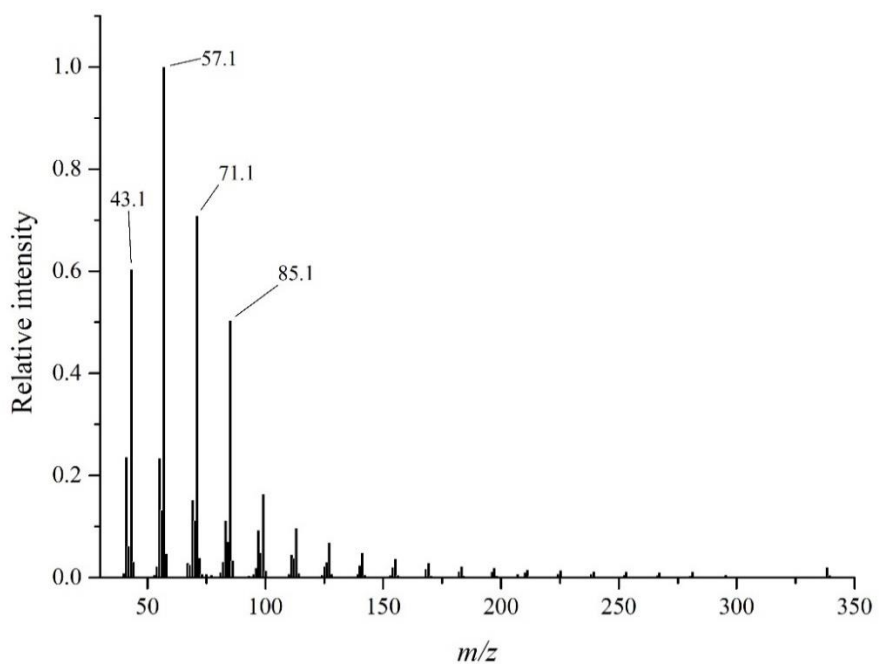
Figure 18 – Tetracosane (internal standard) full–scan mass spectrum obtained from a test sample at RT of 9.02 min.
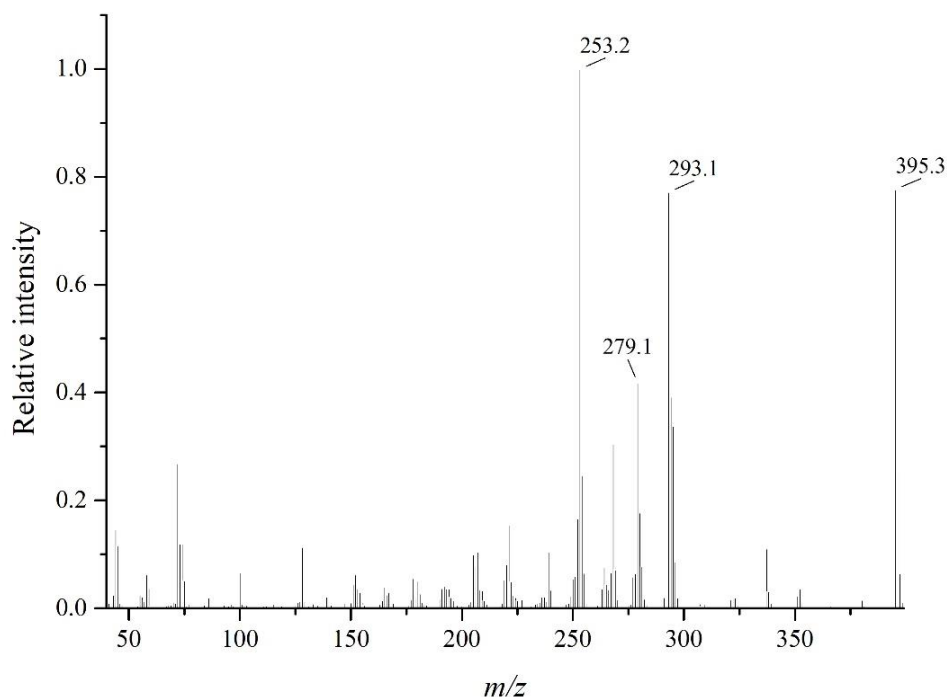
Figure 19 – TMS–LSD full–scan mass spectrum obtained from a test sample at RT of 18.61 min.

The compounds were identified by comparison of spectrum obtained by full scan mode with the 2020 SWGDRUG Mass Spectral Library.[77] The two mass spectra were tabulated to choose the fragments to be monitored. The ions were selected accordingly the follow criteria:

- Unique to the compound (not common in a wide range of compounds).
- More abundant ions (easy to detect at low concentration).
- Higher in mass (usually more specific and separated from interference).

Table 12 shows the ions that matched the above described criteria. The two ions from the IS selected to be more were m/z 71 (quantifier) and m/z 85 (qualifier). The TMS–LSD selected ions to be monitored were m/z 395 (quantifier), m/z 253 (qualifier), and m/z 279 (qualifier). Despite the m/z 57 being the most abundant peak in the IS spectra (base peak), it is common in several other compounds, thus it was not chosen. Likewise, the m/z 293, the second most abundant peak in TMS–LSD spectra, it is also present in several chromatographic column components which unavoidably co–eluate

(column bleed) during the GC–MS run. A previous study on the effects of column bleed identified this potential source of interference and thence the decision not to use this ion was made. Once the monitoring ions were selected, their accurate mass ($\pm$ 0.1 AMU) was determined by measuring the mass centroid.

Table 12: Most relevant ions obtained by scan GC-MS.

Group	Ion	Mass centroid ± 0.1 AMU	Relative intensity
IS[(*)]	57	57.1	1
IS	71	71.1	0.76
IS	43	43.1	0.61
IS	85	85.1	0.50
TMS–LSD[(*)]	253	253.15	1
TMS–LSD [(**)]	395	395.25	0.71
TMS–LSD	293	293.15	0.76
TMS–LSD	279	279.1	0.41

[(*)] base peak, [(**)] molecular ion.

Two SIM groups were formed: one for the two IS ions (IS group), and other for the three TMS–LSD ions (TMS–LSD group). By grouping the ions, the method can monitor the target ions for a list of a compounds by switching from one to another at the appropriate time throughout the chromatographic run. Only one group can be monitored at a given time, and the start time for the second group becomes the end time for the first one. The IS group was set to star at 8.5 min, and to end at 9.5 min. The IS group start time match the solvent delay time.

Once the ions are chosen, the mass centroids are determined, and the ions grouped into SIM groups, the dwell time for each group was set. The central goal in adjusting the dwell time is to optimize cycle time to get 15 to 20 scans across the chromatographic peaks. The SIM group parameters are displayed in Table 13.

Table 13: SIM group parameters.

SIM group	Peak width (ms)	Scan number	Monitored ions number	Dwell time
IS	0.516	15	2	50
TMS–LSD	0.812	15	3	51

4.2 **Method Validation**

4.2.1 _Identification_

For identification purposes, the retention time (RT) of the analytes should agree closely with those of a standard analyzed under the identical conditions, with an allowable error of $\pm 2\%$. Moreover, the areas of peaks obtained by SIM mode at the analyte retention time should have relative intensities which match those of a standard analysed, with an allowable error of $\pm 20\%$. Ion chromatograms obtained for the calibration standards are displayed below:
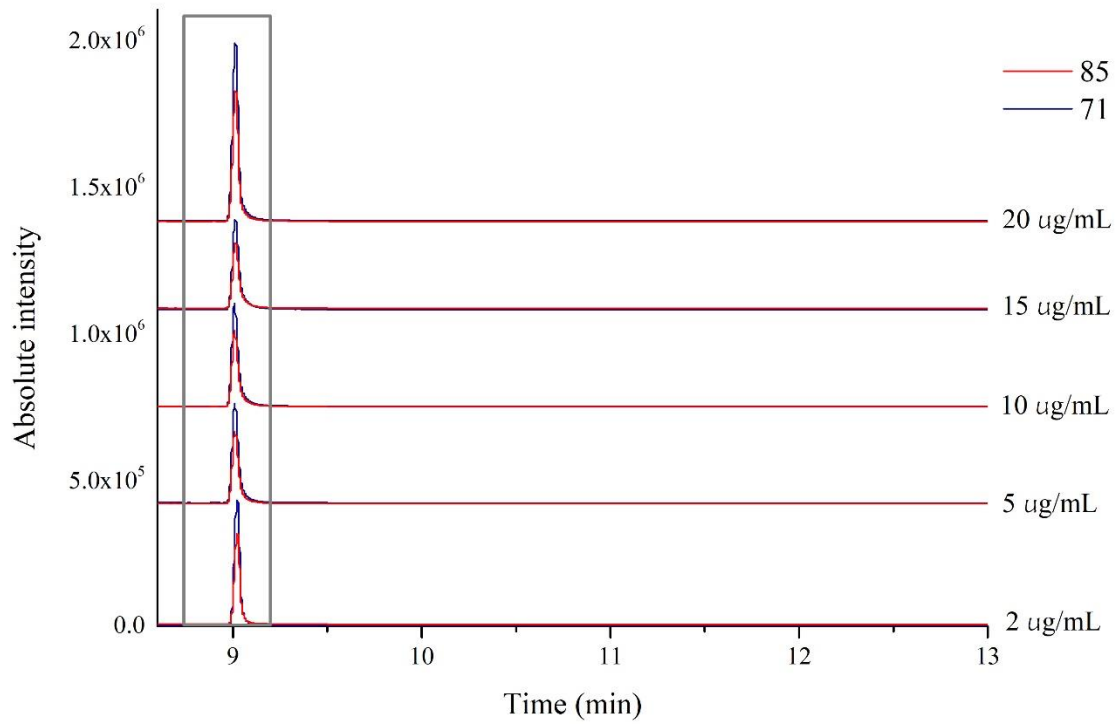
Figure 20: Chromatograms obtained for the calibration standards, analyzed by GC–MS in SIM mode (IS monitored m/z). The grey rectangle delimits the acceptable variation interval.
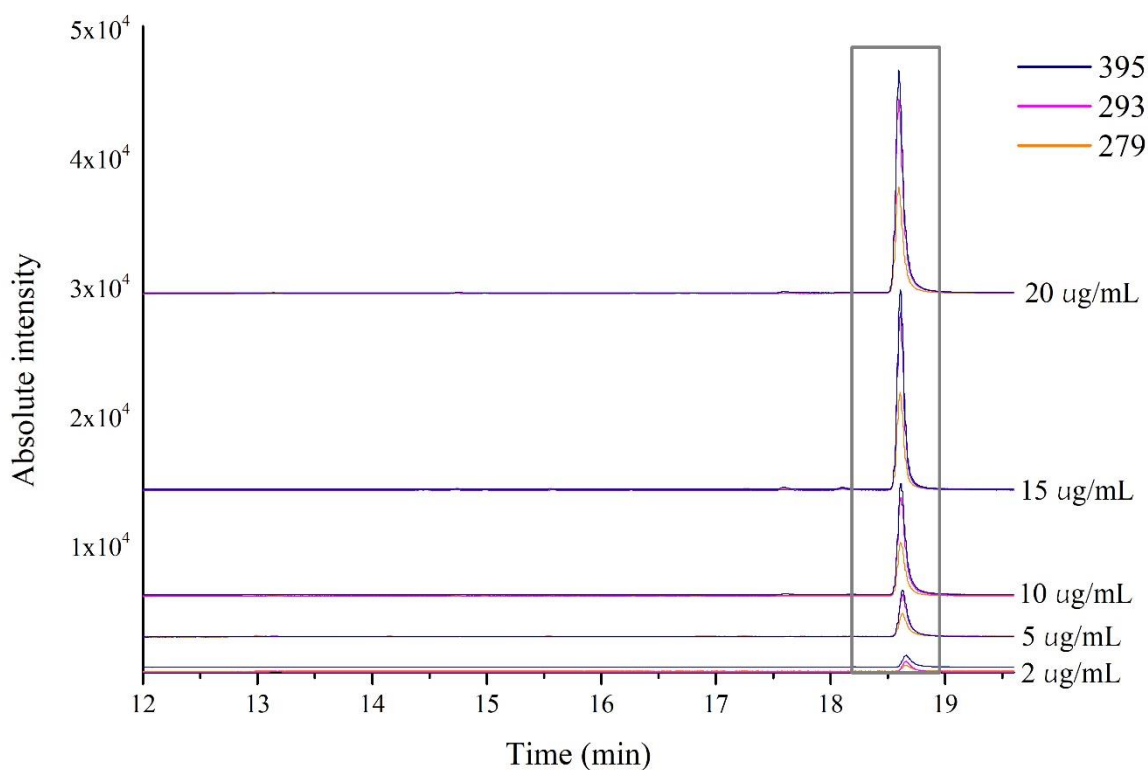
Figure 21: Chromatograms obtained for the calibration standards analyzed by GC–MS in SIM mode (TMS-LSD monitored m/z). The grey rectangle delimits the acceptable variation interval.

As shown in **Figure 20** and **Figure 21**, both the IS and TMS-LSD retention time variation fell within the acceptable interval. Moreover, it is possible to visualize that the TMS-LSD ions relative intensities were proportional regardless the concentration level. The ions relative intensity are better visualized from **Table 14**.

Table 14: Average relative intensity for each method monitoring ion.

Compound	Ion	Average relative intensity
Tetracosane	71	0.58
Tetracosane	85	0.42
TMS-LSD	279	0.20
TMS-LSD	293	0.37
TMS-LSD	395	0.42

4.2.2 *Interference Studies*

To detect the more common matrix interferences ten blank matrix samples (without de addition of internal standard) were analyzed in both full scan and SIM modes, Figure 22 to Figure 24.
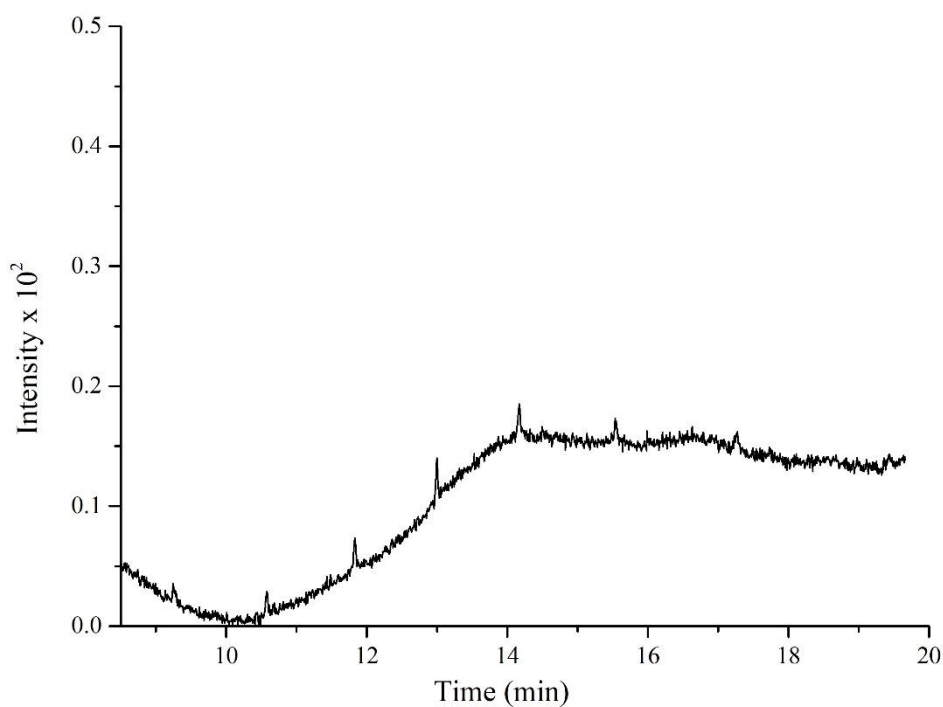
Figure 22 – Representative chromatogram of blank matrix, derivatized with MSTFA/PYR (1:1), and analyzed by GC–MS in TIC mode.
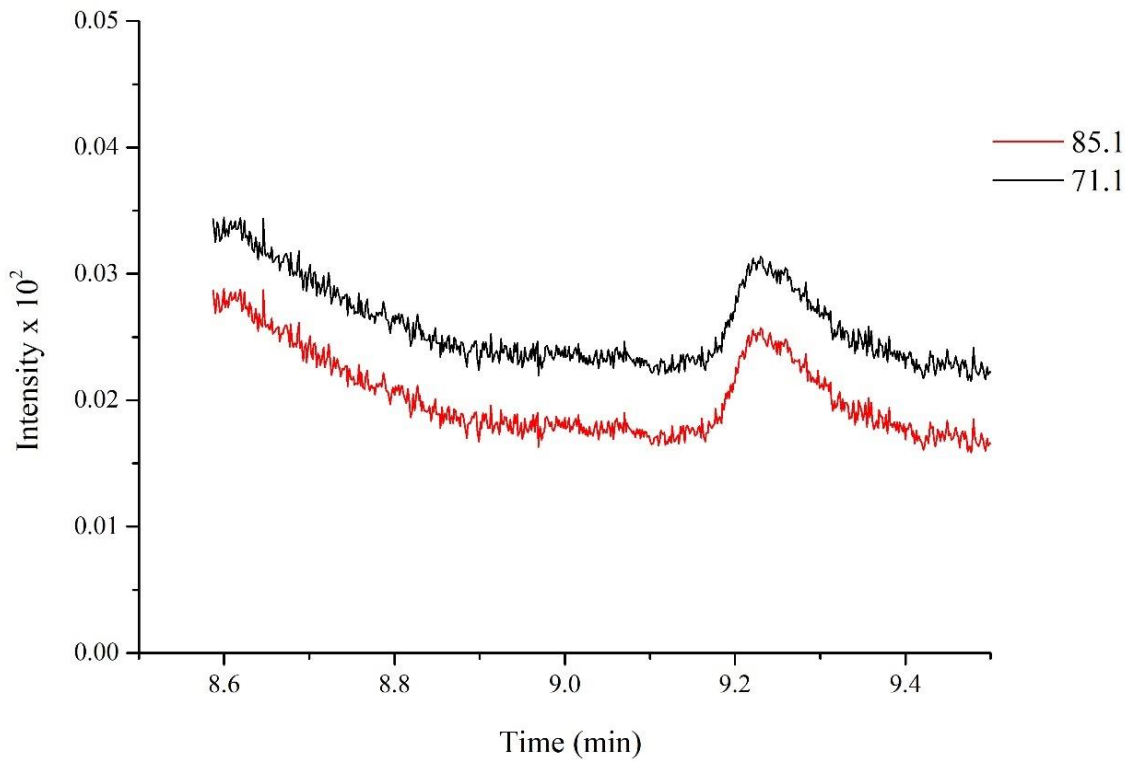
Figure 23 – Representative chromatogram of blank matrix, derivatized with MSTFA/PYR (1:1), and analyzed by GC–MS in SIM mode.
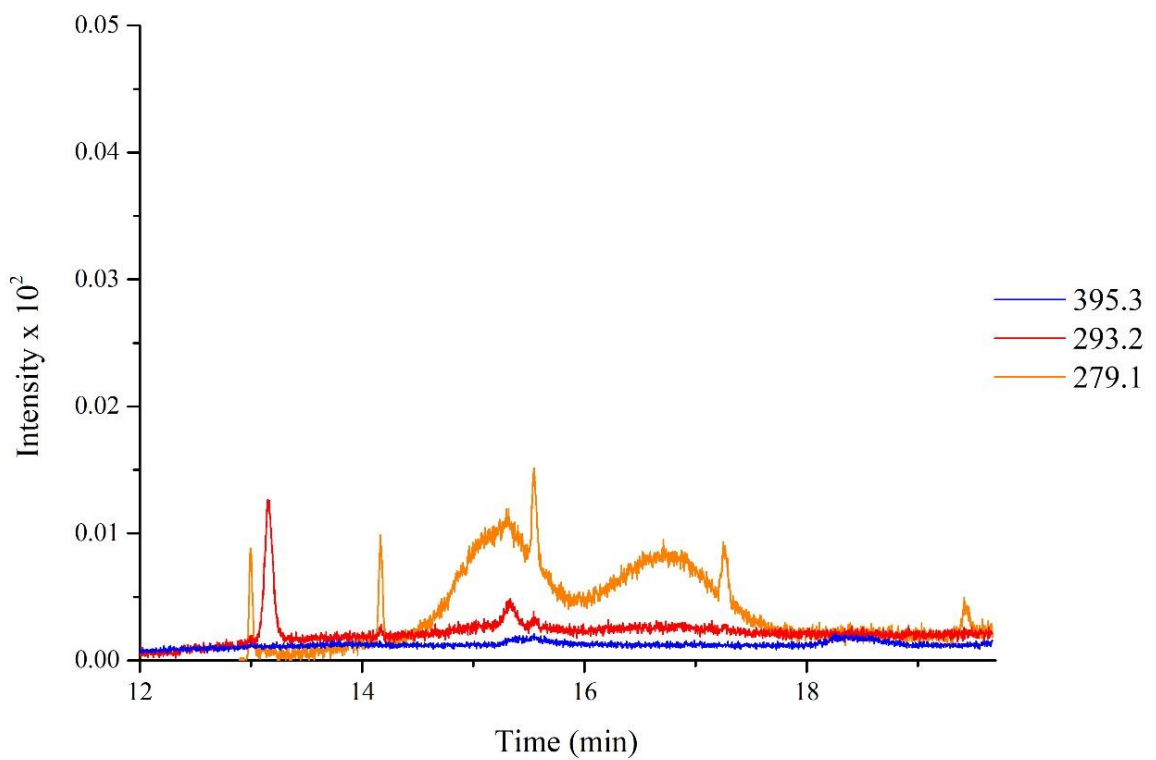
As can be visualized from figures above, there were no detectable signals at neither the IS nor analyte retention times.

To investigate the method ability to measure the analyte in presence of contaminants, real samples known to contain LSD (n = 3) were fortified with a mix solution of substances known to be encountered in street samples. SIM acquisition chromatograms from one randomly chosen sample are showed below to represent the study (Figure 25 and Figure 26).
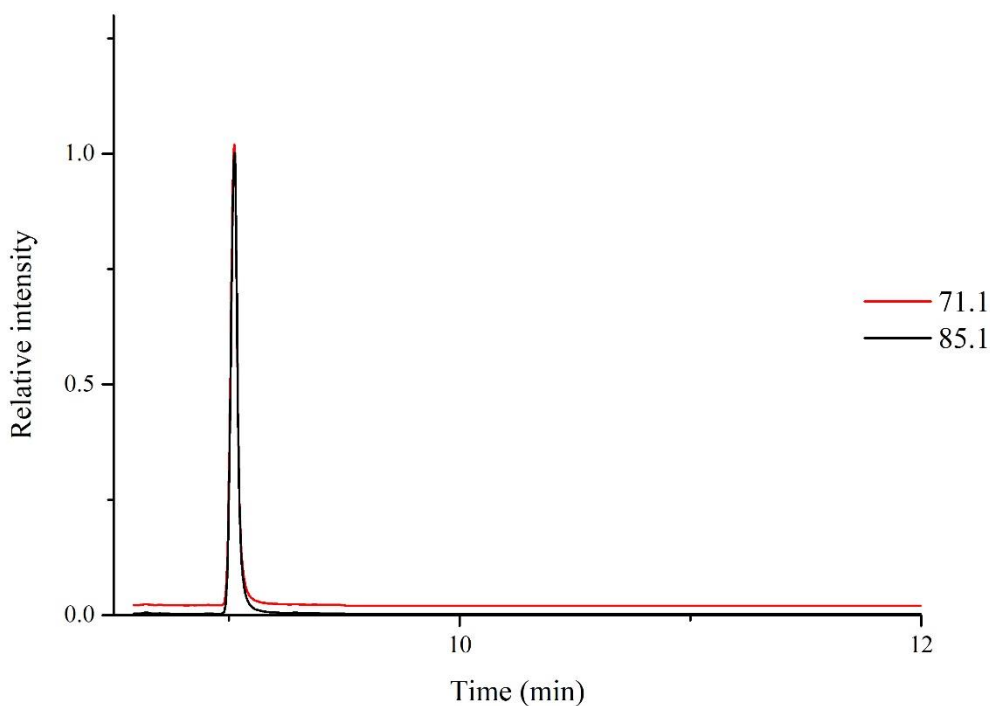
Figure 25 – Representative chromatogram of positive LSD sample fortified with interferent solution, derivatized with MSTFA/PYR (1:1), and analyzed by GC–MS in SIM mode (internal standard monitored m/z).
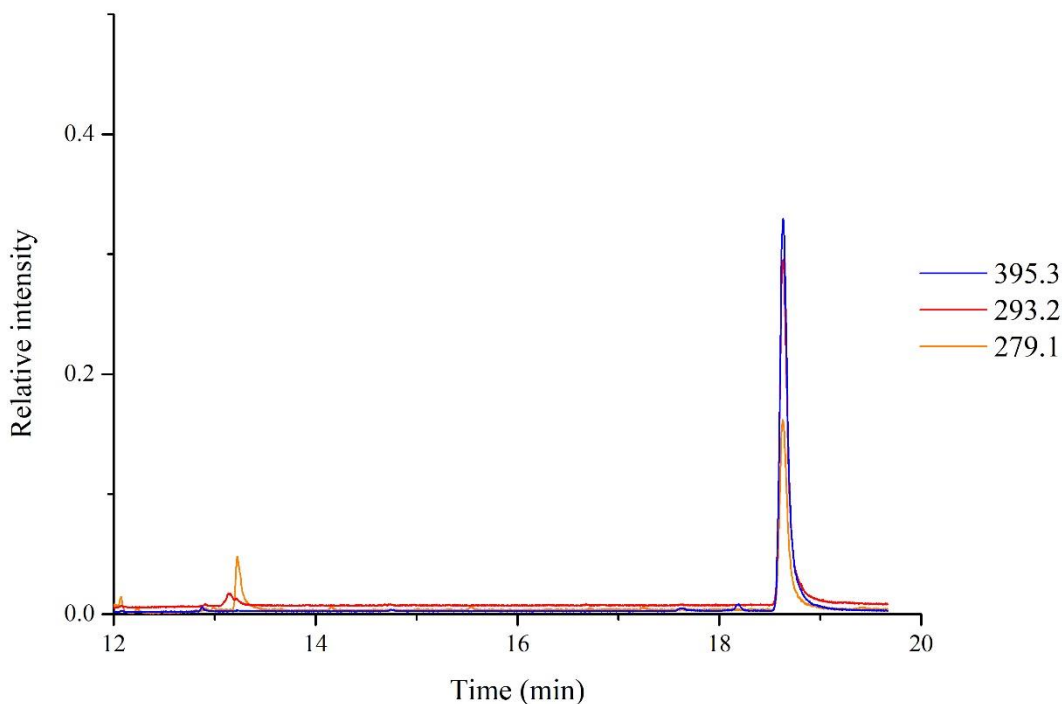
Figure 26 – Representative chromatogram of positive LSD sample fortified with interferent solution, derivatized with MSTFA/PYR (1:1), and analyzed by GC–MS in SIM mode (TMS–LSD monitored m/z).

As visualized from **Figure 26** and **Figure 27**, the deliberately added contaminants interfered neither in the internal standard (RT = 9.01 min) detection nor the TMS–LSD (RT = 18.62 min) detection. Both the IS and the LSD–TMS retention times matched those of the samples prior the contaminants addition. Likewise, the areas of monitored peaks matched those of standard solutions analyzed at the same conditions.

4.2.3 *Working Range and Linearity*

The concentration of LSD in seized material may fluctuate broadly, thus a wide concentration range was firstly analyzed, and several fitting approaches were tested. Since it was not possible to fit both the laboratory demands and the metrological requirements, the range reduced, covering from 2 to 20 µg/mL, which is equivalent to 2 to 20 µg/blotter.

The calibration curves were obtained by plotting the ratios between the area of the quantifier analyte ion (m/z 395) and the area of quantifier internal standard ion (m/z

71) against the standard concentration. Six replicates for each level were analyzed excepted for the first level (x_1) and the last level (x_N), which were analyzed ten times for homoscedasticity study.

It is necessary to check whether the dependent variable (area ratio) is uniform throughout the calibration curve. This parameter was evaluated by means of the Hartley–test (2.3.3.1), given a confidence level of 99% ($\alpha = 0.01$) due its critical importance in calibration curve determination.

Firstly, the measured values were tested for outliers by means of Grubbs test (ISO/IEC 17025 recommendation). The validation of data is demonstrated in **Table 15**. For x_1 and x_N the calculated $TV(G)$ were 1.35 and 0.96, respectively. Since both values were less than the Grubbs critical value (2.29) with $\alpha = 0.05$ and $n = 10$, all measurements were validated. For the other concentration levels the same procedure was followed with a different Grubbs critical value (1.88) with $\alpha = 0.05$ and $n = 6$. All the measurements were validated with no discrepant values detected.

Table 15: Grubbs test for outliers verification on calibration curve measurements.

Level	TV(G)	Grubbs critical value ($\alpha = 0.05$)	$p[H_0]$
x_1	1.35	2.29	0.21
x_2	1.29	1.88	0.23
x_3	1.72	1.88	0.12
x_4	1.43	1.88	0.19
x_N	0.96	2.29	0.36

Table 16 shows that the calculated $TV(f)$ (4.81) was less than f critical value (5.35), which supports the acceptance of null hypothesis ($p - value \geq 0.020$) i.e., there is homogeneity of variance and an ordinary least-squares (OLS) approach could be well suitable.

Table 16: Hartley–test for homoscedasticity.

x_1(µg/mL)	x_N (µg/mL)	$TV(f)$	f critical value ($\alpha = 0.01$)	$p[H_0]$
2	20	4.81	5.35	0.020

As demonstrated in Table 17, the OLS model provides a satisfactory coefficient of determination ($R^2 > 0.0995$). The decision on whether OLS or quadratic model must be adopted was statistically supported by Mandel–test (2.3.3.2).

Table 17: Linearity investigation and choose model.

n	Working range (µg/mL)	Equation	R^2	$TV(f)$	f critical value ($\alpha = 0.01$)	p[H_0]
5	2–20	$y = -4.14 \times 10^{-4}x - 3.98 \times 10^{-4}$	0.0997	4.21	10.56	0.070

The calculated $TV(f_M) = 4.21$ was less than the f critical value (10.56) with $\alpha = 0.01$, which indicates that the quadratic model does not provides a better adjustment than the OLS model, and the null hypothesis must be accepted (p[H_0] = 0.07). Then, the OLS model must be chosen due it has higher number of degrees of freedom.

After the model selection, it is necessary investigating the presence of discrepant values in the calibration curve. One approach to identify suspected point is calculating the difference between experimental and predicted values, in which the greater is the difference more likely the suspected point to be an outlier. It is important to note that the calculated differences must be divided by each standard deviation so that allow the comparison within themselves. The scatter of the standardized residuals is shown in Figure 27.
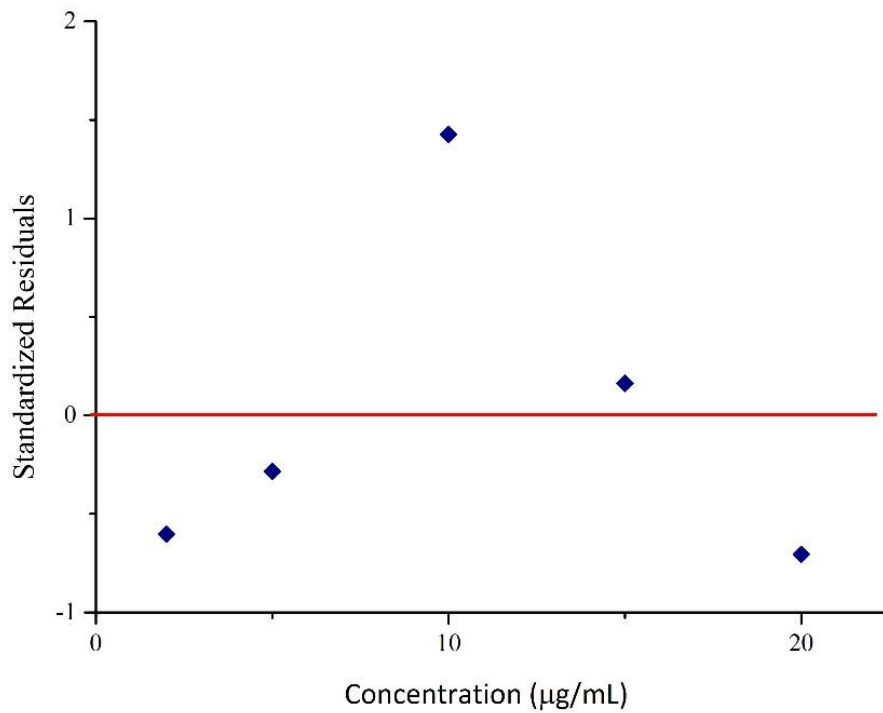
Figure 27 – Standardized residuals plotted against standard concentration.

An initial approach to identify possible discrepant values can be performed by inspecting the points which are more distant from the predicted values (graphically represented by the straight line), or the spreadiest values on the residuals plot. Nevertheless, both the mechanisms described above do not guarantee either the rejection or acceptance of suspected values with statistical safety, therefore the Mandel–test was used to improve the safety of decision making.
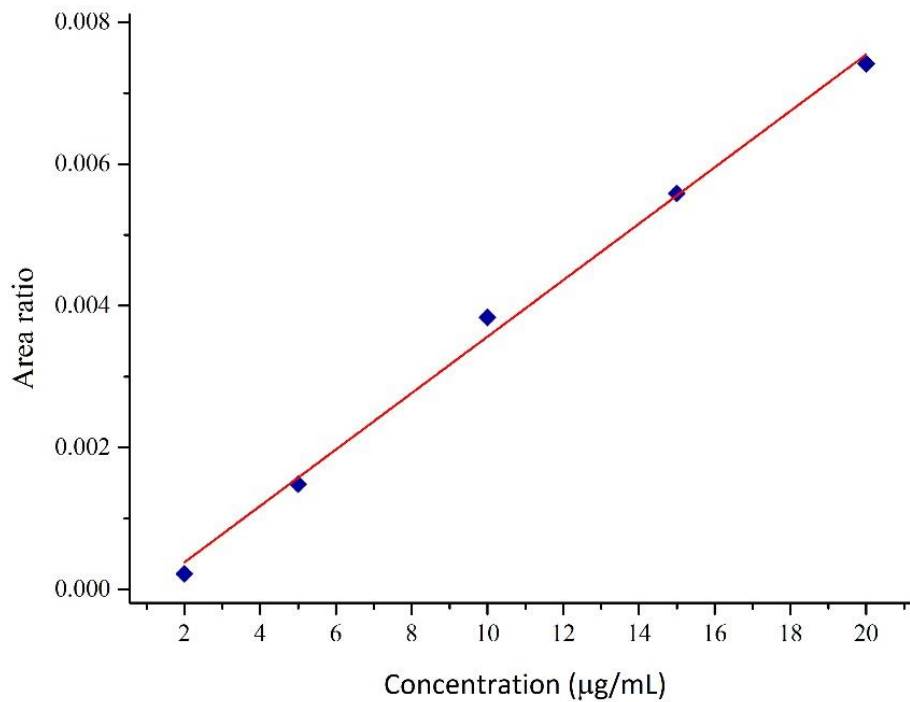
Figure 28 – The ordinary least–squares model.

As can be observed in Figure 28, the point x_3 is the most distant from the adjust line. The obtained $TV(f_M) = 6.61$ did not exceeded the f critical value (12.92), which means that the suspected point is not a discrepant value, thus it shall not be removed. Once the suspected value was kept, it is assumed that the calibration curve is free of outlier i.e., data–points are validated (Table 18).

Table 18: Standard solution concentration levels and the respective area ratios.

Level	Concentration (µg/mL)	Area ratio
x_1	2	0.00030
x_2	5	0.0015
x_3	10	0.0038
x_4	15	0.0056
x_N	20	0.0074

Area ratio = area analyte quantifier ion/area IS quantifier ion.

4.2.4 *LOD and LOQ*

The experimental limit of detection (x_{LOD}) and the experimental limit of quantification (x_{LOQ}) were obtained applying equations in (2–9) and (2–10), respectively, by using data estimated during the linearity study.

Table 19: Limit of detection and limit of quantification.

x_{LOD} (µg/mL)	x_{LOQ} (µg/mL)	x_1 (µg/mL)	x_N (µg/mL)
1.08	3.23	2	20

As demonstrated in Table 19, the obtained x_{LOD} (1.08 µg/mL) was less than the first standard of the calibration curve x_1, however, the x_{LOQ} (3.23 µg/mL) was greater than x_1 (2 µg/mL). Although it is desirable that both x_{LOD} and x_{LOQ} values fall below the first calibration standard value, it is possible to validate the calculated limits while the x_{LOQ} is less than the second level of calibration curve point x_2 (5 µg/mL).

4.2.5 *Precision*

The precision of the measurement was evaluated by analyzing test samples at three concentration levels (low, mid, and upper parts of working range), in triplicate, on the same day (repeatability conditions). The results stated in terms of RSD (**Table 20**). This analysis was repeated on four consecutive days, in which the mean value of each measurement per day was used to access the intermediate precision.

Table 20: Repeatability and intermediate precision on measurements of test samples, expressed as RSD.

Level	RSD	
	Repeatability (n = 3)	**Intermediate precision (n = 4)**
Upper	2.95	8.32
Mid	4.50	7.64
Low	6.96	9.37

It was observed from **Table 20**, that all CV values for minimum level were lower than the recommended limit 20%, and the other control levels were better than 15% as well. As it was expected, the RSD values are higher at intermediate conditions than repeatability conditions. Nevertheless, all the intermediate precision RSD values were within the recommended limits also. As visualized from Figure 29 the RSD values were higher at the minimum concentration level except for day 4.
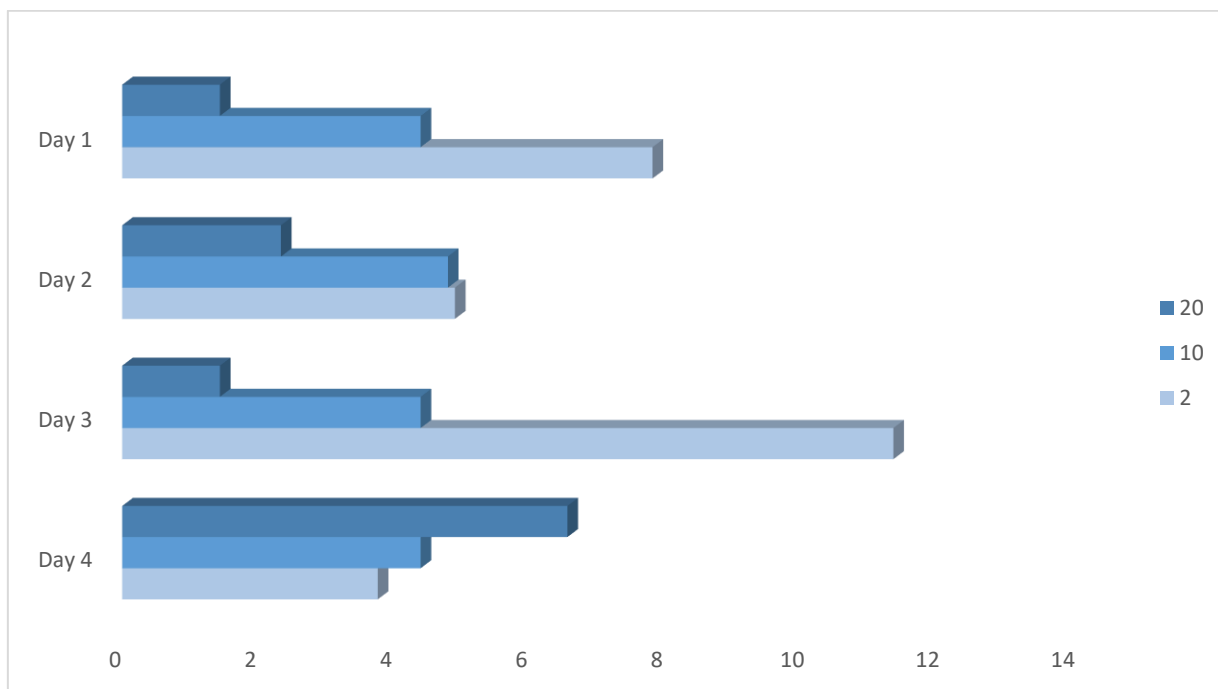
Figure 29 – Repeatability precision (n = 3) on measurements of test samples, expressed as RSD.

4.2.6 *Bias*

For accessing the method trueness, test samples were spiked at known concentrations of LSD. The samples were analyzed in replicate on three consecutive days. The difference between the mean measurements ($\bar{x}$) and the expected result ($\bar{x}_{ref}$) was calculated and expressed as relative standard error $\%RE$. It was also checked the method performance factor by means of t–test.

As showed in Table 21 the $\%RE$ of all three levels of concentration fell within the recommended limits ($\pm$ 20% at the lower concentration level and $\pm$15% at higher concentrations), which states that the method is accurate.

Table 21: Relative standard error %*RE* of each concentration level (n = 3), and the statistical performance evaluation.

Level	%RE	$TV(t)$	t critical value (α = 0.05)	p$[H_0]$
High	9.61	1.46	3.36	0.181
Medium	11.45			
Low	– 6.55			

The trueness was further analyzed by means of performance factor. The calculated $TV(t)$ was less than the t critical value given 95% of confidence level, thus the method performance is ranked as satisfactory i.e., it met expectations in terms of accuracy.

4.2.7 *Uncertainty*

Validation data were used to quantify the measurement uncertainty (intermediate precision and bias). The relative standard uncertainty $u(RSD)$ was calculated by dividing the intermediate standard deviation mean values $\overline{RSD}$ by the number of measurements (n = 3). The estimated relative error uncertainty $u(RE)$ was obtained by dividing the relative error standard deviation by the number of measurements (n = 9). Their values and uncertainties are collected in **Table 22**.

Table 22: The individual uncertainty components estimated from in–house validation development.

Intermediate precision $\overline{RSD}$	$u(RSD)$	S_{RE}	$u(RE)$
0.0844	0.0050	0.048	0.033

It is demonstrated in Figure 30 that *Bias* is the largest contribution to the measurement uncertainty. Since this component is derived from the overall variability in the method, further experiments would be needed to show where improvements could be made.
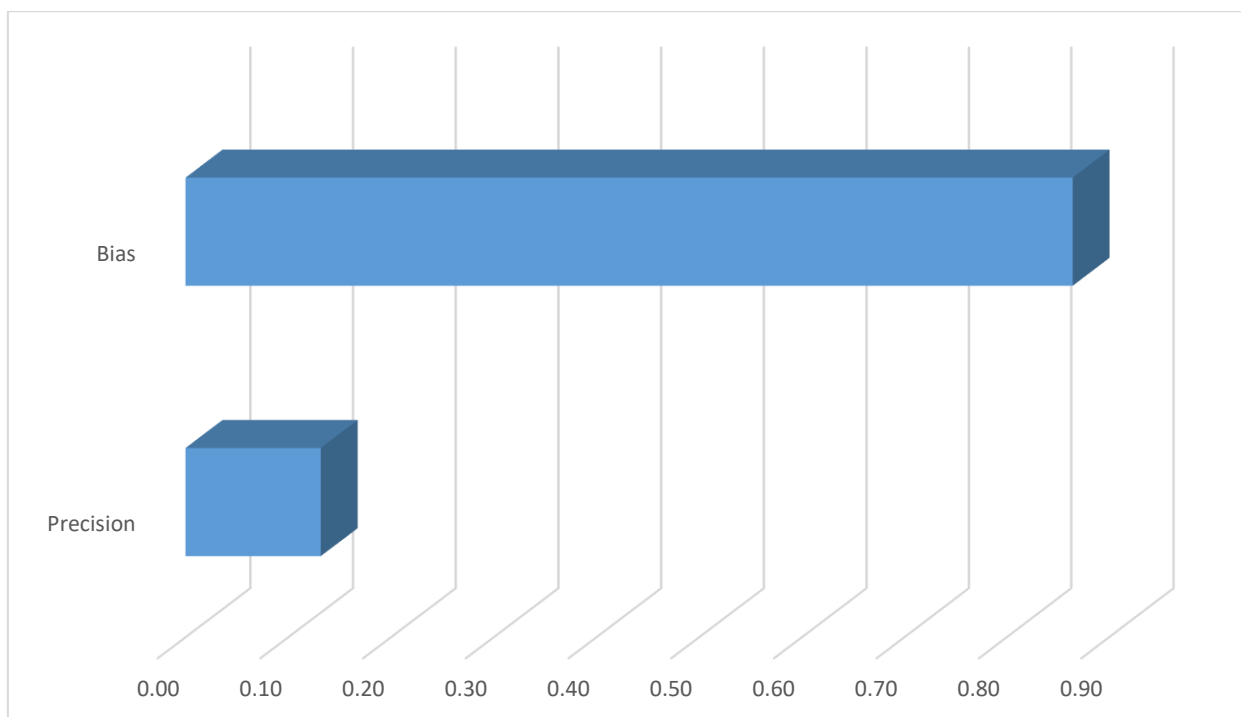
Figure 30 – Normalized individual uncertainty components.

The individual uncertainty components were combined to estimate the combined standard uncertainty (u_c):

$$u_c = \sqrt{(0.0050)^2 + (0.033)^2} = 0.033 \ \mu g/mL$$

The expanded uncertainty was calculated by multiplying the combined standard uncertainty by the chosen coverage factor k. For most of purposes it is recommended that k is set to 2.

$$U = 2 \times 0.033 = 0.067 \ \mu g/mL$$

4.2.8 *Processed Samples Stability*

The laboratory analysts are recommended to manage their schedules to prepare and analyze a sample in a single workday i.e., only start a new exam if there is time enough to have all preparation steps done and the sample ready for GC–MS analysis at the end of the day. Nonetheless, unexpected events may occur in which the GC–MS analysis is

postponed, and it may be necessary to run the sample the following day or later. Thus, it is necessary to evaluate the length of time a processed sample can be maintained before it undergoes unacceptable changes, that may compromise the confidence of the results.

The TMS derivatives are overly sensitive to moisture, thus the derivatized samples were stored in a desiccator at room temperature. The limited sample final volume does not allow having a single sample split into different vials corresponding to each day of study, hence the vial capes were substituted by new ones after each GC–MS analysis to keep the vials properly sealed. The processed sample stability was assessed by analyzing a set of samples fortified at low and high concentration levels of the analyte, in triplicate runs, at different time intervals.

The first set of analysis was performed immediately after sample preparation were concluded to establish the time zero T_0 responses. For the T_1 and T_2, the samples were analyzed after 24 h and 48 h, respectively. Average responses at each time interval are compared to the T_0 responses.
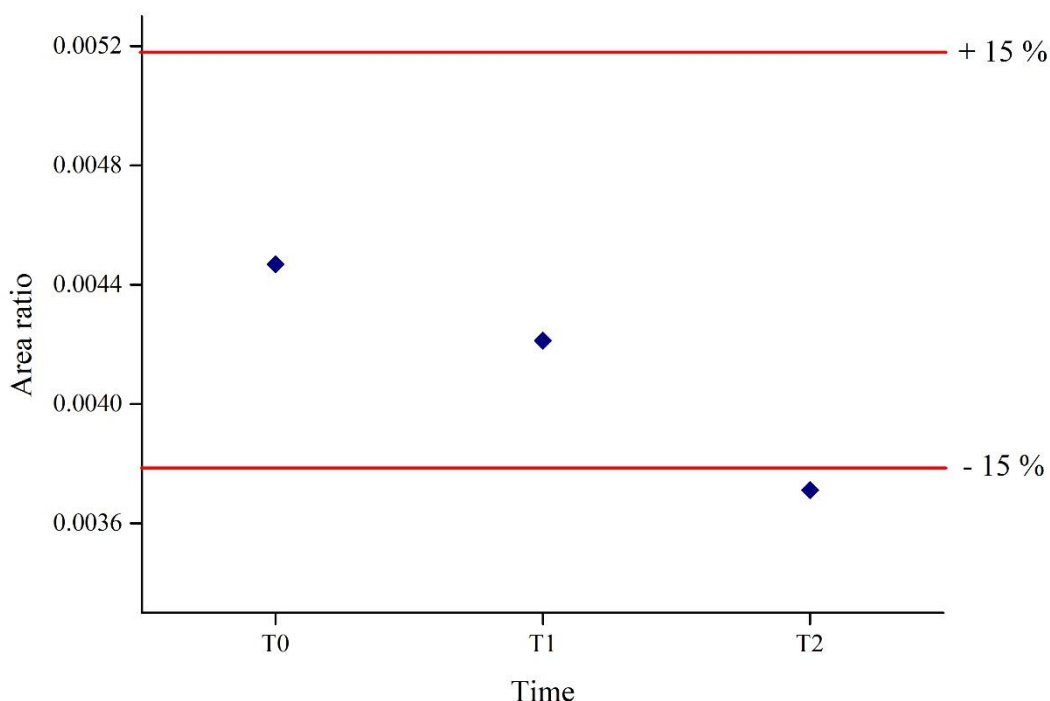
Figure 31 – TMS-LSD and IS chromatographic peaks average area ratio for evaluation of processed sample stability. The red lines indicate the critical limit of acceptance ± 15%.

As visualized in Figure 31, the average response on T_1 was less than 6% lower than the average response on T_0, which falls within of the method´s acceptable bias $\pm15\%$ (red straight lines). One the other hand, the average response on T_2 fell outside the inferior method´s acceptable limit. Considering it, samples analyzed up to 24 h after their preparation are likely to provide reliable results. Given the demonstrated instability after 24 h, samples that were not analyzed within that period must be discarded.

4.2.9 *Real Samples Analysis*

The developed method was applied to 6 real samples. Among them, two showed areas ratio out of the method working range, thus they were diluted with hexane (1:1) and re–analyzed. The results are demonstrated at **Table 23Table 1**. The SIM chromatogram obtained for sample #05 was randomly chosen to represent all analyzed samples (Figure 32).
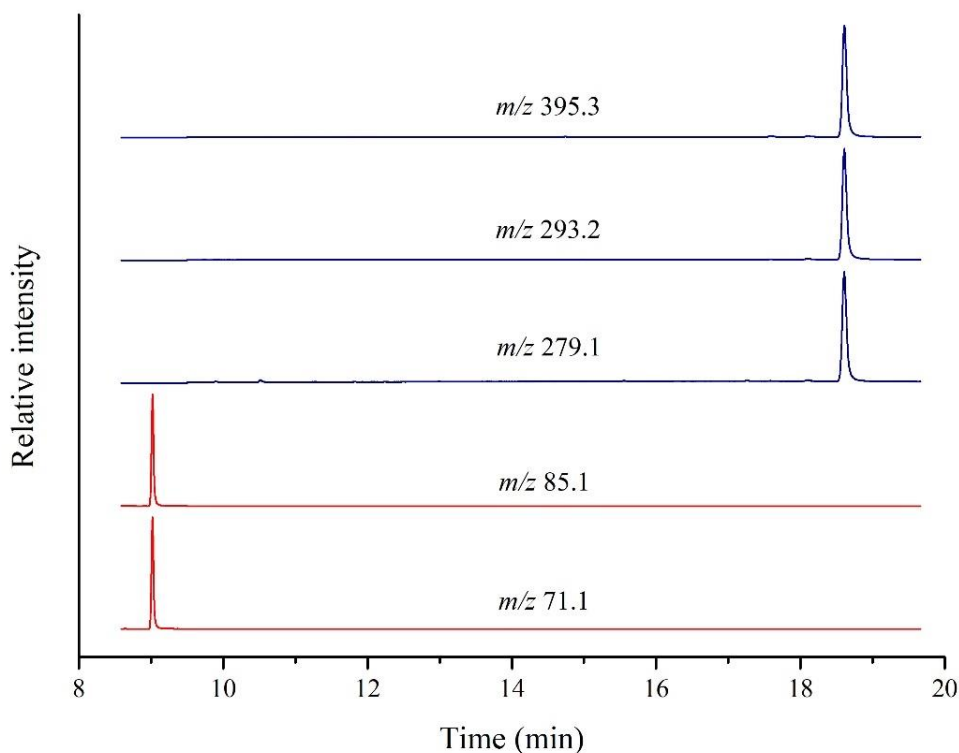
Figure 32 – Representative chromatogram of real sample, derivatized with MSTFA/PYR (1:1), and analyzed by GC–MS in SIM mode.

Table 23: Results from the application of the developed method on real samples.

ID	Blotter mass (g)	Areas ratio	Sample concentration (µg/mL)	Sample concentration (µg/blotter)	Purity % (µg/mg)
#01	0.016	0.00659	35.2 (± 2.7)	23.7 (± 2.7)	0.15 (± 0.03)
#02*	0.016	0.00294	168.6 (± 5.7)	109.1 (± 5.7)	0.70 (± 0.06)
#03	0.015	0.00334	18.9 (± 3.0)	11.6 (± 3.0)	0.08 (± 0.03)
#04*	0.015	0.00249	146.0 (± 6.3)	93.4 (± 6.3)	0.60 (± 0.06)
#05	0.016	0.00699	37.2 (± 3.8)	23.8 (± 3.8)	0.15 (± 0.04)
#06	0.016	0.00279	16.1 (± 4.0)	10.3 (± 4.0)	0.07 (± 0.04)

* sample concentration and its respective standard error were corrected by dilution factor.

As demonstrated above, the SIM mode produces smooth and well–defined peaks for both qualifiers and quantifiers ions, which matched accurately with the chromatogram obtained from the standard solution in terms of both retention time and peaks relative intensity. The concentrations of the real samples analyzed ranging from 10.3 (± 4.0) to 109.1 (± 5.7) µg/blotter.

5   Conclusion and Future Perspectives

The aim of the present study was to develop and validate a reliable analytical method to analyse and quantify LSD from street seized samples by gas chromatography coupled to mass spectrometry.

The efficiency of a simple and fast extraction procedure by soaking the blotter in MeOH was demonstrated. In comparison with the standard protocol, the new protocol yielded higher selectivity to the target analyte. The derivatization protocol employing MSTFA successfully formed the TMS analyte derivative.

The developed chromatographic method performed well in separating the matrix components, chemical interferents and by–products, from the target compounds. The SIM data acquisition method allowed high–selectively to identify and quantify the target analyte, even though at low–concentration levels, which was not reached by full–scan analysis.

International recommended validation parameters were evaluated. It was demonstrated the method selectivity to the target analyte. The fit model selection was supported by meticulous statistical support, and its performance parameters as well. The LOD and LOQ values were low enough to agree with the laboratory needs. The repeatability and the intermediate precision were within acceptance criteria limits. The accuracy measurements at all analyzed levels were less than $\pm$ 15%, and the method performance was ranked as satisfactory. There was also demonstrated that the prepared samples are stable enough to be analyzed up to 24 h.

The method was successfully tested in six real samples. The samples concentration ranged from 10.3 ($\pm$ 4.0) to 109.1 ($\pm$ 5.7) µg/blotter.

As part of this work there was developed a standard operation procedure (SOP), and a spreadsheet, which will support the full implementation of the method into the laboratory routine.

It was not possible to evaluate quantitatively the extraction procedure protocol since there were no two identical samples for comparison purposes. Likewise, recovery studies were not fulfilled. It was initially planned to overcome the lack of certified reference material by spiking known blank sample matrixes at different concentration levels but given the limited available time, it could not be done.

The validated calibration curve is restricted to low concentration levels (2 to 20 µg/mL), which will frequently demand dilutions of the routine samples after an initial GC–MS analysis. Thus, it is important to demonstrate to what extent the diluted samples still provide reliable results. Alternatively, other calibration models may provide wider working ranges, avoiding then dilution, which contribute to increase the analysis error.

6 References

1.   Wacker, D. et al. Crystal Structure of an LSD-Bound Human Serotonin Receptor. Cell 168, 377-389.e12 (2017).

2.   Hofmann, A. Notes and documents concerning the discovery of LSD. Agents Actions 1, 148–50 (1970).

3.   Hofmann, A. The discovery of LSD and subsequent investigations on naturally occuring hallucinogens. In Discoveries in Biological Psychiatry (Ayd, F. J., and Blackwell, B., Eds., 1970).

4.   Hofmann, A. LSD- My Problem Child. (McGraw-Hill Book Company, 1980).

5.   Nichols, D. E. & Grob, C. S. Is LSD toxic? Forensic Sci. Int. 284, 141–145 (2018).

6.   Nichols, D. E. Dark Classics in Chemical Neuroscience: Lysergic Acid Diethylamide (LSD). ACS Chem. Neurosci. 9, 2331–2343 (2018).

7.   Obreshkova, B. et al. Pharmaco-toxicological aspects and analysis of phenylalkylamine and indolylallkylamine hallucinogens (Review). Pharmacia 64, 32–47 (2017).

8.   Stoll, W. A. Lysergsäure-diäthylamid, ein Phantastikum aus der Mutterkorngruppe. Schweiz Arch. Neurol. Psychiatr. 279− 323 (1947).

9.   Grinspoon. No Title[1] L. Grinspoon, J.B. Bakalar, Psychedelic Drugs Reconsidered, Basic Books, New York,.

10.   Yu, A.-M. Indolealkylamines: Biotransformations and Potential Drug–Drug Interactions. Am. Assoc. Pharm. Sci. (2008) doi:10.1208/s12248-008-9028-5.

11.   National Center for Biotechnology Information. PubChem Compound Summary for CID 5761, Lysergide. PubChem https://pubchem.ncbi.nlm.nih.gov/compound/Lysergide. (2019). Accessed in: 2020/10/07.

12.   Stoll, A. & Hofmann, A. Partialsynthese von Alkaloiden vom Typus des Ergobasins. (6. Mitteilung über Mutterkornalkaloide). Chim. Acta 929, (1943).

13. Cerny, A. & Semonsky, M. Mutterkornalkaloide XIX. Ü ber die verwendung von N,N′-carbonyldiimidazol zur synthese der D- lysergsäure-, D-dihydrolysergsäure(I)- und 1-methyl-D- dihydrolysergsäure(I)amide. Czech. Chem (1962).

14. Pioch, R. P. Preparation of Lysergic Acid Amides. (1956).

15. Garbrecht, W. L. Synthesis of Amides of Lysergic Acid. J. Org (1959).

16. Stork, C. M. & Henriksen, B. Lysergic Acid Diethylamide. Encycl. Toxicol. Third Ed. 2, 120–122 (2014).

17. EMCDDA. Lysergide (LSD) Drug Profile. (2020).

18. Cole, M. The analysis of Controlled Substances. (John Wiley & Sons, 2003).

19. Shimron, S., Fenigshtein, E. & Wolf, U. LSD Perforated Blotter Papers. J. Clandestine Lab. Investig. Chem. Assoc. (2019).

20. Passie, T., Halpern, J. H., Stichtenoth, D. O., Emrich, H. M. & Hintzen, A. The Pharmacology of Lysergic Acid Diethylamide : A Review. 14, 295–314 (2008).

21. Dolder, P. C., Schimid, Y., Steur, A. ., Kramer, T. & Retsch, K. M. Pharmacokinetics and pharmacodynamics of lysergic acid diethylamide in healthy subjects. Clin. Pharmacokinet (2017).

22. Dolder, P. C., Schimid, Y., Steur, A. ., Kramer, T. & Retsch, K. M. Pharmacokinetics and concentration-effect relationship of oral LSD in humans. Neuropsychopharmacol (2015).

23. Canezin, J. et al. Determination of LSD and its metabolites in human biological fluids by high-performance liquid chromatography with electrospray tandem mass spectrometry. Chromatogr., Biomed. Appl. 15–27 (2001).

24. Portugal. Decreto-Lei n.º 15/93, de 22 de Janeiro.

25. UN. The UN Drug Control Conventions. 31 (2015).

26. Susan, B. & Drug Policy Alliance. Drug Decriminalization in Portugal Learning from a Health and Human-Centered Approach. Drug Policy Alliance 22, 1–12 (2018).

27. Portugal. Lei n.º 30/2000, de 29 de Novembro.

28.   Portugal. Portaria n.º 94/96, de 26 de março. (1996).

29.   Portugal. Lei n.º 49/2008, de 27 de Agosto. (2015).

30.   Portugal. Decreto-Lei n.º 137/2019, de 13 de setembro. (2019).

31.   Portugal. Decreto-Lei nº 54/2013, de 17 de abril. (2013).

32.   EMCDDA. EU Drug Markets Report 2019. (2019) doi:10.2810/796253.

33.   EMCDDA. European Drug Report 2020: Trends and Developments. (2020) doi:doi:10.2810/420678.

34.   EMCDDA. Statistical Bulletin 2020. https://www.emcdda.europa.eu/data/stats2020_en. Accessed in 2020/03/01.

35.   Europol. Police Seize More Than Eur 4.5 Million In Cryptocurrencies un Europe's Biggest Ever Lsd Bust. (2018). https://www.eureporter.co/frontpage/2018/06/28/police-seize-more-than-e4-5-million-in-cryptocurrencies-in-europes-biggest-ever-lsd-bust. Accessed in 2020/05/25.

36.   SICAD, DMI & DEI. Statistical Bulletin 2018 – Illicit Substances. http://www.sicad.pt (2018). Accessed in 2020/04/10.

37.   Polícia Judiciária. Combate ao tráfico de estupefacientes em Portugal: Relatório anual 2018. https://www.policiajudiciaria.pt/wp-content/uploads/2019/05/RelatorioAnual2018-TCD.pdf (2018). Accessed in 2020/04/10.

38.   APORFEST. Institutional Brochure 2020. https://www.aporfest.pt/english (2020). Accessed in 2020/04/10.

39.   Valente, H. et al. Evaluation of a drug checking service at a large scale electronic music festival in Portugal. Int. J. Drug Policy 73, 88–95 (2019).

40.   EMCDDA. Drugs and the darknet: Perspectives for enforcement, research and policy. (2017).

41.   ENFSI, Team, P. & ENFSI - European Network of Forensic Science Institutes. Guidelines for the single laboratory Validation of Instrumental and Human Based Methods in Forensic Science. Eur. Netw. Forensic Sci. Institutes 1–31 (2014).

42. UNODC - United Nations Office on Drugs and Crime. Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens. 67 (2009).

43. SWGDRUG - Scientific Working Group for Forensic Toxicology. Standard Practices for Method Validation in Forensic Toxicology. 1–52 (2013).

44. UNODC - United Nations Office on Drugs and Crime & UN - United Nations. Recommended Methods for Testing Lysergide (LSD). (1989).

45. Sneddon, J., Masuram, S. & Richert, J. C. Gas Chromatography-Mass Spectrometry-Basic Principles, Instrumentation and Selected Applications for Detection of Organic Compounds. Anal. Lett. 40, 1003–1012 (2007).

46. Hoffmann, E. de & Stroobant, V. Mass spectrometry: Principles and Applications. (2007).

47. Silverstein, Robert and Bassler, G.. Spectrometric Identification of Organic Compounds. Chem. Educ. 1962, 39, 11, 546. Publication Date:November 1, 1962

48. Schug, K. A. Sample Preparation in Mass Spectrometry. Wiley Anal. Sci. (2014).

49. Sleeman, R. & Carter, J. F. Mass Spectrometry - Overview. Encycl. Anal. Sci. Second Ed. 337–344 (2004) doi:10.1016/B0-12-369397-7/00342-3.

50. Prest, H. F. New Approaches to the Development of GC / MS Selected Ion Monitoring Acquisition and Quantitation Methods Technique / Technology Lock the Compound Retention Times for. Agil. Technol. Inc. 1–6 (2001).

51. Agilent Technologies. Strategies for Developing Optimal Synchronous SIM-Scan Acquisition Methods AutoSIM / Scan Setup and Rapid SIM. Tech. Overv. 7 (2006).

52. Agilent Technologies. Setting up a SIM Acquisition Method. 1–2 (2015).

53. Skoog, D. A., West, D. M., Holler, F. J., Crouch, S. R. & SKOOG. Fundamentals of Analytical Chemistry. Journal of Chemical Information and Modeling vol. 53 (Cengage Learning, 2013).

55. Lynch, K. L. Toxicology: liquid chromatography mass spectrometry. in Mass Spectrometry for the Clinical Laboratory 109–130 (Elsevier, 2017).

doi:10.1016/B978-0-12-800871-3.00006-7.

56.   Molnár, B. & Molnár-Perl, I. The role of alkylsilyl derivatization techniques in the analysis of illicit drugs by gas chromatography. Microchem. J. 118, 101–109 (2015).

57.   Blau, K. & Halket, J. Handbook of derivatives for chromatography. Appl. Biochem. Biotechnol. 43, 161 (1993).

58.   Segura, J., Ventura, R. & Jurado, C. Derivatization procedures for gas chromatographic-mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents. J. Chromatogr. B Biomed. Appl. 713, 61–90 (1998).

59.   Sigma-Aldrich. BSTFA - Product Specification. 1–16 (2008).

60.   Knapp, D. R. Handbook of Analytical Derivatization Reactions. (1979).

61.   Magnusson, B. & Örnemark, U. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics. 62 (2014).

62.   IUPAC - Internation Union of Pure and Applied Chemistry. Harmonized guidelines for single-laboratory validation of methods of analysis. 74, 835–855 (2002).

63.   IUPAC - Internation Union of Pure and Applied Chemistry. Guideline for calibration in analytical chemistry- Part 1 . Fundamentals and single component. 70, 993–1014 (1998).

64.   Topics, R. The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics.

65.   ISO - International Standard Organization. ISO 8466-1:1990, Water quality — Calibration and evaluation of analytical methods and estimation of performance characteristics — Part 1: Statistical evaluation of the linear calibration function. vol. 1990 (1990).

66.   OIV - International Organisation of Vine and Wine. OIV-MA-AS1-12:R2005, Practical guide for the validation , quality control , and uncertainty assessment of an alternative oenological analysis method. (2005).

67. ISO - International Standard Organization. ISO 8466-2:2001, Water quality — Calibration and evaluation of analytical methods and estimation of performance characteristics — Part 2: Calibration strategy for non-linear second-order calibration functions. vol. 2001 (2001).

68. Rawski, I., Skital, P. M. & Saletnik, D. E. Regression Analysis in Analytical Chemistry . Determination and Validation of Linear and Quadratic Regression Dependencies. 166–173 (2016).

69. Ellison, SLR Williams, A. Eurachem/CITAC guide: Quantifying Uncertainty in Analytical Measurement. (2012).

70. Reichenbächer, M. & Einax, J. W. Challenges in analytical quality assurance. Challenges in Analytical Quality Assurance (2011). doi:10.1007/978-3-642-16595-5.

71. RESPONSE Project. NPS and Related Compounds - Analytical Reports. https://www.policija.si/apps/nfl_response_web/seznam.php (2020). Accessed in 2020/10/25.

72. Nelson, C. C. & Foltz, R. L. Chromatographic and mass spectrometric methods for determination of lysergic acid diethylamide (LSD) and metabolites in body fluids. J. Chromatogr. B Biomed. Sci. Appl. 580, 97–109 (1992).

73. Musshoff, F. & Daldrup, T. Gas chromatographic/mass spectrometric determination of lysergic acid diethylamide (LSD) in serum samples. Forensic Sci. Int. 88, 133–140 (1997).

74. Francom, P. et al. Determination of lsd in urine by capillary column gas chromatography and electron impact mass spectrometry. J. Anal. Toxicol. 12, 1–8 (1988).

75. Clarkson, E. D., Lesser, D. & Paul, B. D. Effective GC-MS procedure for detecting iso-LSD in urine after base- catalyzed conversion to LSD. Clin. Chem. 44, 287–292 (1998).

76. Mendoza-Valencia, C. Y. et al. Mix of new substances psychoactive, NPS, found in blotters sold in two Colombian cities. Forensic Sci. Int. 304, 109969 (2019).

77.   SWGDRUG - Scientific Working Group for Forensic Toxicology. 2020 SWGDRUG Mass Spectral Library. https://swgdrug.org/ms.htm (2020). Accessed in 2020/11/22.

Annex

Procedimento Operacional Padrão

Lisboa, 2020.

Objetivo

O objetivo deste trabalho é desenvolver e validar um método de cromatografia gasosa acoplada à espectrometria de massas para identificação e quantificação da dietilamida do ácido lisérgico (LSD) em amostras provenientes de apreensões.

Escopo

Especificação do processo de medição

Processo de medição	Análise quantitativa através de cromatografia em fase gasosa acoplada à espectrometria de massas (GC-MS)
Analito	LSD
Mesurando	Concentração de LSD em amostras provenientes de apreensões
Unidade de medida	µg/mg
Matriz da amostra	• Micro-selos de papel (*blotters*)

Justificação

O artigo 2º da Lei Nº 30/2000 estipula os limites para que a posse de substâncias controladas seja considerada para uso pessoa. De acordo com a Lei, a quantidade máxima não pode ultrapassar àquela necessária para dez dias de consumo com base na dose média individual (DMI). A DMI para cada planta, substância ou preparação listadas nas tabelas I a IV estão estipuladas através da Portaria Nº 94/96. Uma vez que o LSD está listado na tabela II-A e sua DMI corresponde à 50 µg, materiais apreendidos com quantidades de LSD superiores à 500 µg serão enquadradas à luz da lei Penal (Art. 21º da Lei 15/93).

Portanto a quantificação é fundamental para a verificação da materialidade da infração penal.

Campo de Aplicação

Este procesimento é aplicavél ao sector de Drogas e Toxicologia do Laboratório da Polícia Científica (STD-LPC) da Polícia Judiciária.

Documentos de referência

- UNODC – Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in seized Material and Biological Specimens
- ENFSI - Guidelines for the single laboratory Validation of Instrumental and Human based Methods in Forensic Science
- Eurachem – The Fitness for Purpose of Analytical Methods: *A Laboratory Guide to Method Validation and Related Topics*
- UNODC - Guidelines on Representative Drug Sampling

Amostragem

Realizar a amostragem de acordo com as recomendações do *United Nations Drug Control Programme* (UNDCP), adotado pelo LPC-PJ:

$para\ N < x \therefore n = N$;
$para\ x \leq N \leq y \therefore n = z$;
$para\ N > y \therefore n = \sqrt{N}$
onde, $x = 10$, $y = 100$ e $z = 10$

- Aferir a massa bruta da amostra e registar o número total de doses individuais (*blotters);*
- Aferir a massa da amostra (um *blotter)*

Extração

- Acondicionar a amostra em um tubo de vidro e adicionar 1 mL de metanol;
- Posicionar o tubo de vidro com a amostra dentro do banho de ultrassom, com a temperatura previamente ajustada para 30 ºC. Colocar a tampa do equipatamento para poteção à luz UV;
- Deixar em banho de ultrassom por 30 minutos.

Após a extração, reservar, para cada amostra, dois vials de 2 mL.

- Remover o *blotter* do tubo de vidro;

- Transferir 500 µL do extrato para um vial e reserva-lo sob abrigo de luz UV para *screnning* de novas substâncias psicoactivas (NSPs).

- Transferir o extrato restante (500 µL) para o vial.

Derivatização

- Ao extrato, adicionar 100 µL da solução de padrão interno e agitar em vortex por 30 segundos;Evaporar completante os solventes sob fluxo de azoto;

- Adicionar 50 µL de MSTFA/PYR (1:1) e voltar a agitar em vortex por 1 minuto;

- Transferir a solução para um insert de 250 µL e tampar o vial. É fundamental que o vial esteja totalmente selado;

- Acondicionar o vial na estufa, com a temperatura previamente ajustada em 60 ºC, por 30 minutos.

- Após a reação de derivatização é extremamente crítico que o vial seja acondicionado dentro do dessecador para arrefecimento por, no mínimo, 30 minutos.

Análise por GC-MS

A primeira alíquota do extrato deve ser analisada directamente (sem derivatização) para pesquisa de NSPs. No decorrer da desenvolvimento deste protocolo, foi evidenciado que o método denominado "LSD_FS" foi capaz de identificar todas as substâncias deliberadamente adicionadas as amostras teste, enquanto que algumas substâncias não foram detetadas através dos métodos "MS" e "ANF", pelo que recomenda-se que análise seja feita atravé do método "LSD_FD".

A analíse da segunda alíquota do extrado (que fora derivatizada) deve ser analisada através do método validado para análise qualitativa e quantitativa de LSD, denominado "LSD_SIM". Recomenda-se que, sempre que possível, imediatamente após a injeção da amostra, a tampa do vial seja substituída por uma nova e que o mesmo seja acondicionado dentro do dessecador, sob o abrigo de luz UV. Este procededimento visa garantir a estabilidade do analito para quando re-análises se façam necessárias.

A faixa linear validada do método é estreita e cobre apenas concentrações relativamente baixas do analito. É preciso, portanto, verificar se a resposta instrumetal

(razão entre as áreas do analito e do padrão interno) encontra-se dentro da gama analítica validada. Os valores de referência econtram-se na folha de cálculos, em que LOD corresponde ao limite de deteção do método, LOQ o limite de quantificação e Y_N o maior valor instrumetal aceitável. Caso a razão das áreas seja superior ao valor de Y_N a amostra deve ser diluida em hexano e re-analizada.

Tratamento dos Dados

O tratamento dos dados deve ser feito através do software Agilent MassHunter Workstation – Qualitative Analysis. O procedimento descrito abaixo deve ser seguido para análise de todas as amostras e soluções padrão de LSD.