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Cannabis and methodological aspects in mass spectrometry: clinical applications

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A J. e J. e a Giacomo, entrambi

Summary

1. Introduction

1.1 Cannabis history

The use of *cannabis* (Indian hemp) has been reported for thousands of years and about 147 million people, 2.5% of the world population, consume *cannabis* (annual prevalence) in the world [1].

Cannabis is surely among the most ancient cultivated plants, it is due to its adaptability in a wide range of habitats and to its several properties: it has been used as a food, fiber and as a drug plant [2]. This plant can be classified into three species: *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis*. *C. Sativa* is taller and more fibrous, while *C. Indica* is shorter and more psychoactive; *C. ruderalis* is the only wild [3]. Botanical taxonomists reported the presence of a further specie: *Cannabis afghanica*.

The principal one used in western society is *Cannabis Sativa*, of which there are several chemical phenotypes which express different cannabinoid compositions [4].

In the literature, studies attested the presence of this plant already about 11700 years ago, in the territories of Central Asia (Mongolia, Kazakhstan, and Siberia) and East Asia [5]. Afterwards, it has spread all over the world, thanks to human domestication.

The first evidence of its utilization by humans date about 10.000 years ago: *cannabis* fruit and seeds fossils have been found in Okinoshima archeological site of Mesolithic Age (Boso Peninsula, central Japan) [6].

Cannabis use as a fiber, food source but also for medicinal and ritualistic functions was documented in ancient China (4000 years BCE) [2,7].

In addition to these and others archaeological discoveries, several written documents of *cannabis* use were found in ancient texts, in particular in Chinese ones.

Hemp cultivation was reported as one of the principal cultivations during the emperor Shen Nung (2700 BCE), in the ancient agricultural treatise "Xia Xiao Zheng" written more than 3000 years ago [8].

Regarding *cannabis* therapeutic use, the first historical presence in the traditional medicine has been reported in the "Shen Nung Pen Ts'ao Ching", the ancient Chinese Pharmacopoea, written in the first century BCE [9]. According to this text a preparation based on female *cannabis* flowers, named MáFěn, was used in condition of Yin loss, for example constipation, gynaecological disorders, rheumatic pain and malaria [7,10]. This preparation was also capable to purify blood and low body temperature, as described in the text "Chêng Lei Pen Ts'ao," written in 1108 CE.

Analgesic properties of *cannabis* are reported, for the first time, in the text "Ho Han Shu": surgical operations without pain were performed thanks to the administration of máyóu, an oily preparation obtained by mixing *cannabis* resin, Datura, and wine, by the surgeon Hua Tuo (110–207 CE) [2].

On the other side, very few documents among the ancient Chinese texts regarding *cannabis* psychoactive properties were found: in the "Pen Ts'ao Ching" the effects of a large amount of this plant ingestion are reported: "see demons" or "communicate with spirits" were the observed symptoms. In fact, the use of *cannabis* for psychoactive purposes was mainly restricted to shamans [10].

Hemp use was also documented in other country of Asia, such as India, Tibet and Japan.

In India it was used especially for sacred purposes: it was employed as a source of joy and freedom in many religious rituals, as documented in the sacred text "Atharva Veda" written in Sanskrit by Arii, a nomadic Indo-European people arrived in India two thousand years BCE [7].

The therapeutic use of *cannabis* in India was closely related to religion and ritual practices. Its properties were described in "Susrita Samhita," one of the foundational Ayurvedic medicine texts (800 BCE), it was analgesic, anaesthetic, antiparasitic, antispastic and diuretic.

It was also used as aphrodisiac agent, as anti-convulsant, as an expectorating agent, as an appetite stimulant and as a tonic [11]; its use was also documented in the treatment of otitis, diarrhoea, constipation, gonorrhoea, cough, asthma, headache and insomnia [12].

Considering its "sacred properties", in Tibet *cannabis* was used in Tantric Buddhism as a holy plant able to facilitate meditations [7].

In addition, *cannabis* was used also in traditional Japanese medicine. It was mainly employed as a laxative, as a body tonic in the treatment of asthma, in skin pathologies against parasites and poisonous bites [13].

The diffusion of *cannabis* from Asia into Europe occurred through Scythians people, moving from Central Asia through Russia about 3500 years ago. Indeed, it was found in Scythians graves in Germany, Siberia and Ukraine; as reported by the Greek historian Herodotus form Halicarnassus (484–425 BCE), they used hemp during funeral ceremonies and in banquets for its relaxing properties [7].

It is reported in the literature that Scythians could have known *cannabis* use from Assyrians and Thracians (2600 BP), who used it to facilitate visions and trances for ritualistic purposes.

Cannabis was largely employed by Assyrian for its main properties, both psychoactive and not: it was used for swellings and bruises treatment, arthritis, kidney stones, depression, impotence and gynaecological disorders [14].

It was also reported as the most important medicinal plant in the "Avasta", the religious texts of Zoroastrianism, one of the world oldest religions founded by the Iranian prophet Zarathustra [15].

Cannabis was known also in ancient Egypt: its use and several ways of administration (oral, rectal, vaginal, by fumigation and topic) were described in the Egyptian Pharmacopoeia. As reported in the

"Ebers papyrus" (1550 CE), it was used in childbirth contractions induction, while in the "Papyrus Ramesseum III" (1700 BCE) it is described for the treatment of eye pathologies [16].

Regarding Africa, the use of this interesting plant was documented in the medical texts of Avicenna (980–1037 CE). He suggests *cannabis* use for the treatment of degenerative bone and joint disease headache, ophthalmitis, edema, gout and uterine pain [17].

In addition, in Arabic medicine, the knowledge on *cannabis* was advanced with respect to Europe and it was considered a sacred medicine: hemp seeds oil was largely used to treat ear affections, locally for skin diseases but also for intestinal diseases, fever, vomiting and neurological pain[16,18].

Another diffusion step occurred during African slave deportation in South America: in this context hemp use as a medicine and also for religious rituals was spread [19].

In these countries new applications were introduced in *cannabis* field: roots were used as laxative scope and stems and seeds were used against insomnia, while *cannabis* juice was employed in seizures and urinary affections treatment, while alcoholic tinctures were used to relieve neuralgia [20].

Also, Greek and Latin civilisations used *cannabis*, mainly as a fiber. Its use as a medical plant with analgesic properties is documented by physicians Pliny (ca. 23–79 CE), Galen (ca. 129–201 CE, and Dioscorides (ca. 40–90 CE) [2].

During Medieval Age in Europe *cannabis* use in medicine progressively decreased, due to its psychoactive properties: its use was strongly condemned by the pope Innocent VIII in 1484 [21].

On the other side, during Medieval Age ad also during Renaissance it was employed in the manufacturing of textile fiber.

In the XIX century pharmacological and toxicological properties of *cannabis* has been studied with scientific method. First experimentations in this field where conducted in India by the Irish physician

William Brooke O'Shaughnessy: he diffused pharmacological benefits of *cannabis* among the European medical community [7,22].

He described the differences between the variety of *cannabis* cultivated in India, *C. indica*, and the European one, *C. sativa*, used for fiber manufacturing. Differences regarded also the pharmacological properties [2].

In 1890, Sir J. Russell Reynolds, the personal physician of Queen Victoria, described his medical observations on *cannabis* properties on the medical journal The Lancet, reporting the usefulness of this plant in pain treatment [2].

The French psychiatrist Jacques Joseph Moreau, considered as the father of modern psychopharmacology, employed hemp inflorescences and resin as psychoactive drugs.

In 1840 ca. he administered *cannabis* to his patients to increase appetite, calm down and facilitate sleep [23].

In Italy, the first experimentations on *cannabis* was carried out by Carlo Erba, pharmacist and founder of a famous Italian Pharmaceutical Company and Giovanni Polli, physician and Director of the scientific journal "Annals of Chemistry applied to Medicine" [24].

The second part of the XIX century was the Golden age of *cannabis*, characterized by large use in medicine of this plant and a wide number of publications about this field.

Cannabis was mainly prescribed as analgesic, anti-inflammatory, and antispastic [21].

Regarding the discovery of cannabinoids, the active substances of *cannabis*, occurred for the first time in 1964. In particular, the identification of the chemical structure of Δ9 -tetrahydrocannabinol (Δ9 - THC), the most abundant and psychoactive cannabinoid, was performed in Israel by the scientists Gaoni and Mechoulam [25].

Finally, in 1988 also the endocannabinoid system, its receptors and the endocannabinoids were discovered [2,26].

1.2 Cannabis Pharmacology

Cannabis plant contains approximately 540 natural compounds [4] including about 120 phytocannabinoids, presenting a chemical structure with a skeleton of oxygenated 21 carbon atoms, with a common fragment that includes the hydrophobic alkyl chain and a dibenzopyran ring [27].

In the phytocannabinoids biosynthetic pathway Acetyl-CoA and Malonyl-CoA are the precursor building blocks of the aromatic fragment from the polyketide intermediate, which undergoes sequential cyclization, aromatization and reduction in order to originate olivetolic acid.

Simultaneously, isopentenyl diphosphate (IPP) and dimethylalyl diphosphate (DMAPP) lead the geranyl pirophosphate formation [28].

The olivetolic acid and geranyl pirophosphate lead to the formation of cannabigerolic acid (CBGA), that undergoes decarboxylation to generate CBG. GBGA have also a role in the formation of the carboxylated phytocannabinoids THCA, CBCA and CBDA (Δ9 -tetrahydrocannabinolic acid, cannabichromenic acid and (canabidiolic acid, respectively) [28-30].

THC is the major constituent in *cannabis*, it is thermodynamically unstable and undergoes isomerization in the presence of acids leading the formation of Δ8 -THC [4].

Regarding pharmacokinetics (PK) of cannabinoids, following inhalation, the principal route of *cannabis* administration, it quickly passes into circulation from the lungs and is therefore rapidly absorbed by the tissues; it is first metabolized by the liver by cytochrome P450 and C-11 is the major

site attacked: THC is converted into 11-hydroxy-THC, a psychoactive compound, that is further oxidized in THC-COOH, which may be glucuronidated to 11-nor-9-carboxy-THC glucuronide.

Inhaled cannabinoids show similar PK to intravenously administered ones [31], exhibiting higher maximum concentrations compared to oral ingestion [31-37]. After smoking, THC and CBD peak plasma exposures are reached rapidly, about 3 and 10 min [31,36].

The inhaled THC bioavailability ranges from 10% to 35% [31], due to intra- and inter- subject variability, the inhalational characteristics and the used inhalational device [33-35,37,38]. Inhaled CBD presents bioavailability of 31%, and a plasma concentration–time profile similar to THC [31,36].

In the context of medical *cannabis*, the use of a vaporizer for cannabinoids administration is favourable: the PK of vaporized and smoked cannabinoids are comparable, and vaporization avoids toxic pyrolytic compounds exposure and the smoked cannabis respiratory risks [39].

In addition, inhalation avoids or reduces the extensive first-pass metabolism characteristic of the oral administration. This is also a characteristic of the oromucosal delivery; oromucosal preparations are rapidly absorbed via the oral mucosa, producing higher plasma drug exposure then the oral ones, but reduced then inhaled THC [40].

Regarding cannabinoids oral administration, THC and CBD absorption is variable: it suffers extensive hepatic first-pass metabolism [41], showing lower peak plasma concentration than inhaled administration [42] and reaching peak concentration after ca. 120 min [31,43].

Transdermal cannabinoids administration also avoids first-pass metabolism but higher hydrophobic nature of these molecules limits the diffusion across the skin aqueous layer [44]. This route of administration can be productively used by a permeation enhancement [45].

Anyway, in the literature studies with human skin showed CBD permeability 10-fold higher than that of Δ9 - THC [44,46], because of its lower lipophilicity [46].

Regarding distribution, cannabinoids rapidly distribute into lung, heart, brain and liver [42,47,48], and subsequently into the less vascularized tissue [48]. They accumulate in adipose tissues in patients under chronic treatment [37,49].

CBD and THC volumes of distribution (Vd) are high, Vd_{β} ~32 l kg^{-1} (calculated following intravenous administration) [36] and Vd_{ss} 3.4 l kg^{-1} (calculated following inhaled administration) [37], respectively.

Composition and size of the body and disease influencing blood–tissue barriers permeability could affect the Distribution of these molecules [50].

As reported above, THC metabolism is mainly hepatic, via CYP 450 isozymes CYP2C19, CYP2C9 and CYP3A4. Also, extra-hepatic tissues that express CYP450, such as small intestine and brain, have a role in cannabinoids metabolism [37].

It is important to know, with regard to toxicity, that THC is able to cross the placenta [43] and is also present in human milk [51].

CBD is metabolized by CYP450 isozymes: CYP2C19, CYP3A4, CYP1A1, CYP1A2, CYP2C9 and CYP2D6 [45]. Regarding its metabolism, it is hydroxylated to 7-hydroxy cannabidiol (7-OH-CBD), A few data are available in the literature about the pharmacological activity of CBD metabolites [52].

As concerns elimination, *cannabis* heavy users show a relatively longer elimination half-life [34], it is due to the slow redistribution from deep compartments e.g. adipose tissues [34,35].

Indeed, in the heavy user blood it is possible to measure THC concentrations >1 μg l^{-1} more than 24 h after the last cannabis use [34,53,54]. Also, CBD has a long terminal elimination half-life, with the (24 ± 6 h following intravenous administration and 31 ± 4 h post-inhalation) [36].

As reported in the study of Conroe *et al.* a repeated CBD daily oral administration presented an elimination half-life between 2 to 5 days [55].

Regarding pharmacodynamic (PD), in 1988 Allyn Howlett and W.A. Devane discovered the Endocannabinoid System: a complex biological and molecular system that plays a central role in several physiological processes such as neurogenesis, neuroprotection, nervous functions, depression, eating and emotional behaviour, recompense, cognition, memory, learning, painful sensation and also in fertility and pregnancy [56-58].

The interest in the endocannabinoid system role in health and disease processes increased over recent years: indeed, it results an interesting pharmacological target for many diseases including: pain, headache, anxiety and depression, neurodegenerative diseases, metabolic and appetite disorders, glaucoma and cardiovascular diseases [59-61].

Considering endocannabinoid system structure, its components include receptors, their ligands, and enzymes involved in their biosynthesis and degradation [62]. In particular: G-Coupled Protein Receptors (GPCRs) (Cannabinoid-receptor type 1 and 2, CBR1 and CB2R) [63], Ligand-sensitive ion channels (Transient Receptor Potential Vanilloid 1, TRPV1) and nuclear receptors (e.g., PPARs) [64,65].

The endogenous ligands of the endocannabinoid system are anandamide or N-arachidonoyl ethanolamine and 2-arachidonoylglycerol; diacylglycerol lipase isozymes α and β, fatty acid amide hydrolase, monoacylglycerol lipase, and N-acylphosphatidylethanolamine-selective phospholipase D are some of the endocannabinoid synthesis and degradation enzymes [66]. All these components are largely distributed throughout mammalian tissues and cells.

In details, CB1 and CB2 have been identified in the central nervous system and peripheral nervous system. CB1 is the most abundant GPCR in the central nervous system and it is expressed in pre-synaptic neurons in the neocortex, cerebellum and limbic system (amygdala, hippocampus). It is also present in the peripheral nervous system, where it activates K^+ channels and causes inhibition of neurotransmitter release.

CB2 has been identified in the immune system, such as in lymphocytes, mast cells and macrophages, and in the CNS on microglia cells and astrocytes [67] and this appears to mediate the anti-inflammatory and immune-regulating properties of these compounds.

Activation CB1 receptor mediates drug rewards and natural retributions: sexual activity, social interaction and food consumption [68]. Favourable effects on an individual mental health are produced by Δ 9 -THC interacting with this receptor [69].

However, as reported in the literature, CBD shows anxiolytic activity reverting Δ9 -THC psychotic and anxiogenic effects, by a CB1 receptor-independent mechanism [70]. It plays a role in decreasing condition of fear, reducing chronic stress and autonomic arousal [71] through the interaction with TRPV-1, serotonin 5-HT1A and, in a lower degree, CB1 receptors [71].

A role of CBD in reducing depression by increasing brain-derived neurotropic factor (BDNF) has also been suggested [72]. Indeed, BDNF protein promotes neurogenesis and the nerve cells growth, maintenance and survival.

Regarding the principal indication of *cannabis* use, the endocannabinoid system has an important role in pain management and cannabinoids show to target its components [73] such as the CB1 and CB2 receptors, non CB1R/CB2R cannabinoid G protein-coupled receptor 55 [74], opioid/serotonin (5-HT) receptors [75-77], N-arachidonoyl glycine receptor [78], TRPV1 [79,80], and PPARα and γ [65].

In a study on murine model [74], the GPR55 receptor modulates proinflammatory cytokines (IL-4, IL-10, IFN gamma…) reducing hyperalgesia.

As reported in the study of Anand *et al.*, CB_2 receptor antagonists showed antinociceptive activity in inflammatory and nociceptive pain, probably by inhibiting anandamide metabolism or by modulating TRPV1 antagonists, peroxisome proliferator-activated receptor α agonists and α2- adrenoceptor modulators [81].

Sometimes, this is obtained by opioid system activation of μ-opioid receptor agonists: cannabinoid and opioids or non-steroidal anti-inflammatory drugs have been demonstrated to have a synergistical activity [81].

In details, several studies highlight that CBD could have therapeutic advantages in treating fibromyalgia, rheumatoid arthritis, arthritis, chronic pain, headache and facial pain [58,82].

Cannabinoids also showed activity against thermal and noxious pain, cancer pain, postoperative pain, pain related to spinal cord injury and traumatic nerve injury and toxic insults [83,84].

Cannabinoids shows potent anti-inflammatory activity: inflammation occurs in many pathologies, such as cancer, asthma, rheumatoid arthritis, multiple sclerosis, hepatitis, colitis and dermatologic diseases [58].

As reported by Nagarkatti *et al*. endocannabinoids and phytocannabinoids Δ9 -THC and CBD showed anti-inflammatory and immune-suppressive properties interacting with CB1 and CB2 receptors [85]. Cannabinoids are able to downregulate the production of cytokine and chemokine, suppressing inflammatory responses [85]: as an example, CBD could modulate inflammation by controlling the release of pro-inflammatory cytokines such as TNF-α, inhibiting GPR55 receptor activity [86].

1.3 Therapeutic indications and regulatory aspects

In Italy, the Ministerial Decree of 9 November 2015 regulates the authorization, cultivation, import, export and distribution of *cannabis* [87].

The therapeutic indications for *cannabis* medical use concern:

- analgesia in pathologies involving spasticity associated with pain (multiple sclerosis, spinal cord lesions) refractory to treatment with traditional therapies;

- analgesia in chronic pain (with particular reference to neurogenic pain) in which treatment with non-steroidal anti-inflammatories or with cortisone or opioid drugs has demonstrated ineffective;
- anti-kinetic and anti-emetic activity in nausea and vomiting, caused by chemotherapy, radiotherapy, HIV therapies, which cannot be obtained with traditional treatments;
- the appetite stimulating effect in cachexia, anorexia, loss of appetite in cancer patients or in people living with HIV and in anorexia nervosa, which cannot be obtained with standard treatments;
- the hypotensive effect in glaucoma refractory to treatment with traditional therapies;
- the involuntary body and facial movements reduction in Gilles de la Tourette syndrome patient refractory to treatment with traditional therapies;

As mentioned above, different phenotype of *cannabis* present different amount of cannabinoids: in some plants Δ9 -THC is the predominant one, while in other plants is CBD and in other variety a mixtures of this two molecules [4].

This is a fundamental aspect for medical *cannabis*: in details, preparations with high CBD content are indicated for inflammation, mental disorders, epilepsy, migraine, depression, and anxiety, while preparations with high THC for pain, muscle spasticity, glaucoma, insomnia, low appetite, cachexia, nausea [71,88,89].

Regarding *cannabis*-based herbal medicine authorized for the Italian market, Sativex® is a spray formulation of a mixture of CBD and THC; it is only indicated in patients with moderate to severe spasticity refractory to treatment with traditional therapies.

Epidyolex® is a highly purified CBD oral solution approved by European Medicines Agency (EMA) [88]: it is indicated for seizures associated with Lennox–Gastaut syndrome (LGS) or Dravet syndrome in patients aged > 2 years in combination with clobazam.

The Decree of the Ministry of Health of 9 November 2015 [90] authorizes the Military Chemical and Pharmaceutical Works of Florence to cultivate and product medical *cannabis*. *Cannabis* FM2 was the first preparation was, available since January 2017, with standardized concentrations of THC and CBD at 5–8% w/w and 7.5–12% w/w, respectively. The second product, was FM1 (July 2018), with a declared THC and CBD content of 13–20% w/w and <1% w/w, respectively.

FM1 and FM2 are distributed to authorized pharmacies for the preparation of magisterial galenic preparations.

In addition, *cannabis* varieties imported in Italy from Holland are: Bedrocan® (THC concentrations 22% w/w and CBD <1%), Bediol® (6.5% THC and 8% for CBD), Bedrobinol® (13.5% THC and <1% CBD) and Bedrolite® (0.4% THC and 9% CBD w/w).

Indeed, to satisfy product demand, non-registered Dutch Cannabis-based products are imported in relation to the export availability of the Office of Medicinal *Cannabis*, with the application of the directives of the Ministry of Health, Welfare and Sport Dutch on the export of such products [89].

Ministerial Decree also indicates recommended routes of administration for medical *cannabis*: oral administration (decoctions and oil extract in drops), and vaporization.

The recovery of the different cannabinoids in the decoction is very limited and with high variability between THC and CBD [91-93]. Moreover, the cannabinoids stability in aqueous solution is very low [94]. Oil extracts have a better recovery of THC and CBD and a greater stability.

In the literature several methods for galenic product preparation are reported and the absence of standardization contribute to the high variability in the active compound concentrations that are observed [95].

1.4 Use of cannabis in pain management

Regarding the neuropathic and chronic pain management (NP, CP), NP is described as "pain caused by a lesion or disease of the somatosensory system" by The International Association for the Study of Pain (IASP) [96]. The overall prevalence of NP in the population is between 7 and 10% [97] reaching 20–25% in patients subjects with CP [98].

Particularly, it may be spontaneous or as a consequence of mechanical or thermal stimuli (hyperalgesia or allodynia) and it appears in noxious stimulus absence [99].

Allodynia and hyperalgesia represent the symptoms that most limit the quality of life of patients suffering from chronic pain, in particular neuropathic pain.

Added to these painful symptoms are co-morbidities such as anxiety, depression, cognitive dysfunction and memory loss, which make neuropathic pain a neuropsychiatric pathology whose pharmacological treatment is still a challenge today, due to the adverse effects that often limit many pharmacological options.

NP n is characterised by a combination of positive and negative phenomena: positive phenomena are defined as various painful symptoms such as hyperalgesia and/or allodynia, and abnormal non-painful symptoms such as paraesthesia and/or dysaesthesia.

Negative ones are represented by neurological sensory deficits in the painful area, in association with other motor or vegetative deficits, depending on the location of the lesion [98,100,101].

NP is classified by the diseases aetiology affecting the peripheral or central nervous system. Metabolic (e.g. diabetes mellitus), inflammatory (e.g. post-herpetic neuralgia) or traumatic nerve injury are involved in painful peripheral neuropathies development, that are aetiologies of peripheral NP [99].

Central NP can occur after a stroke, spinal cord injury or in patients with multiple sclerosis [98]. Often neuropathic and non-neuropathic mechanisms are involved in pain syndromes, such as cervical or lumbar radiculopathies.

Neuropathic pain can negatively affect quality of life: it is often associated with loss of function, depression, anxiety, sleep and cognitive disorders [102].

The management of NP is mostly focused on symptom treatment.

Recommended therapeutic options are: anti-epileptic drugs (pregabalin, gabapentin), serotonin norepinephrine reuptake inhibitors (duloxetine) and tricyclic antidepressants.

For peripheral neuropathic pain management high-concentration capsaicin patches, lidocaine patches and tramadol are recommended as second-line treatments, while strong opioids and botulinum toxin are recommended as third-line treatments [102].

Despite these neuropathic options NP management remains a critical clinical point: pain relief with classic medications is reported by less than 50% of patients and several adverse effects occurs [103]. In addition, the current alarming addiction rates and deaths from opioid abuse have shown that there is a need to explore other treatment options safer [104].

Many individuals with chronic pain are seeking alternative medications for pain management and *cannabis* and its metabolites seem to be a safer alternative to opioids, non-steroidal anti-inflammatory drugs, and most treatments.

Data are available in the literature about this topic: a review by Vučković *et al*. [73] analysed scientific studies performed between 1975 and March 2018 on CBD use in the treatment of pain associated to cancer, neuropathic pain and fibromyalgia suggesting the efficacy of medical *cannabis* use in pain management [73]. CBD could have analgesic activity with different mechanisms of action: activating pain inhibition pathways, reducing inflammation, inhibiting neuropeptide and neurotransmitter release and regulating neuron excitability (in particular in neuropathic pain).

Cannabinoids effectiveness on multiple sclerosis related pain compared with placebo were observed in the CAMS study [105], regarding 630 patients treated with THC and in the MUSEC trial focused on stiffness management [106].

In addition, greater pain reduction was observed in patients treated with nabilone than ones who were treated with placebo in a trial studied nabilone as an additional treatment to gabapentin for neuropathic pain in multiple sclerosis [107].

On a study published by Blake *et al*. *cannabis* (Sativex) improved significantly pain and sleep quality in patients with rheumatoid arthritis, without reporting adverse effects [108].

Regarding cancer-related pain, in 2010 in a randomized double blinded study the effects of nabiximol spray (THC:CBD) in the treatment of advanced cancer patients refractory to opiate management was investigated.

Patients treated with nabiximol showed improved pain compared with ones treated with placebo and, among them, 23 subjects demonstrated a reduction of more than 30% of baseline pain [109].

In light of all these evidences, *cannabis* has demonstrated its efficacy in pain treatment and in reducing opioid consumption; more studies in order to ensure its effectiveness and safety in patient pain management are urgently needed.

All medical staff involved in the management of patients with chronic and neuropatic pain treated with *cannabis* should monitoring them, and base treatment decisions on scientific evidence to provide safety and efficacy in the management of this fragile population.

1.5 Therapeutic drug monitoring in patients treated with medical cannabis

Various administration and delivery forms have been tested for the therapeutic use of medical *cannabis*. The most common administration routes are inhalation (smoking or vaporization) and oral

administration (tablet, edibles containing *cannabis* extract or oils). Other minor strategies include oromucosal (oil/lozenge), sublingual, rectal, transcutaneous, and intravenous [110].

PK profile of cannabinoids, particularly the adsorption step, vary significantly depending on the route of administration (20-30% cannabinoids adsorption for the oral routes against 10-60% adsorption for the inhalation) [111].

Smoking remain most common administration route between medical *cannabis* users. Inhalation of combusted-dried flowers allows a faster onset of action combined with a much higher peak serum concentration relative to most of the other administration routes. However, the combustion process implies the production of toxic products such as tar, PAH (polycyclic aromatic hydrocarbons), carbon monoxide (CO) and ammonia (NH3) commonly associated with respiratory symptoms (bronchitis, cough, phlegm) [88].

The oral route of administration overcomes many of the drawbacks of inhalation with relatively stable serum concentrations [110]. However, the extensive first-pass metabolism effect complicates the dosing of orally administered cannabinoids [111].

Few data are available in the literature regarding both the PK properties and toxicity of the several medical *cannabis* preparations; also, optimal dosing in different populations data are scarce.

In light of this, therapeutic drug monitoring (TDM) in patients treated with this plant has an important role for personalized therapy and pharmacovigilance in the development of medical *cannabis* products.

TDM is the clinical practice used to measure drugs concentration in patient bloodstream and optimize individual dosage regimens. It is used primarily for drugs with narrow therapeutic ranges, drugs with high pharmacokinetic variability, drugs with significant adverse effects and substances with target concentrations difficult to monitor; it allows to use difficult-to-manage medications appropriate concentrations and to optimize clinical outcomes in patients [112].

In the context of medical *cannabis*, TDM may be used to fix dosing schedules, currently lacking, and optimizing individual therapy [113].

To date, few data are available in literature concerning *cannabis* PK although a large number of methods for cannabinoids detection have been published over the last few years [114-119], including plasma and urine analysis using HPLC and gas chromatography coupled with mass spectrometry [120].

The gold standard for cannabinoids quantification and for utilization in TDM analysis is represented by plasma analysis by LC-MS/MS.

2. Aims of the study

Since no method performs an optimal chromatographic separation between Δ9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC) and, in addition, in a few minutes, primary aim of the present study was the development and validation of an ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method for the quantification of principal cannabinoids and their metabolites in human plasma, as a useful tool for PK studies.

Furthermore, since no data are available in the literature, this study could be helpful in evaluating the efficacy and safety of medical *cannabis* in treated patients, laying the foundations for the therapeutic range definition.

Indeed, another aim was to evaluate the cannabinoids and their main metabolites PK in a cohort of patients suffering from neuropathic pain, treated with inhaled medical cannabis and decoction, as a galenic preparation administered in clinical practice.

Finally, other aims of the present study were to evaluate the impact of pharmacogenetics and neurological biomarkers, for example neurofilaments (NFL), on the therapeutic outcome.

3. Materials and methods

3.1. Method Validation

3.1.1. Chemicals

Δ9-THC, Δ8-THC, CBD, Cannabinol (CBN), Tetrahydrocannabinolic acid (THCA), Cannabidiolic acid (CBDA), 11-OH-THC, 11-COOH-THC, THC-gluc, cannabidiol–D_3 (CBD-D_3), tetrahydrocannabinol–D_3, (THC-D_3), 11–Hydroxy–Δ9–tetrahydrocannabinol–D_3 (11-OH-THC- D_3), 11–Nor–9–carboxy–Δ9–tetrahydrocannabinol (11-COOH-THC-D_3), THC–glucuronide-D_3 solutions in methanol (≥99% purity) were obtained from Sigma-Aldrich (Milan, Italy).

HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy).

HPLC grade acetonitrile was obtained from VWR International (Radnor, PA, USA).

Formic acid was purchased from Sigma-Aldrich (Milan, Italy).

Blank plasma from healthy donors were kindly supplied by the Blood Bank of the "Città della Salute e della Scienza" of Turin.

3.1.2. Standard and Quality Control

Stock solutions were used to independently spike blank plasma to obtain 6 levels of calibration standard and 2 different quality control samples (QCs): high and low (QC H and L, respectively). Calibration and QCs concentrations are: 250 ng/ml (ULOQ – upper level of quantification), 100 ng/ml, 50 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml (LLOQ – lower level of quantification) for standards and 150 ng/ml (QC H) and 7 ng/ml (QC L) for QCs, respectively.

3.1.3. Standards, QCs and Patients' Samples Extraction

After thawing at room temperature, each sample was treated as follows: 10 µl of internal standard (IS) working solution were added to a volume of 100 µl of samples, standards and QCs and then, analytes extraction was obtained by addition of 300 µl of frozen-acetonitrile (-20°C). Following, samples were stored at -20°C for 10 minutes to improve protein precipitation.

The samples were vortex-mixing for at least 10s. Subsequently, all samples were centrifuged at 4000 rpm for 10 min at 4 °C.

Then, 180 µl supernatant were diluted with 120 µl of water : acetonitrile 70:30 v/v, transferred in total recovery vials and 10 µl were injected in the chromatographic system.

3.1.4. Chromatographic conditions

The chromatographic system was an Acquity H-Class PLUS ® (Waters), composed of a Sample Manager FTN-H® autosampler and a column manager Acquity UPLC® column oven. The chromatographic separation was performed on a KINETEX® 2.6 µm Polar C18 100 Å LC column 100 x 2.1 mm (Phenomenex, Italy) at 40°C.

The flow rate was settled at 0.5 mL/min; the mobile phases used are 0.1% v/v formic acid in water (Phase A) and 0.1% v/v formic acid in acetonitrile (Phase B), according to the gradient shown in **Tab.**1.

Time (min)	Flow (mL/min)	Phase A (%)	Phase B (%)
0.0	0.500	82.0	18.0
0.60	0.500	82.0	18.0
5.50	0.500	28.0	72.0
6.50	0.500	28.0	72.0
7.30	0.500	21.0	79.0
7.80	0.500	21.0	79.0
8.80	0.500	5.0	95.0
9.20	0.500	5.0	95.0
10.0	0.500	18.0	82.0

Tab. 1: Gradient phases concentration (%v/v) in chromatographic elution.[121]

The total run time was 10 minutes. The temperature of the sample manager was set at 15°C. Water:acetonitrile 30:70 v/v was adopted as washing solution.

The separation efficiency was evaluated by Van Deemter model through N (number of theorical plates) and HETP (height equivalent to a theoretical plate) calculus, as follow: $N = 16 \cdot \left(\frac{t_R}{W_b}\right)^2$ and $H = \frac{L}{N}$ where t_R was retention time expressed in minutes, W_b was the width calculated at the base of peek and L was the length of column in millimetres.

These parameters were monitored for control analytical process and derives was prevented.

3.1.5. Mass spectrometry conditions

Tandem mass spectrometry detection was carried out by means of tandem mass spectrometry XEVO TQ-S micro ® (Milan, Italy) with an electrospray ionization (ESI) interface. The ESI source was set in positive ionization mode (ESI+) for most of the analytes and in negative ionization (ESI-) for THC-COOH-glucuronide, CBN, THCA and CBDA (see Tab.2) Optimization of the MS conditions was obtained by infusion of reference standards of each drug (100 ng/mL in acetonitrile and HPLC grade water 60:40) at 5.0 μL/min into the mass spectrometer, combined with the flow from the

chromatographic system at medium concentrations phases (Phase A and Phase B 50% v/v) as reported in **Tab.2**.

Analyte	PARENT MRM [m/z]	Ion QUANTIFIER MRM [m/z]	Ion QUALIFIER MRM [m/z]	Cone Voltage [V]	Collision Energy [V]
CBN	311	293	223	-25	-18
CBD	315	193	259	25	20
Δ9-THC	315	193	259	25	20
Δ8-THC	315	193	259	20	20
11-OH- THC	331	313	175	30	12
THC-COOH	345	299	327	25	15
THCA	357	313	245	-30	-30
CBDA	357	313	245	-36	-12
THC-glucuronide	519	343	113	-25	-22
7-OH-CBD	331	175	201	25	12
THC-D_3	318	196	262	36	20
CBD-D_3	318	196	262	64	22
11-OH- THC-D_3	334	316	178	35	25
THC- COOH-D_3	348	302	330	25	30
THC-glucuronide-D_3	522	346	116	-25	-12

Tab. 2: MRM transitions. The Analytes detected in negative ionization presents negative collision energy and capillary voltage value [121].

Nitrogen (>99.9%) produced with a Nitrogen LCMS 40-1 nitrogen generator (Claind, Italy) was used as nebulising and heating gas, while argon was used as collision gas.

The general conditions for positive ionisation are: electrospray voltage at 4.0 kV; source temperature at 600°C; nebulizing gas flow at 1000 L/h.

3.1.5. Validation

3.1.6.1. Analytical selectivity and specificity

The selectivity was evaluated analyzing blank sample (plasma sample without addition of analyte or IS) obtained from ten different lots of plasma. The detected response was evaluated through percent

deviation from LLOQ concentration level: these results were accepted with gap ≤ 20% (absolute value) for analytes responses and ≤ of 5% (absolute value) for IS responses.

3.1.6.2. Calibration Curve and Range

The calibration curve was performed with six concentrations levels (measure range 5 – 250 ng/ml), in addition to blank sample, in order to represent the relationship between analyte concentration and peak area normalized for its IS. The interpolation of Area/IS Area and concentration was calculated by least square method. Correlation factor (R^2) >0.995 was considered as acceptable criteria for linearity. The Δ% for each concentration level was accepted with value including to ±20% for LLOQ and ±15% for other concentration levels [122].

The limit of detection (LOD) was estimated by Hubaux-Vos algorithm [123].

The limit of quantification (LOQ) was defined theoretically as three times of the LOD. The LOQ corresponded to LLOQ for each analyte in calibration curve [124].

3.1.6.3. Repeatability and Reproducibility

The repeatability and reproducibility were evaluated through ten repetition measures at four levels of concentrations in different sessions, as reported in results paragraph. The statistical analysis of this data set was executed at 97.5% level of confidence t-student distribution. In this context, the intra-laboratory precision with limit repeatability was evaluated as follow $r = \sqrt{2} \times t \times s_r$, where t represented the t-student at $(1 - \alpha) = 0.975$ with $\nu = 9$ (degrees of freedom for ten repetition of experiment intra-day), then s_r was the standard deviation in repeatability conditions. The assessment of precision in repeatability and reproducibility conditions were evaluated by relative standard deviation $RSD\% = \frac{s}{\overline{x}} \times 100$, where s represented standard deviation in repeatability or reproducibility conditions, $\overline{x}$ is the mean value of ten measures executed by single operator on the same sample. The

repeatability measures were conducted on the same day, while the reproducibility measure on three different days.

3.1.6.4. Accuracy, precision, recovery and Uncertainty

The method extraction efficacy, identification and quantification of analytes was demonstrated by spiking plasma samples with standard solution at four levels of concentrations: LLOQ, 20% ULOQ, 40% ULOQ, ULOQ. These samples were processed as ten replicates on three different days in order to evaluate precision. The condition of acceptability for coefficient of variation (CV)% was: ±15% for each level, except for the LLOQ ±20%.

In these experiments the relative recovery was evaluated and described as $R(\%) = \frac{C_f - C_s}{C_a} \times 100$, where C_f was the mean concentration of spiked sample, C_s was the mean concentration of not spiked sample and C_a was the concentration of spiked sample. The calculation of relative recovery was performed through ten experiments executed by a single operator. The acceptability conditions were been the same reported for precision test.

The evaluation of accuracy in accordance with the ICH guidelines [125] was performed through Bias % calculus as $b(\%) = \frac{\overline{x} - x_{ref}}{x_{ref}} \times 100$, where $\overline{x}$ was the mean of the results and x_{ref} was the reference value obtained by proficiency test specimens (EQA, external quality assessment). The evaluation of measurement uncertainty was performed by Horwitz heuristic model. The conditions of applicability of Horwitz equation were verified on data distribution as follow: ratio between s_r and sR was to be comprised between 0.50 and 0.67. The sR (standard deviation in reproducibility conditions calculated by Horwitz equation) was calculated by $sR = \frac{C}{100} \cdot 2^{(1 - 0.5 \operatorname{Log} C)}$ where C was the concentration level of single analytes reported as mass ratio.

3.1.6.5. Blood sampling and Matrix- effect

We tested the following types of vacutainer tubes for plasma and serum collection: lithium heparin, ethylenediaminetetraacetic potassium salt 1:3 (EDTA-3K) and without additive. The evaluation of interactions between sampling tubes contents and the instrumental response was conducted by response factor (RF) calculated as follow: $RF_i = \frac{A_i}{C_i}$, where A_i was the single analyte area and C_i was the related concentration. The deviations by mean RF for each collection type and analyte were evaluated with percent difference of RF (*ΔRF%*) as follow: $\Delta RF\% = \frac{RF_i - \overline{RF}}{\overline{RF}} \times 100$, where the mean RF was calculated by relation $\overline{RF} = \frac{\sum_{i=1}^{n} RF_i}{n}$. In the last equation, the n = 6 was the number of concentration levels in calibration curve of each analyte. Therefore, the $\Delta RF\%$ was computed for single concentration level related to single analyte respectively in three different collection conditions as reported before.

The study of the matrix was carried out by comparing the solubility chemical equilibrium and analytical distribution of single specimens in solvent and in plasma matrix. The quantitative deviations of matrix effect were evaluated by slope comparing between calibration curve executed in solvents and in plasma matrix. Furthermore, in accordance with ICH guideline the matrix effect was evaluated by three replicates of QC L and QC H in ten different plasma lots. The analytes average response was compared to theorical concentration with percent deviation in acceptability range of ±15% for each concentration level upper LLOQ and ±20% at LLOQ. The same acceptability values were considered for CV% in precision evaluation of response data in these experimental sessions.

3.1.6.5. Stability and Incurred Samples Reanalysis

Stability study was conducted on bank plasma spiked at three different concentrations (5, 50 and 250 ng/mL), in order to evaluate the feasibility of samples and standards collection. Two different batches of blank plasma have been used also to assess matrix-effect.

Samples were stored at –20°C and –80°C and the selected timings for the stability study included 10, 30 and 90 days.

Stability was calculated as the percent difference between analytes concentrations found in samples freshly extracted and samples collected at –20°C and –80°C.

We also performed incurred samples reanalysis on authentic patient samples in three indipendent analytical sessions. The condition of acceptability for coefficient of variation (CV)% was ±20%.

3.2. Clinical Application

3.2.1. Characteristics of Enrolled Patients

A number of 67 patients with a diagnosis of neuropathic and chronic pain were enrolled at the "SC Terapia del Dolore – ASL Città di Torino" at the "Oftalmico" hospitals (Turin, Italy).

Inclusion criterion was Medical Cannabis assumption.

The study was performed in compliance with the Declaration of Helsinki and local review board regulations; all patients gave written informed consent, according to the local ethics committee standards ("Cannabis terapeutica nei pazienti affetti da dolore neuropatico: studio osservazionale", approved by Ethical Committee "A.O.U. CITTA' DELLA SALUTE E DELLA SCIENZA DI TORINO - A.O. ORDINE MAURIZIANO DI TORINO – A.S.L. CITTÀ DI TORINO", n° 0131170 del 25/11/2022).

3.2.2. Magistral Preparations of Cannabis Plant Derivatives

The used drug was the dried flower tops of the cannabis plant.

Dried flower tops with different concentrations of THC and CBD were used:

Bedrocan® medical *cannabis* with THC level standardized at 19% and with a CBD level below 1%.

Bediol® medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8%, respectively.

Cannabis was administered as a decoction in 47 patients, while 11 patients as inhaled cannabis.

3.2.3. Pharmacokinetic Analyses

Pharmacokinetic analysis was conducted before the new dose assumption (C_{trough}) at the steady state. Plasma samples were isolated after whole blood centrifugation at 1400 x g for 10 min at 4°C and stored at -80°C until the analysis.

Cannabis plasma concentrations in patients were obtained using a previously published fully validated method [121].

3.2.4. 25-OH-vitamin D Analyses

VD plasma determination (25-OH-vitamin D) was performed with the MSMS Vitamin D kit (Perkin Elmer, Wallac Oy, Finland).

Samples analysis was carried out with a LX50 UHPLC (Perkin Elmer). The chromatographic separation was obtained through a gradient run on an Acquity UPLC® BEH C18 1.7 µm 2.1 x 50 mm column.

3.2.5. Genetic polymorphisms analyses

DNA extraction was realized with the 'QIAamp DNA mini kit' (Qiagen, Valencia, CA). These kits contain columns allowing the DNA purification starting from 200 µL of blood or plasma.

Allelic discrimination was assessed through the RT-PCR (BIORAD, Milan, Italy).

The following allelic variants were analyzed:

VDR ApaI C>A (rs 797523219), *VDR TaqI* T>C (rs 731236), *VDR BsmI* G>A (rs 1544410), *VDR Cdx2* A>G (rs 11568820), *VDBP* GC1296 A>C (rs 7041), *CYP27B1* -1260 G>T (rs 10877012), *CYP24A1* 3999 T>C (rs 2248359), *CYP24A1* 22776 C>T (rs 927650), *CYP24A1* 8620 A>G (rs2585428), *CYP27B1* +2838 C>T (rs 4646536), *ABCB1* 3435 C>T (rs1045642), *ABCB1* 2677 G>A (rs2032582), *ABCB1* 1236 C>T (rs1128503), *ABCG2 421* C>A (rs2231142), *ABCG2* 1194+928 T>C (rs13120400), *CYP2D6* 4180 C>G (rs 1135840), *CYP1A1* 7294 C>A (rs 2606345), *CYP1A2* 890 C>T (rs2470890), *CYP2C19*2* G>A (rs4244285), *CYP1A2* 32035 A>C (rs762551), *BSEP* T>C (rs2287622), *CYP3A4*1B* G>A (rs2740574), *CYP2C9*3* 1075 A>C, *CYP2C9*2* 430 C>T, *COMT* 680 T>C (rs4680), *GHC1* 841 T>C (rs841), *OPRM1* 971 T>C (rs1799971), *TRPV1* 080 G>A (rs8065080).

3.2.6. ELISA tests

In this study, BT LAB kits (Bioassay Technology Laboratory, Birmingham, United Kingdom) were used. In particular, the direct method was used and the antibody is found on the bottom of the various wells.

Interleukin 6 (IL-6), Interleukin 10 (IL-10) and Tumor Necrosis Factor (TNF-α) were analysed as inflammation biomarkers.

3.2.7. Simoa tests

Plasma specimens were analysed through Single Molecule Array (Simoa SR-X, Quanterix®) for markers of neuronal damage and signalling and plasticity.

In order to evaluate neuronal damage and signalling and plasticity NFL and Brain-derived neutrophic factor (BDNF) were analysed.

3.2.8 Statistical Analyses

All of the continuous variables were tested for normality with the Shapiro–Wilk test. The correspondence of each parameter was evaluated with a normal or non-normal distribution through the Kolmogorov–Smirnov test. Non-normal variables were described as median values and interquartile range (IQR); categorical variables as numbers and percentages. Kruskal-Wallis and Mann-Whitney tests were adopted for differences in continuous variables between seasons, considering a statistical significance with a two-sided p-value < 0.05.

Correlations among drug concentrations at different days were evaluated through Pearson test.

In conclusion, the predictive capability of the investigated variables was assessed through univariate ($p < 0.05$) and multivariate ($p < 0.05$) linear regression analysis. All of the tests were performed with IBM SPSS Statistics for Windows v.28.0 (IBM Corp., Chicago, IL).

4. Results

4.1 Analytical validation

The analytical method was fully validated in accordance with the recommendations of ICH Harmonised Guideline for bioanalytical method validation [126] and the results were published [121]. The following parameters were investigated: specificity and selectivity, linearity range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, intra-day and inter-day precision, robustness, matrix-effect and sampling mode.

4.1.1 Chromatographic separation

The whole chromatographic run was completed in 10.0 minutes.

Retention times of the selected analytes were reported in **Tab.** 3.

Analyte	RT	Wb	N	H
THC-glucuronide	4,40	0,55	1024	0,0977
11-OH- THC	5,24	0,48	1951	0,0513
7-OH-CBD	5,25	0,52	1668	0,0599
THC-COOH	5,40	0,34	4036	0,0248
CBDA	5,80	0,42	3147	0,0318
CBD	5,30	0,36	4489	0,0223
Δ8-THC	6,10	0,31	6692	0,0149
CBN	6,48	0,3	7327	0,0136
Δ9-THC	6,75	0,32	6972	0,0143
THCA	7,06	0,34	6899	0,0145

Tab. 3: Chromatographic parameters according to van Deemter model. RT= retention time; Wb= width calculated at the base of peek; N= number of theorical plates; H= height equivalent to a theoretical plate [121].

Fig. 1 showed a chromatogram recorded from the middle point of the calibration curve (100ng/mL) and the chromatographic parameters are reported in **Tab**.3.

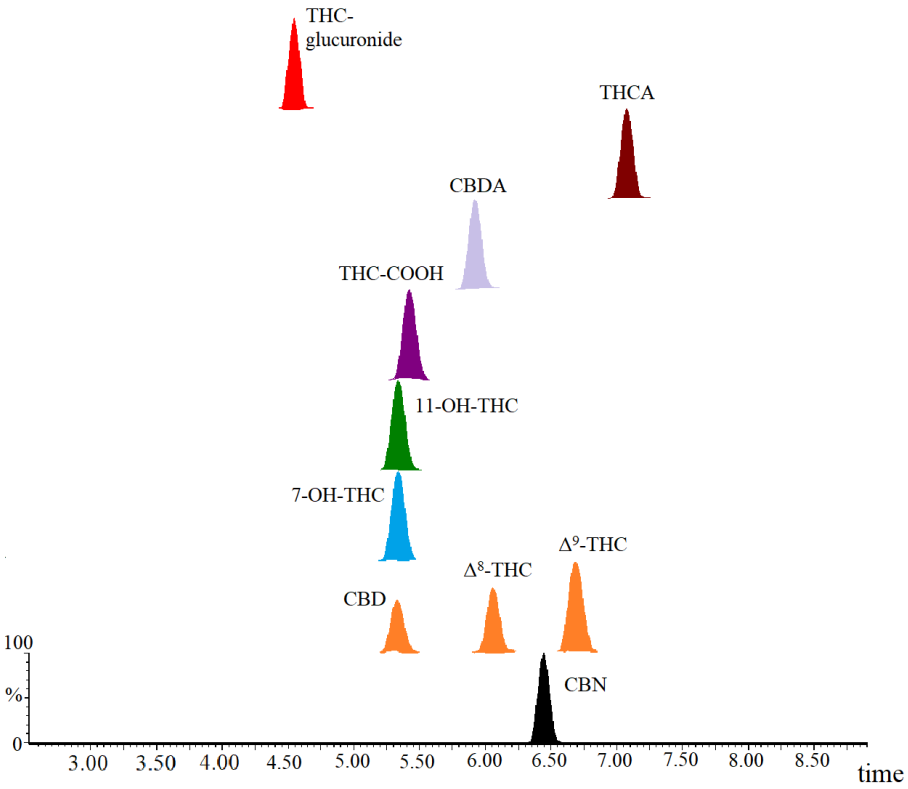


Fig.1: Chromatogram recorded from the middle point of the calibration curve (100ng/mL) reporting the considered analytes[121].

4.1.2 Analytical selectivity, specificity

The response detected in the processed blank samples did not report significative percent deviation (<2.5% as absolute value) compared to LLOQ default amount. While simultaneously, the chromatograms and mass spectra in SCAN mode of blank sample did not report interfering signal in analytes and IS acquisition window.

4.1.3 Calibration Curve and Range

The calibration curve was analysed through samples processed in three replicates on three different analytical sessions. The linearity results were consistent with acceptability criteria as reported in **Tab.** 4. The LOD and LOQ were calculated and reported in **Tab.**4.

A	THCA			THC-COOH			CBDA			Δ9-THC			Δ8-THC		
	LEV 1	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*	*LEV 1*	*LEV 2*	*LEV 3*
mean value of calculated concentration (Xm)	5.45	49.26	239.94	5.20	47.70	245.86	4.81	51.45	243.95	4.80	50.01	244.04	5.08	48.12	243.54
standard deviation on repeatability (Sr)	0.70	4.60	22.70	0.55	4.80	9.34	0.75	7.50	33.00	0.72	6.95	26.30	0.82	5.72	38.25
confidence interval (CI)	0.50	3.29	16.24	0.39	3.43	6.68	0.54	5.36	23.61	0.51	4.97	18.81	0.59	4.09	27.36
repeatability coefficient (r)	2.24	14.72	72.62	1.76	15.35	29.88	2.40	23.99	105.57	2.30	22.23	84.13	2.62	18.30	122.37
relative standard deviation on repeatability (RSDr)	12.85	9.34	9.46	10.58	10.06	3.80	15.60	14.58	13.53	14.98	13.90	10.78	16.14	11.89	15.71
relative standard deviation on reproducibility (RSDR)	13.20	11.23	11.74	11.70	9.10	7.11	10.50	5.50	12.00	13.01	17.80	16.42	15.02	12.62	13.30
correlation factor (R^2)		0.998			0.997			0.998			0.997			0.997	
angular coefficient of solvent calibration curve (m)		0.12739			0.04391			0.22623			0.05658			0.04722	
angular coefficient of matrix calibration curve (m')		0.13359			0.04192			0.21685			0.05402			0.04913	
matrix deviation in percentage; (Δm%)		4.87			-4.53			-4.15			-4.52			4.04	
limit of detection (LOD)		0.45			0.45			0.22			0.87			0.86	
limit of quantification (LOQ)		5.00			5.00			5.00			5.00			5.00	
recovery (R%)		92.0			95.0			93.2			94.6			94.3	
uncertainty of measure (U(x))	35.1	25.2	19.8	35.3	25.3	19.8	35.7	25.0	19.8	35.7	25.1	19.8	35.4	25.3	19.8

B	CBD			CBN			11-OH-THC			THC-COOH-gluc			7-OH-CBD		
	LEV 1	***LEV 2***	***LEV 3***	***LEV 1***	***LEV 2***	***LEV 3***	***LEV 1***	***LEV 2***	***LEV 3***	***LEV 1***	***LEV 2***	***LEV 3***	***LEV 1***	***LEV 2***	***LEV 3***
mean value of calculated concentration (Xm)	4.86	54.69	250.15	5.45	48.53	245.37	4.99	53.23	220.72	4.87	53.85	239.79	5.56	47.72	245.39
standard deviation on repeatability (Sr)	0.74	8.81	26.95	0.84	6.93	30.65	0.61	4.50	23.83	0.72	6.94	22.84	0.76	5.55	24.25
confidence interval (CI)	0.53	6.30	19.28	0.60	4.95	21.93	0.44	3.22	17.04	0.52	4.96	16.34	0.55	3.97	17.34
repeatability coefficient (r)	2.38	28.19	86.20	2.70	22.15	98.06	1.95	14.40	76.22	2.30	22.19	73.08	2.44	17.74	77.56
relative standard deviation on repeatability (RSDr)	15.26	16.11	10.77	15.47	14.27	12.49	12.23	8.45	10.79	14.79	12.88	9.53	13.70	11.62	9.88
relative standard deviation on reproducibility (RSDR)	15.91	14.90	15.00	8.20	14.00	15.11	9.60	8.90	7.03	9.74	13.40	15.07	12.00	12.89	13.50
correlation factor (R^2)		0.997			0.997			0.998			0.999			0.997	
angular coefficient of solvent calibration curve (m)		0.07658			0.06335			0.09658			0.05568			0.26273	
angular coefficient of matrix calibration curve (m')		0.07292			0.06642			0.09198			0.05842			0.25221	
matrix deviation in percentage; (Δm%)		-4.78			4.85			-4.76			4.92			-4.00	
limit of detection (LOD)		0.86			0.15			0.55			0.24			0.17	
limit of quantification (LOQ)		5.00			5.00			5.00			5.00			5.00	
recovery (R%)		95.1			95.0			96.2			96.5			92.4	
uncertainty of measure (U(x))	35.7	24.8	19.7	35.1	25.2	19.8	35.5	24.9	20.1	35.7	24.8	19.8	35.0	25.3	19.8

Tab. 4 (A and B): Evaluated parameters for analytical validation.
Xm= mean value of calculated concentration; Sr= standard deviation on repeatability ; CI= confidence interval; r= repeatability coefficient ; RSDr= relative standard deviation on repeatability ; RSDR= relative standard deviation on reproducibility; R2= correlation factor; m= angular coefficient of solvent calibration curve; m'= angular coefficient of matrix calibration curve; Δm%= matrix deviation in percentage; LOD= limit of detection; LOQ= limit of quantification; R%= recovery; U(x)= uncertainty of measure[121].

4.1.4 Repeatability and Reproducibility

The results showed the stability of the analytical process over time and good compliance of precision and accuracy. The overall report of measures was reported in **Tab.4**.

4.1.5 Accuracy, precision, recovery and Uncertainty

The results of precision were reported in **Tab.4**. The percent deviation compared to the default amount and the CV% were in accordance with acceptability criteria.

The recovery for each compound was included between 92.0% and 96.5% (absolute value), while the Bias% was comprised between 8.00% and 3.5% (absolute value). The uncertainty of measure was evaluated with Horwitz equation and its values were comprised between 35.7% and 19.7% calculated with coverage factor as k = 1.0 (**Tab.4**).

4.1.6 Blood sampling and Matrix- effect

The sampling method for optimal results was chosen with lower ΔRF% for each analyte. The ΔRF% of sample collected in vacutainer tubes added with EDTA-3K was < 5.0% (see **Fig. 2**).

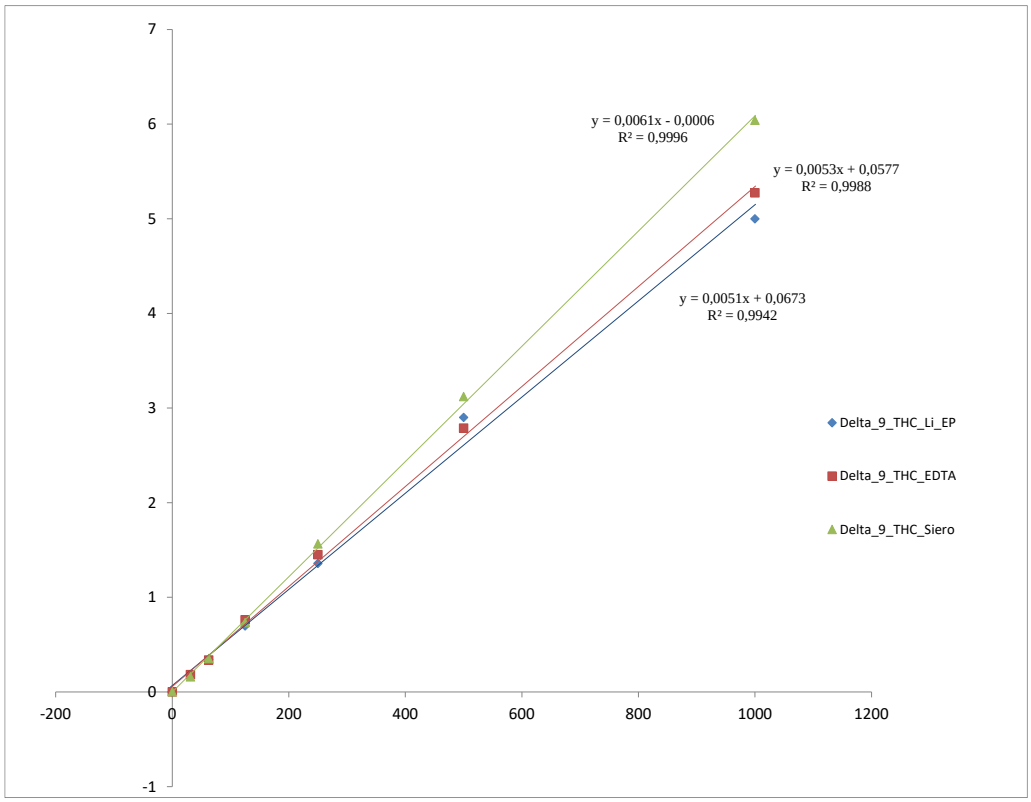


Fig.2: The matrix effect study for Δ9-THC, calibration curves in lithium heparin, EDTA-3K and without additive [121].

The matrix effect was quantified in percent deviation of linear slope in matrix calibration compared to solvent calibration: the analytical protocol was efficient and suppressed matrix effect down to 5.0%. The CV% of QC H and QC L in evaluation of precision in matrix effect study was compliant with acceptability criteria (see **Tab.4**).

4.1.7 Stability

Analysis showed that samples were stable for 10 days when kept at -20°C and up to 3 months when stored at -80°C. As reported in EMA and FDA guidelines, samples have been re-analysed to evaluate incurred samples reanalysis.

They showed acceptable bias: 14% for Δ9-THC, 17% for THC-COOH and 4% for THC-glucuronide.

4.2 Clinical application

4.2.1. Characteristics of enrolled patients

A number of 67 patients were enrolled in the present study; 9 patients were excluded from the statistical analysis since cannabis decoction was incorrectly assumed. Characteristics of enrolled patients were reported in **Tab.5**.

Participants had a median age of 61 years (interquartile range 52 – 67 years), 20 (34.5%) were male and the median body mass index was 20.6 (interquartile range 17.9; 23.4) Kg/m2. Participants were all Caucasian.

Patients enrolled at the "SC Terapia del Dolore – ASL Città di Torino" at the "Oftalmico" hospitals (Turin, Italy) were affected of neuropathic and chronic pain caused by several pathologies: 46.6% (n=27) of patients had fibromyalgia, 8.6% (n=5) had headache, 6.9% (n=4) were oncologic patients, 65.5% (n=38) had other concomitant pathologies, such as diabetes, hypertension, HIV or were polytraumatized subjects.

Characteristics	
No. of patients	58
Cigarettes smoke, n (%)	19 (32.8%)
Gender (Male), n (%)	20 (34.5%)
Caucasian, n (%)	100%
BMI (Kg/m2), median (IQR)	20.6 (17.9; 23.4)
Age, (years), median (IQR)	61 (52;67)
Fibromyalgia, n (%)	27 (46.6%)
Headache, n (%)	5 (8.6%)
Cancer, n (%)	4 (6.9%)
Other pathologies, n (%)	38 (65.5%)

TNF alfa (ng/mL), median (IQR)	110.40 (98.43; 140.90)
IL-6 (ng/mL), median (IQR)	73.8 (68.1;88.4)
IL-10 (pg/mL), median (IQR)	245.3 (222.7; 307.3)
Vitamin D (ng/mL), median (IQR)	18.3 (9.3; 26.2)
BDNF (pg/mL), median (IQR)	1672.6 (912.4; 5384.5)
NFL (pg/mL), median (IQR)	6.96 (4.53; 9.72)

Tab. 5: Characteristics of enrolled patients. IQR= Interquartile range

Considering their diagnosis, most of patients had polypharmacy: 41.2% of patients (n=21) were treated with opioids and 39.2% (n=21) with antidepressants.

All concomitant class of drugs were reported in **Tab. 6**.

Drugs	***Number of patients (%)***
Antidepressant, n (%)	20 (39.2%)
Anti-inflammatory drugs, n (%)	16 (31.4%)
Opioids, n (%)	21 (41.2%)
Analgesics for neuropathic pain, n (%)	16 (31.4%)
Cardiovascular system drugs, n (%)	15 (29.4%)
Vitamin D supplementation, n (%)	9 (17.6%)
Anti-anxiety medications, n (%)	17 (33.3%)
Other, n (%)	26 (89.7%)

Tab. 6: Concomitant class of drugs in enrolled patients.

Regarding *cannabis* administration, it was given as a decoction in 47 patients, while 11 patients as inhaled *cannabis*.

Concerning variety of administered medical *cannabis*, 51.7% (n=30) of patients were treated with *cannabis* with THC level standardized at 19% and with a CBD level below 1%, while 48.3% (n=28) with medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8%, as reported in **Tab. 7**.

Treatment characteristics	***Number of patients***
***cannabis* with THC level standardized at 19% and with a CBD level below 1% (e.g. Bedrocan®), n (%)**	30 (51.7%)
***cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8% (e.g. Bediol®), n (%)**	28 (48.3%)
Inhaled cannabis, n (%)	11 (19%)

Tab. 7: Characteristics of enrolled patients.

Patients were treated with different *cannabis* dosages.

The majority of patients were treated with 300 and 200 mg of cannabis/die: 22.4% (n=13) and 20.7% (n=12), respectively.

All the dosages were reported in **Tab. 8** and in **Fig. 3** and **4**.

Cannabis mg	Number of patients
100	8 (13.8%)
1100	1 (1.7%)
1200	2 (3.4%)
1400	1 (1.7%)
150	2 (3.4%)
1500	2 (3.4%)
200	12 (20.7%)
250	2 (3.4%)
300	13 (22.4%)
400	8 (13.8%)
450	1 (1.7%)
50	1(1.7%)
500	1 (1.7%)
600	2 (3.4%)
900	2 (3.4%)

Tab. 8: *Cannabis* dosages (mg).

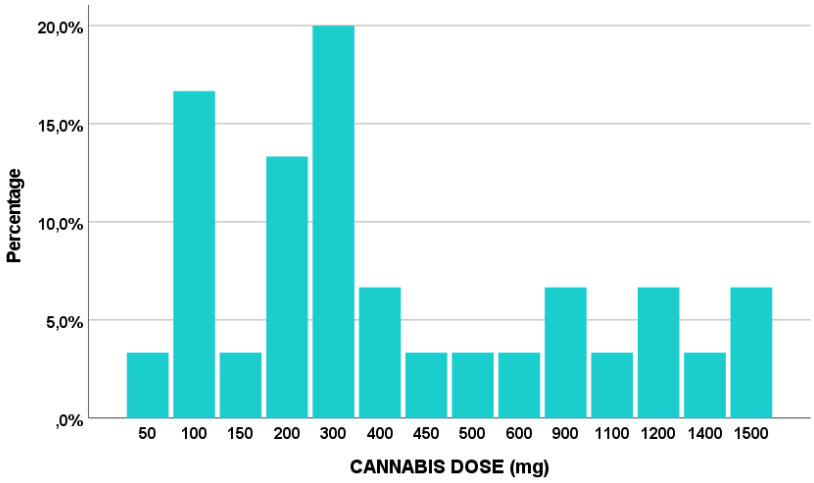


Fig. 3: Dosage distribution of patients were treated with *cannabis* with THC level standardized at 19% and with a CBD level below 1%.

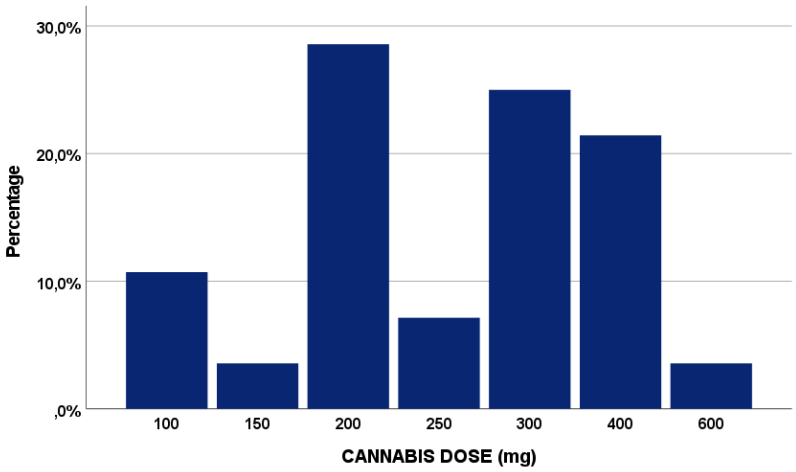


Fig. 4: Dosage distribution of patients treated with medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8%.

4.2.2. Pharmacokinetic analysis

Concerning cannabinoids pharmacokinetic analysis, all samples were successfully quantified for each drug. Cannabinoids plasma exposures (expressed as ng/mL) in patients treated with medical *cannabis* with THC level standardized at 19% and with a CBD level below 1% and medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8% were reported in **Tab. 9** and **10**, respectively.

Statistically significant differences were found in cannabinoids plasma exposure between inhaled and oral assumption (decoction) of medical *cannabis* with THC level standardized at 19% and with a CBD level below 1%, except for CBD, THCA and CBD.

Regarding medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8%, no statistically significant differences between inhaled or oral *cannabis* were observed.

Cannabinoids plasma concentrations as median and IQR and *p*-value were reported in **Tab. 9** and **10**.

Medical *cannabis* with THC level standardized at 19% and with a CBD level below 1%			
Metabolite	**ng/ml Median (IQR) inhaled cannabis**	**ng/ml Median (IQR) decoction**	***p*-value**
Δ9-THC	14.26 (5.70; 23.99)	5.08 (4.53; 11.04)	**0.011**
OH-THC	0 (0; 11.34)	0 (0; 0)	**0.017**
COOH-THC	62.99 (27.85; 248.33)	10.53 (6.62; 23.59)	**0.004**
COOH-THC-glucuronide	511.35 (103.44; 1076.27)	47.92 (7.32; 80.01)	**0.003**
CBD	5.26 (1.45; 11.45)	2.94 (0.56; 5.73)	0.364
7-OH-CBD	2.26 (0.79;9.82)	0 (0; 0)	**<0.001**
THCA	0 (0;2.11)	3.35 (0; 11.75)	0.127
CBDA	0 (0;0.41)	0 (0; 0.95)	0.546

Tab. 9: Median and IQR of plasma cannabinoids in patients treated with medical *cannabis* with THC level standardized at 19% and with a CBD level below 1%: differences between inhaled cannabis and oral (decoction) assumption. IQR= interquartile range.

Medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8%			
Metabolite	**ng/ml Median (IQR) inhaled cannabis**	**ng/ml Median (IQR) decotion**	***p*-value**
Δ9-THC	5.85 (4.60;/)	4.52 (4.18;5.48)	0.326
OH-THC	0 (0;0)	0 (0;1.39)	0.412
COOH-THC	43.76 (5.21;/)	11.43 (4.91; 21.70)	0.517
COOH-THC-glucuronide	197.70 (17.81;/)	35.07 (10.35; 63.88)	0.404
CBD	7.83 (3.44;/)	2.12 (0;3.72)	0.104
7-OH-CBD	0.96 (0;/)	0 (0;1.67)	0.667
THCA	0 (0;0)	4.89 (0;9.04)	0.100
CBDA	0 (0;0)	1.05 (0;5.76)	0.118

Tab. 10: Median and IQR of plasma cannabinoids in patients treated with medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8%: differences between inhaled cannabis and oral (decoction) assumption. IQR= interquartile range.

4.2.3. Role of gender and cigarette smoke on cannabinoids pharmacokinetics

Regarding the role of gender in influencing cannabinoids plasma concentrations, statistically significant differences were observed between male and female considering COOH-THC, COOH-THC-glucuronide, THCA and CBDA plasma exposure, as shown in **Tab. 11** and in **Fig.5**.

Analyte (ng/ml)	p-value
Δ9-THC	0.259
OH-THC	0.929
COOH-THC	**0.033**
COOH-THC-glucuronide	**0.008**
CBD	0.078
7-OH-CBD	0.444
THCA	**0.002**
CBDA	**0.027**

Tab. 11: Gender influence on cannabis metabolites plasma exposure.

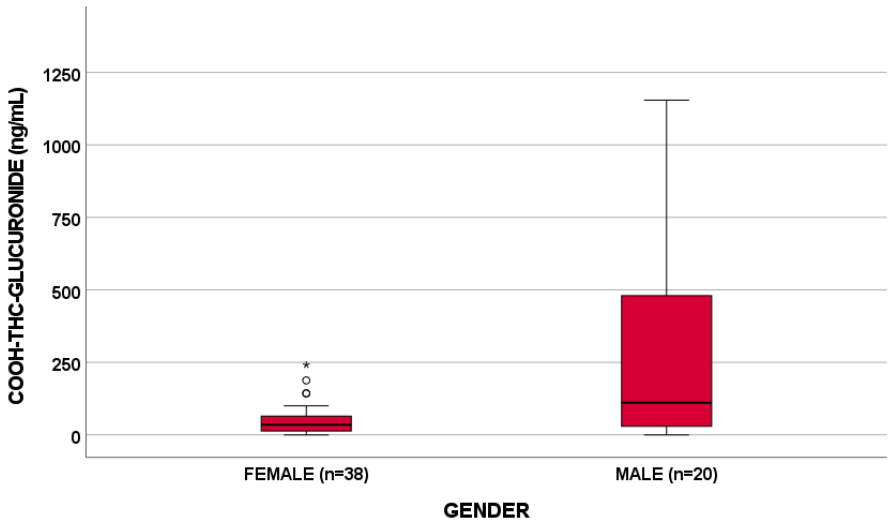
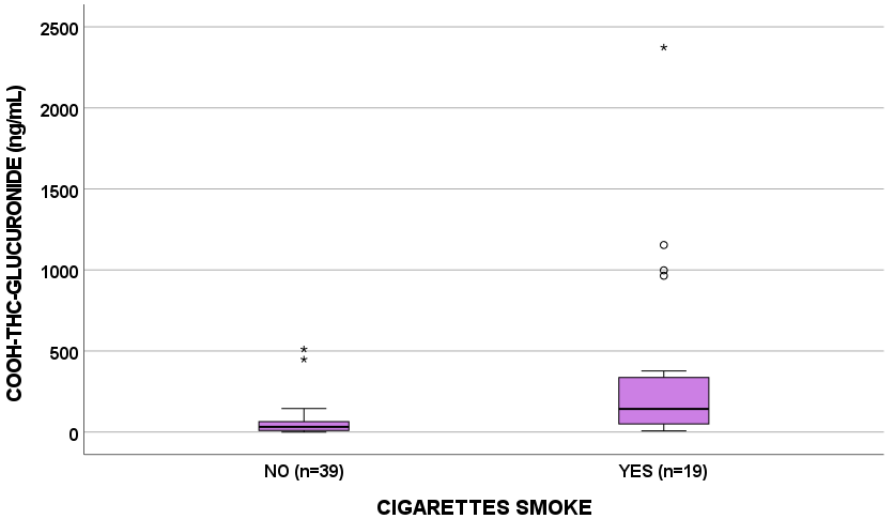


Fig. 5: Influence of gender on COOH-THC-glucuronide exposure (p = 0,008). Outliers are represented by little circles, and extreme outliers are represented by little stars.

Concerning the influence of cigarette smoke on cannabinoids plasma exposure, statistically significant differences were observed between smokers and no smokers, regarding all cannabis metabolites, except for 11-OH-THC, CBD, THCA and CBDA, as reported in **Tab. 12** and in **Fig. 6**.

Analyte (ng/ml)	*p*-value
Δ9-THC	**<0.001**
OH-THC	0.058
COOH-THC	**<0.001**
COOH-THC-glucuronide	**<0.001**
CBD	0.264
7-OH-CBD	**0.021**
THCA	0.979
CBDA	0.163

Tab. 12: Cigarette smoke influence on cannabis metabolites plasma exposure.



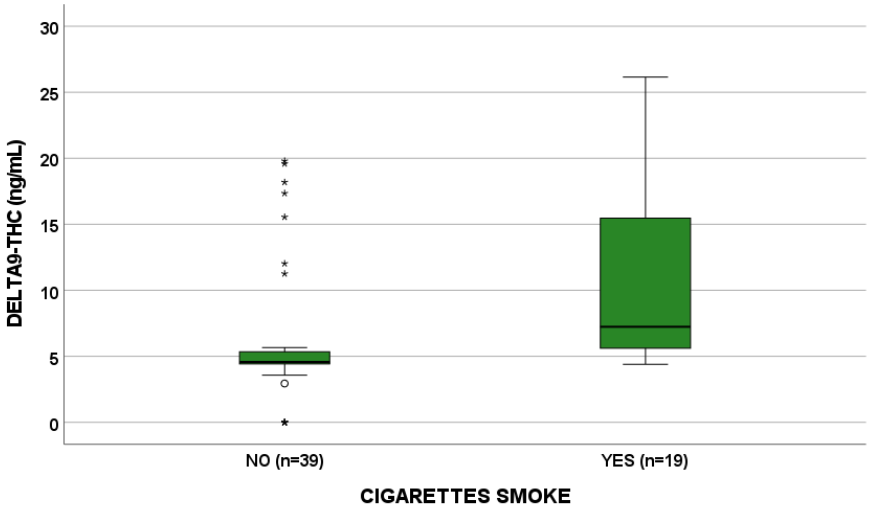


Fig. 6: Influence of cigarette smoke on COOH-THC-glucuronide exposure (p = <0.001) and Δ9-THC (p <0.001). Outliers are represented by little circles, and extreme outliers are represented by little stars.

4.2.4. Correlations

Possible correlations among inflammation biomarkers and cannabinoids plasma exposure were evaluated: plasma TNF-alfa and CBDA plasma levels (p = 0.013, S= 0.346) and plasma NFL with THCA plasma levels (p = 0.000, S = 0.572) and plasma BDNF with 11-OH-THC, COOH-THC, COOH-THC-glucuronide and 7-OH-CBD plasma levels (p = 0.005, S = 0.367; p = 0.023, S = 0.307; p = 0.001, S = 0.447).

Moreover, CBDA plasma levels were correlated with IL-10 levels (p<0.001, S = 0.431), as reported in **Fig. 7**, and with IL-6 levels (p = 0.011, S = 0.337).

A correlation between BMI (Kg/m2) and Δ9-THC plasma levels (ng/mL) was observed (p= 0.032, S = -0.300), as illustrated in **Fig. 8**.

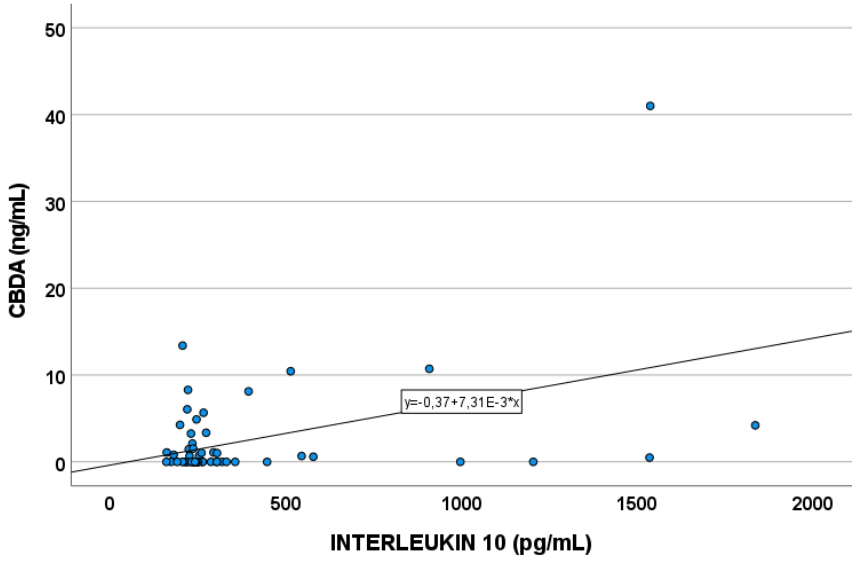


Fig. 7: Correlation between CBDA plasma levels (ng/mL) and IL-10 levels (pg/mL) (p<0.001, S = 0.431).

SNPs role in affecting cannabinoids plasma concentrations (ng/mL) was evaluated and reported in Tab. 13.

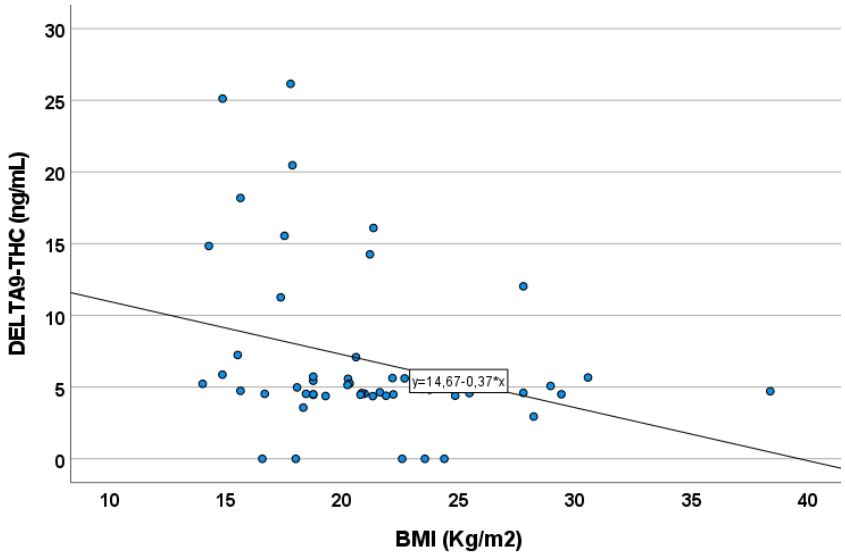


Fig. 8: Correlation between BMI (Kg/m^2) and Δ9-THC plasma levels (ng/mL) (*p*= 0.032, S = -0.300).

4.2.5. Genetics

Single nucleotide polymorphisms (SNPs) role in affecting cannabinoids plasma concentrations (ng/mL) was evaluated and reported in **Tab. 13**.

	Cannabis metabolites							
	Δ9-THC	**OH-THC**	**COOH-THC**	**COOH-THC-glucuronide**	**CBD**	**7-OH-CBD**	**THCA**	**CBDA**
***CYP2D6* 4180 CG/GG**							0.023	
***CYP1A1* 2794 AA**		0.045						0.020
***COMT* 680 TC/CC**	0.017	0.031	0.019	0.031		0.035		
***BSEP* TC/CC**					0.037			
***BSEP* CC**								0.047
***ABCB1* 1236 CT/TT**						0.040		
***CYP24A1* 8620 A>G**					0.035			
***CYP24A1* 3999 CC**	0.020				0.004	0.019		
***CYP24A1* 22776 TT**						0.003		
***CYP1A2* 890 CT/TT**	0.033		0.033					

Tab. 13. Statistically significant influence (*p*-values) of genetic variants on cannabinoids plasma exposure (ng/mL).

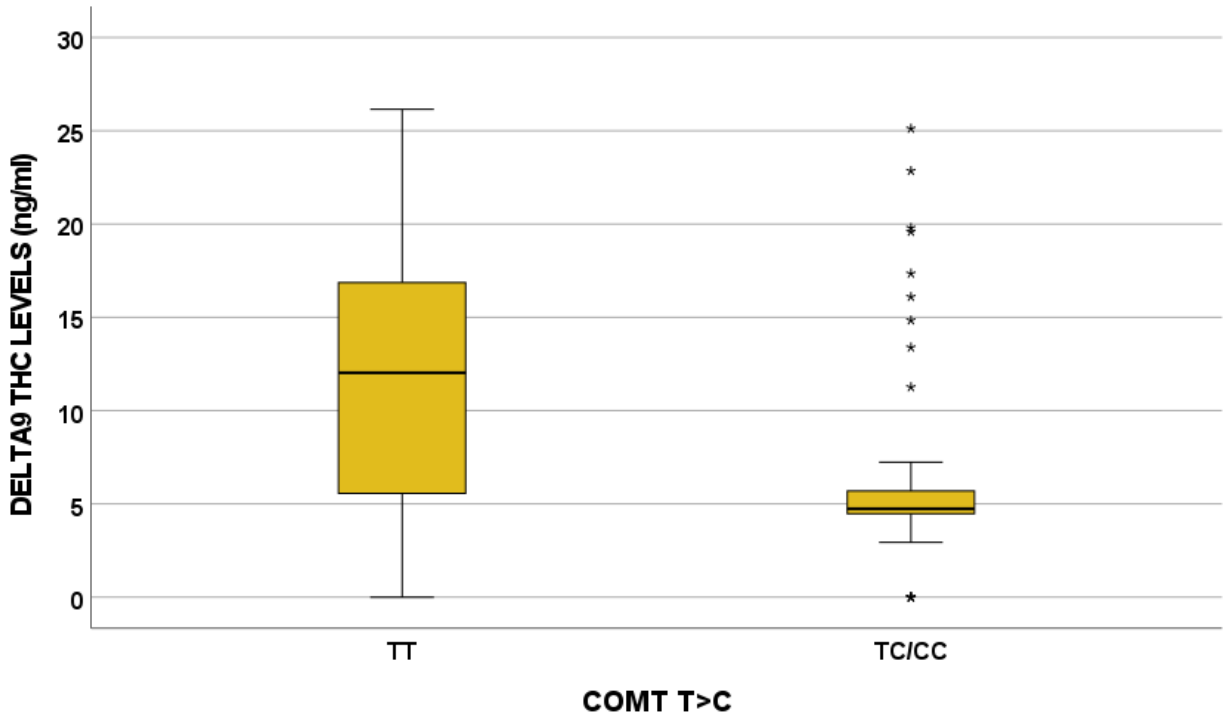


Fig 9: Influence of *COMT* 680 T>C on Δ9-THC plasma levels (ng/mL) (p = 0.017). TT N=11; TC/CC N= 47. Outliers are represented by little circles, and extreme outliers are represented by little stars.

In addition, a statistically significant difference among patients treated with decoction (n=47) and smokers (n=11) in IL-10 (p=0.009) and BDNF (p=0.004) levels was observed, as reported in **Fig. 10** and **11**.

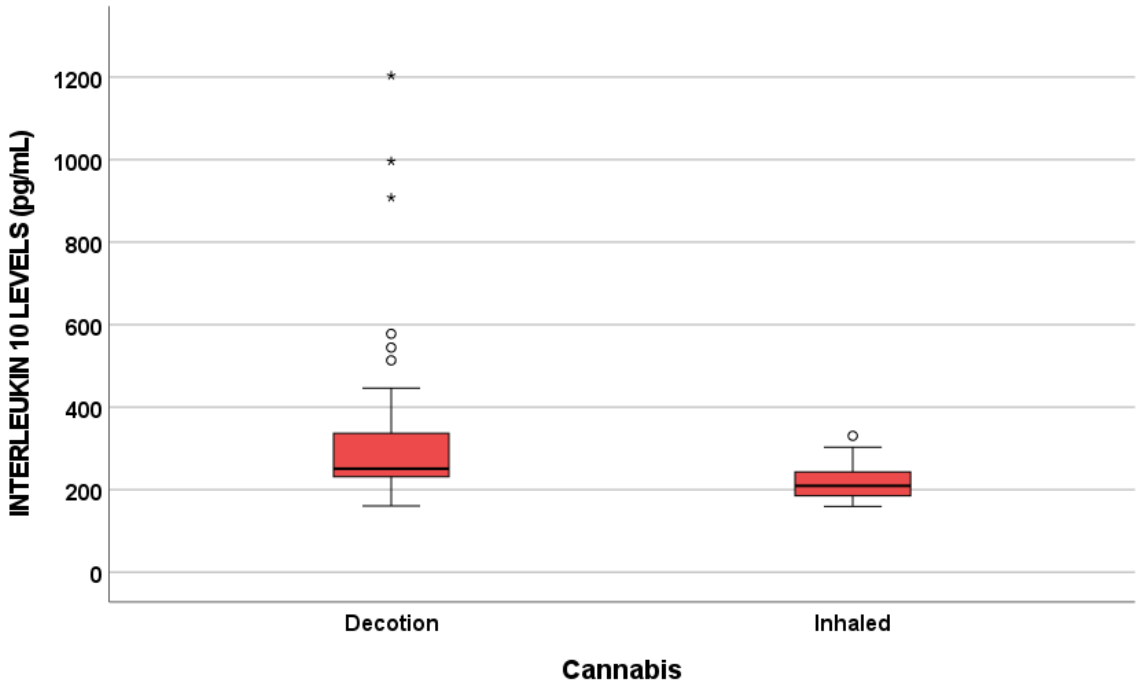


Fig. 10: Influence of the route of administration (decoction and inhaled cannabis) on Interleukin-10 levels (pg/mL) (p = **0.009**). Outliers are represented by little circles, and extreme outliers are represented by little stars.

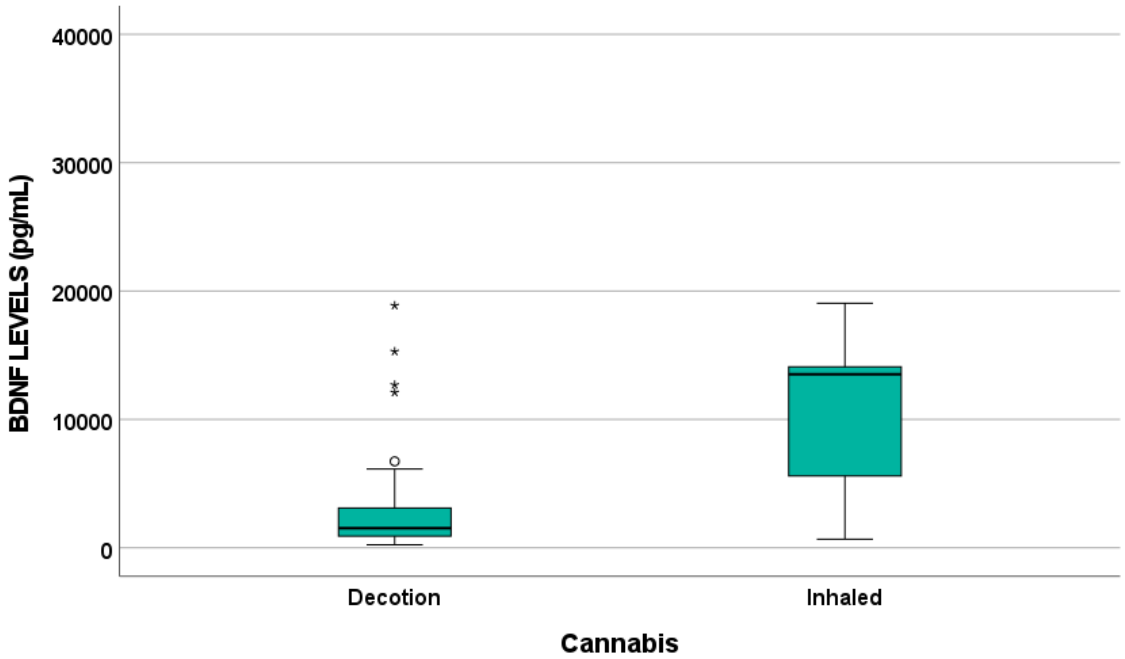


Fig. 11: Influence of the route of administration (decoction and inhaled cannabis) on BDNF levels (pg/mL) (p = **0.004**). Outliers are represented by little circles, and extreme outliers are represented by little stars.

4.2.6. Regression analysis

Finally, genetic, demographic, biochemical and pharmacological factors able to predict the effective cannabinoids plasma exposure were analyzed in a logistic regression analysis table: only gender, cigarettes smoke (**Fig.12**) and inhaled *cannabis* and cardiovascular system drugs remained in the final multivariate regression model.

Cannabinoids plasma exposure				
	Univariate regression		**Multivariate regression**	
Predictive factors	***p*value**	**OR (95% IC)**	***p*value**	**OR (95% IC)**
Gender	**0.006**	**5.200 (1.616; 16.731)**	**0.028**	**16.205 (1.343; 195.581)**
Age >65	0.093	0.960 (0.916; 1.007)		
BMI	0.107	0.889 (0.770; 1.026)		
Mg *cannabis*	**0.015**	**1.002 (1.000; 1.004)**	0.188	1.003 (0.999; 1.006)
Inhaled cannabis	**0.005**	**10.607 (2.027; 55.497)**	**NC**	
Cannabis preparation	**0.031**	**3.429 (1.123; 10.470)**	0.957	0.941 (0.105; 8.397
Antidepressants	0.573	1.400 (0.435; 4.508)		
Anti-inflammatory drugs	0.104	0.308 (0.074; 1.276)		
Opioids	0.402	0.600 (0.181; 1.984)		
Analgesics for neuropatic pain	0.303	0.500 (0.134; 1.868)		
Cardiovascular system drugs	**0.047**	**0.192 (0.038; 0.979)**	**NC**	
Vitamin D supplementation	0.374	0.464 (0.086; 2.517)		
Anti-anxiety medications	0.535	0.673 (0.193; 2.353)		
Cigarettes smoke	**<0.001**	**9.333 (2.637; 33.034)**	**0.022**	**8.516 (1.358; 53,419)**
TNF-alfa levels	0.941	1.000 (0.997; 1.003)		
Interleukin-6 levels	0.762	1.001 (0.997; 1.004)		
Interleukin-10 levels	0.925	1.000 (0.998; 1.001)		
Vitamin D levels	0.510	0.985 (0.942; 1.030)		
***CYP2C19*2* AA**	**NC**		NC	
CYP2D6 4180 GG	0.104	0.370 (0.112; 1.224)		
CYP1A1 7294 AA	0.422	0.606 (0.179; 2.055)		

COMT 680 TC/CC	0.080	0.295 (0.075; 1.159)		
CYP1A2 32035 CC	0.365	0.352 (0.037; 3.370)		
BSEP TC/CC	0.370	2.172 (0.398; 11.844)		
ABCB1 2677 CC	0.107	0.314 (0.077; 1.285)		
ABCB1 3435 TT	0.562	0.694 (0.202; 2.382)		
ABCB1 1236 TT	0.057	0.208 (0.041; 1.046)		
VDR BsmI AA	0.880	0.898 (0.222; 3.639)		
VDR ApaI AA	0.169	0.412 (0.017; 1.457)		
VDR TaqI TC/CC	0.581	0.007 (0.206; 2.423)		
VDBP GC1296 *CC*	0.578	1.474 (0.375; 5.790)		
CYP24A1 3999 *CC*	0.060	3.281 (0.953; 11.292)		
CYP27B1 +2838 TT	0.159	0.421 (0.126; 1.404)		
CYP27B1 -1260 GT/TT	0.159	2.375 (0.712;7.90)		
VDR Cdx2 AG/GG	0.437	0.615 (0.181; 2.091)		
CYP24A1 22776 *TT*	0.244	2.404 (0.550; 10.151)		
ABCG2 421 C>A	0.848	0.786 (0.067; 9.215)		
*CYP 3A4*1B* G>A	0.262	3.571 (0.387; 32.962)		
ABCG2 1194+928 CC	0.387	0.369 (0.039; 3.537)		
CYP1A2 890 CT/TT	0.063	7.615 (0.894; 64.894)		
GHC1 841 T>C	0.177	0.438 (0.132; 1.453)		
OPRM1 971 TC/CC	0.352	1.678 (0.564; 4.994)		
TRPV1 080 AA	0.598	0.670 (0.151; 2.974)		
*CYP2C9*3* 1075 A>C	0.973	1.111 (0.305; 4.042)		
*CYP2C9*2* 430 C>T	0.920	0.938 (0.264; 3.327)		

Tab. 14: Logistic regression analysis: factors able to predict the effective cannabinoids plasma exposure. Bold represents statistically significant values. NC, statistically not classifiable since one statistical group is not present

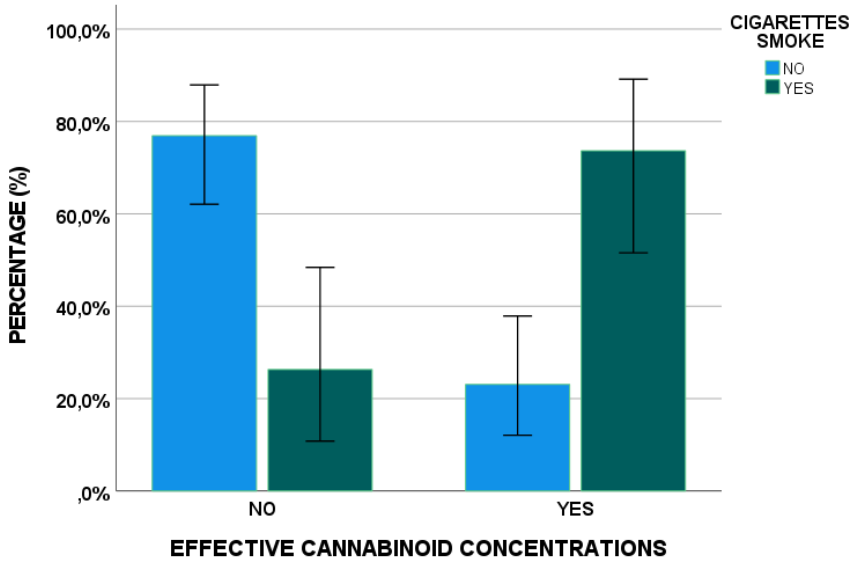


Fig. 12: Influence of smoking cigarettes on cannabinoid plasma exposures.

5. Discussion

The present work aims at developing and validating an analytical method in UHPLC-MS/MS for cannabinoids quantification in plasma for the investigation of cannabinoids PK in a cohort of patients suffering from neuropathic pain, treated with medical cannabis decoction as a galenic preparation administered in clinical practice.

In addition, the evaluation of a possible role of genetic and inflammation biomarkers in cannabinoids plasma exposure was investigated.

The first step of this research was the development and validation of an analytical method performing the identification and quantification of cannabinoids and its metabolites.

The method was fully validated in accordance with the ICH Harmonised Guideline for bioanalytical method validation recommendations [126] and the following parameters were investigated: specificity and selectivity, LOD, LOQ, linearity range, accuracy, intra-day and inter-day precision, robustness, matrix-effect and sampling mode.

The novelty of the present method is represented by a simple extraction method, low plasma requirement (100 µL) and shorter run-time (10 minutes) than previous published LC–MS/MS assays [121].

In details, for the first time, an optimal Δ9-THC and Δ8-THC chromatographic separation was obtained from what previously reported in the literature. For example, in the work of Crippa *et al*. [127] a partial co-elution between Δ8-THC and Δ9-THC (RT 1.90 and 1.97, respectively) was suggested.

The capability of this novel method in Δ9-THC and Δ8-THC separation could be useful for clinical practice; indeed, Δ8-THC is not well studied yet in patients, but it is marketed in cannabis

preparations, making its chromatographic separation from its Δ9 analogue and inclusion in panel analyses useful.

As reported by Tagen and Klumpers [128], the Δ8-THC oral absorption in humans is virtually unstudied: no pharmacokinetic studies have been performed with oral dosing.

Considering the linearity range of calibration curve, since one of the purposes of the present work was the plasma cannabinoids concentration evaluation in patients treated with medical cannabis, we expected higher concentrations than those observed in toxicological analysis. Consequently, 5 mg/ml was fixed as LLOQ: it is a higher value compared to what reported in the scientific literature.

A blood collection study was mandatory in order to clarify the potential variations in analytes measures, since the method was developed as a tool for TDM analysis. In details, the vacutainer sampling types contain different anticoagulant substances probably interfering for mass spectrometer detection, confirming what reported in the previous study published by Jamwal *et al.* [129].

A signal suppression during instrumental analysis with lithium heparin tubes used for blood collection was highlighted in our study.

Moreover, the method was tested on biological samples: 58 patients with a diagnosis of neuropathic and chronic pain. In this work, 11 samples were obtained from patients using inhaled Marijuana and 47 treated with cannabis decoction. Blood sampling in patients and volunteers was performed at the C_{trough} and this study deepens the comprehension of cannabinoids pharmacokinetic.
Our data are regarding inhaled cannabis are comparable with the literature [130].

THC-COOH and THC-COOH-glucuronide were the principal observed metabolites, while CBD and Δ9-THC also featured at low concentrations in blood collected samples, both in patients who smoked and in those who took cannabis decoction.

Δ9 -THC plasma concentrations could be useful for the recent *cannabis* use verification, but becomes problematic in cases of chronic use. In detail, after frequent cannabis exposure, Δ9 -THC accumulates in adipose tissue, from which it is subsequently released [37]: plasma exposures in a chronic *cannabis* user who has been abstinent for some time can be comparable to those of an unusual user recently exposed to cannabis products [34].

Regarding CBD, a missing detection of this analyte does not exclude recent intake [131].

In their work, Schwope *et al.* suggested a maximum plasma CBD concentration of 3.4 ng/mL after consumption of a *cannabis* cigarette (2 mg CBD) *ad libitum* by an experienced user [131]. In our study, we observed concentrations between 5.26 and 7.83 ng/mL of CBD in smoker patients and about 2 ng/mL in subjects treated with decoction.

As reported in the literature by Daldrup *et al*., plasma samples with THC-COOH higher than 75 ng/mL are markers of frequent *cannabis* use, while samples with THC-COOH concentrations ≥75 ng/mL and THC concentrations <5 ng/mL probably indicate a chronic user, confirming our results [132].

Regarding Δ8-THC was n.d. in all samples; 11-OH-THC, CBDA and THCA concentrations <5 ng/mL were observed in all patients. THCA was present at low concentrations only in patients treated with decoction: mean value of 4.89 ng/mL in patients administered medical *cannabis* with THC and CBD level standardized at the similar concentration of 6.5% and 8% and 3.35 ng/mL with medical *cannabis* with THC level standardized at 19% and with a CBD level below 1%.

As reported in literature, the active Δ9 -THC metabolite 11-OH-THC could be an indicator of recent *cannabis* use: indeed, it could be detected at < 1 ng/mL concentrations in blood 8 hours after smoked marijuana consumption by occasional *cannabis* users [132].

Confirming our data, also Pellesi *et al*. observed in their work 11-OH-THC showing significantly lower concentrations than THC and, in their study, two patients treated with *cannabis* decoction had no detectable 11-OH-THC in blood [133].

Regarding medical *cannabis* with THC level standardized at 19% and with a CBD level below 1%, statistically significant differences in cannabinoids plasma exposure between inhaled and oral assumption were found. THC-COOH showed a mean value of 62.99 ng/mL and 10.53 ng/mL in smokers and in subjects treated with decoction, respectively, while THC-COOH-glucuronide was detected at mean concentration of 511.35 ng/mL in smokers and 47.92 ng/mL in oral administration. This investigation highlights some important differences between the *cannabis* routes of administration: patients taking *cannabis* decoction had a higher bioavailability of CBDA and THCA, confirming data published by Pellesi *et al*. [133]. Patients treated with inhaled *cannabis* showed a higher concentration of THC and its metabolites 11-OH-THC and THC-COOH.

It is important to consider that several factors influencing cannabinoid plasma concentrations are present: the drug use form, interindividual differences (such as BMI and gender), life style and pharmacogenetics might have a role in this field. The conversion of the acid precursors to the corresponding cannabinoids, depending on the reaction temperature, could have an impact on plasma concentration variability [132].

Regarding BMI and gender, an inverse correlation between BMI (Kg/m^2) and Δ9-THC plasma levels (ng/mL) was observed ($p= 0.032$, $S = -0.300$): lower Δ9-THC levels occurs in patients with higher BMI. It is probably due to lipophilic properties of this cannabinoid: as reported above, Δ9 -THC accumulates in adipose tissue and it results less available in plasma.

Regarding the role of gender in influencing cannabinoids plasma concentrations, statistically significant differences were observed between male and female regarding COOH-THC, COOH-THC-glucuronide, THCA and CBDA plasma levels. Also in this case, we can suppose the role of lipophilic properties of these molecules: sex differences in the cannabinoid levels could be related to

differences in drug disposition and body fat distribution. Women present a higher body fat percentage than men, suggesting cannabinoids are sequestered in fat cells and less in plasma in women.
In the literature, gender differences in cannabinoid-induced effects related to *cannabis* dependence, were suggested [134]: male *cannabis* smokers exhibit higher circulating levels of Δ9-THC [135], showing higher cardiovascular and subjective effects than female [136].

Regarding the influence of cigarette smoke on cannabinoids plasma exposure, statistically significant differences were observed between smokers and no smokers, considering the principal *cannabis* metabolites. These singular data need to be clarified: in the literature several studies reported that smoking increases the activity of CYP1A2 and CYP2D6 enzymes [137-139].
As an example, fluvoxamine concentrations in smoker patients was significantly lower than non-smokers after a 50 mg single oral in a study of healthy volunteers [140].
In addition, a negative correlation between smoking and clozapine plasma exposure was observed, in accordance with the induction of *CYP1A2* by cigarettes [141].

Regarding genetic analysis, SNPs encoding enzymes and transporters associated with cannabinoids metabolism and elimination were explored. The primary metabolic pathway of THC involves *CYP2C9* [142], converting this cannabinoid 11-OH-THC and COOH-THC, as reported above. In the literature, genetic studies suggested that *CYP2C9**2 and *3 genotypes have high frequencies in Caucasians (>18%) and 15–20% of the population have the *CYP2C9*3* allele [142,143]: this gene variant is associated with slower THC conversation to its metabolites, when compared to the *CYP2C9*1* allele variant [143]. These subjects can accumulate THC levels higher than those with the normal allele (200–300%) [142].

In the present study, variations in cannabinoids concentration associated to *CYP2C9* genetic variants were not found, confirming the data of Papastergiou *et al*. [144].

Other enzymes involved in cannabinoids metabolism are CYP3A4 and CYP2C19: the first is implicated in THC and CBD metabolism, while CYP2C19 has an important role the metabolism of CBD in its active metabolite 7-OH-CBD [145]. Its formation was positively correlated with enzyme activity, but was not associated with *CYP2C19* genotype [145].

However, in the literature no association between 7-OH-CBD levels and *CYP2C19* genotype was reported [145], confirming our results.

CYP2D6 is abundant in brain and it is involved in psychotropic drugs metabolism such as antipsychotics, antidepressants and anticonvulsants. In the literature, few data investigating associations between *CYP2D6* and cannabinoids are available. In this study, a role of *CYP2D6* 840 CG/GG influencing THCA plasma exposure was observed: THCA lower levels were observed in patients with CG/GG genotype.

Regarding drugs transporters, the P-glicoprotein is an efflux protein belonging to the ATP-binding cassette subfamily B member 1 (ABCB1) encoded by the *ABCB1* gene [146].

ABCB1 polymorphisms were studied for their role in *cannabis* addiction [147,148]: the *ABCB1* 3435 SNP was correlated with *cannabis* dependence [143,149]. In a study focused on cannabinoids blood levels influenced by *ABCB1* 3435 T allele carriers (TT/CT) had lower plasma THC concentrations than non-T carriers. However, the exact mechanisms were not clarified [150].

In this study, *ABCB1*1236 CT/TT showed a role in affecting 7-OH-THC levels (p=0.040).

COMT encodes for catechol-O-methyltransferase (COMT) [151] and plays a critical role in dopamine metabolism.

In the literature, COMT impact on *cannabis* response and psychosis risk and cognitive impairment was observed [143,148]. A study published by Henquet *et al*. showed the role of COMT Val158Met genotype in modulating THC effect on cognition and psychosis: high activity associated with GG

genotype (Val/Val) was related with more sensitivity to THC-induced memory impairments compared to the Met allele [151].

COMT 680 T>C showed an impact on all cannabinoids monitored in the presents study, except for CBD, CBDA and THCA: in particular, Δ9-THC plasma levels (ng/mL) are higher in TT genotype and lower in patients with TC/CC genotype ($p = 0.017$).

Regarding patient inflammatory status, evaluated by measuring IL-6, IL-10 and TNF-alfa levels in plasma, a statistically significant difference among subjects treated with decoction (n=47) and smokers (n=11) in IL-10 levels (p=0.009) was observed. In details, smoker subjects showed IL-10 lower levels. Immune cells express cannabinoid receptors and cannabinoids have a role in affecting cytokine production in immune cells [152]. Cannabinoids, in particular CBD, have powerful anti-inflammatory and immunosuppressive properties. We can suppose that, cannabinoids higher levels observed in smoker patients than in decoction treated ones are responsible of anti-inflammatory effects.

Furthermore, a statistically significant BDNF difference among patients treated with decoction (n=47) and smokers (n=11) was observed (p=0.004): higher BDNF levels were observed in concomitant of higher cannabinoids plasma exposure, in smokers.

In the literature, a reduction of neurotrophic factors, particularly BDNF, levels are present in neurodegenerative disorders [153,154], such as Parkinson's disease [155], Alzheimer's disease [156,157], multiple sclerosis [158,159]. BDNF shows trophic effects and a decrease in its plasma levels is associated with an impaired brain health [160,161]: based on our results, we can suppose protective role of cannabinoids.

Finally, genetic, demographic, biochemical and pharmacological factors able to predict the effective cannabinoids plasma exposure were analyzed in a logistic regression analysis table: only gender, cigarettes smoke, inhaled *cannabis* and cardiovascular system drugs remained in the final multivariate regression.

As effective cannabinoid plasma levels samples with THC-COOH glucuronide higher than 60 ng/mL and THC higher than 5 ng/mL were considered.

Limitations of this study are a relatively small cohort of patients treated with medical cannabis and some information lacks, including clinical outcome. In addition, the role of genetics and cigarettes smoke has to be evaluated in further studies in order to clarify their role in cannabinoids plasma exposure.

Since the present study highlights that the treatment with decoction does not allow the achievement of effective cannabinoids plasma concentrations, future perspective could be the treatment of patients with cannabis oil, in order to evaluate the PK and PD parameters.

6. Conclusions

The developed method highlighted difference in cannabinoids metabolites exposures between different galenic formulations. The great variability in cannabinoids formulations results in patient inter-individual difference in concentrations. Thus, in this situation, TDM could be useful to allow for dose adjustment and our study could help to identify therapeutic range and to guide this practice.

In addition, this is the first study showing a possible impact of some inflammation-related factors and factors associated with neuronal impairment on patients treated with medical *cannabis*. These data could be useful in order to clarify the protective role of cannabinoids.

7. References

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