INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO

MESTRADO EM ENGENHARIA QUÍMICA

isep

EVALUATION OF A PYRETHROID METABOLITE (3-PBA) IN URINE OF CHILDREN AFFECTED BY ASD (autism spectrum disease)

MATTIA ORAZI

Julho de 2015

POLITÉCNICO
DO PORTO

"EVALUATION OF A PYRETHROID METABOLITE (3-PBA) IN URINE OF CHILDREN AFFECTED BY ASD (autism spectrum disease)"

Abstract

The described method permits the determination one of the most common metabolite of the pyrethroids permethrin, cypermethrin, deltamethrin, λ-cyhalothrin, fenvalerate, phenothrin and β-cyfluthrin in human urine: The 3-phenoxybenzoic acid (3-PBA) . After alkaline hydrolysis to release the conjugated carboxylic acid metabolite, the analytes were separated from the matrix by means of solid-phase extraction using a reversed-phase/strong cation exchange column. The components of the eluate were converted to their Hexafluoroisopropane esters and extracted in hexane. Separation and quantitative analysis of this pyrethroid metabolite was carried out by capillary gas chromatography and mass selective detection. 2-Phenoxybenzoic acid served as an internal standard. The detection limits was 0,0038 µg per ml urine. The relative standard deviations of the within-series imprecision were always less than 10%. Using this method we determined the elimination of 3-PBA in 46 children(24 autistics,22 controls), the average values in urine of each population were 1,48 µg/ml in ASD (Variance 4,94) and 0,63 µg/ml in controls (Variance 0,22).

1. Introduction

"All the airplane that can land in Chicago along a day, landing all together in the same moment".

With this picture Nancy Minshew, researcher in Pittsburgh University, describe the intricate network and consequently, the abnormal communication through the different brain parts in children affected by Autistic Spectrum Disease.

*Autistic spectrum disease (ASD) are a group of disorders characterized by qualitative impairment in social interaction and communication, and restricted repetitive and stereotyped patterns of behaviour, interests and activities (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (**DSM-IV**);[1] International Classification of Diseases-10 (**ICD-10**)[2]).*

1.1 Autism

Autism was first outlined in 1943 by Leo Kanner, an Austrian-US-American Professor of Child Psychiatry. He described children with mental retardation and severe social isolation not

explained by the developmental level of the children[3]. Kanner referred to Eugen Bleuler by naming the syndrome 'infantile autism' based on Bleulers schizophrenia criterion describing the loss of social interest in schizophrenia. At the same time, Professor Hans Asperger in Vienna, Austria, noticed similar patients with 'autistic psychopathy' and normal intellectual abilities[4] Hans Asperger noted that fathers of these children seemed aloof and socially isolated. Both, Kanner and Asperger, suspected a biological or even genetic origin of the disorder.However, this knowledge was lost during the 1950–1960s, until Michael Rutter[5] and Lorna Wing[6] resumed discussion on diagnostic concepts, differential diagnosis and aetiology of AD in the 1970s and 1980s.

These disorders seems increasing in their number in the last years; in fact recent studies spokes about 400.000 children only in Italy with an ASD diagnosis, this rapid increasing could cause some worries, but for the most of that the merit is a better knowledge and precise classification about the clinical picture.

The most commons features of ASD are divisible in **three core**:

- Socializing Difficulty;
- Monotonous and repetitive game playing in early ages;
- Low interest on the external environment.

Are also notified a particular interest on moving things, especially round movements, and a low weight in birth age.

The ***Autism Spectrum Disease*** can be divided in three different clinical pictures:

Autism and Autism at high functionality show impairments in all three areas and an abnormal development before age 3 years. With a high cognitivity of the external environment thanks to the high functionality, but a still inability in socializing. Common in the high functionality phenotype a particular ability in calculation and repetitive pattern movements.

Asperger Syndrome is characterized by qualitative impairment in social interaction and restricted repetitive and stereotyped patterns of behaviours, interests and activities with an apparently normal language and cognitive development before age 3 years. Interesting is also speaking about the predominance of male children affected, and about the character of the subject that in the measure of the man-kind mental difference is classifiable to be bizarre, naïve, literal in the meaning of communication inducing to think about a leak of archness, in fact their language commonly, is polished. The children most of the time shown good ability and interest for uncommon hobbies, those could increase the isolation-state of the subject, causing possible comorbidity in the adolescence age like Auto-aggressive behaviour, schizophrenia, anxiety, obsession…

PDD-**nos** (pervasive developmental disorder not otherwise specified) is diagnosed in individuals who meet autism criteria, but show a late age of onset, or in individuals who show severe and pervasive impairment in one or two of the three core areas with or without cognitive or language delay. The central difference lies in an apparently normal development

until age 2 years and a clinically significant loss of skills before age 10 years. Owing to the rarity of the disorder (15/10 000)[8] systematic studies regarding its aetiology are missing.

The therapy commonly used for the treatment of this large spectrum disorder is an association by a Neuro-cognitive and a Pharmacological approach, that should be actuated as soon as possible in the child affected by this kind of disorder been it predominantly classified as a leak, or preferably known, as a difference, in network and communication through the different part of the brain. This differences could be changed or reduced by an adequate neuro-cognitive approach therapy in this age (0-6 years), where the neuroplasticity and sinapto-genesis are predominantly. Following with a cognitive-behavioural approach in the adolescence period eventually coadiuvated by a pharmacological approach, to reduce the co-morbidity with other disturb, working for the social adaptation in a sort of alliance through Family-Teacher-Specialist.

1.2 Etiology:

Neuro-Imaging (fMRI-BOLD, sMRI, PET) Approach:

Recently, a great deal of attention has been focused on the delineation of neural systems for brain-behaviour relationships in autism. At the brain circuit level, most of what we understand about autism and its biological abnormalities come from technological advances in diagnostic brain imaging with positron emission tomography (PET) and magnetic resonance structural (sMRI) and functional (fMRI) that have had a profound impact on our understanding of the neurobiological basis of autism through the study , anatomical and functional connectivity of the brain, greatly improving the ability of researcher to examine the neural substrates of cognitive processes.

Biological research results:

Although many of the cognitive and behavioral features of ASD are thought to arise from dysfunction of the central nervous system (CNS), many fields of medicine has documented suggesting that in some individuals, ASD arises from systemic, rather than organ-specific abnormalities. Specifically in the recent decades, research and clinical studies has been implicated with physiological and metabolic systems that transcend specific organ dysfunctions, such as immune dysregulation, inflammation, impaired detoxification, environmental toxicant exposure, redox regulation/oxidative stress and energy generation/mithocondrial systems.
For each of that we have a large percentage of publication that implicate an association between ASD and immune disregulation/inflammation (416 out of 437 publications, 95%), oxidative stress(all 115), mitochondrial dysfunctions (145 of 153, 95 %) and toxicant exposure (170 of 190,89%).In this context, ASD may arise from, or at least involve , systemic physiological abnormalities rather than being a purely CNS disorder, at least in a subset of individuals with ASD.[9]

Research studies in a wide variety of psychiatric disorders, including ASD, have started to investigate gene-environment interactions and epigenic factors, rather than fixed genetic defects. Other research studies examining the etiology of psychiatric disorders have

embraced the study of pathophysiological mechanism that could more directly result in cellular dysfuction and the subsequent development of psychiatric disorders have embrace the study of pathophysiological mechanism that could more directly result in cellular dysfunction and the subsequent development of psychiatric disorders. Like ASD, pathophysiological mechanism identified in some psychiatric disorders include immune dysregulation, inflammation, impaired detoxification, enviromental toxicant exposures, redox regulation/oxidative stress and mitochondrial dysfunctions. Immune dysregulation and inflammation has been implicated in several psychiatric disorders. For example, neuroinflammation, which generally refers to CNS-specific, chronic glial reactions that may not demonstrate typical peripheral evidence of inflammation, has been implicated in several psychiatric disorders, including Alzheimer's disease, schizophrenia, bipolar disorder, and depression as well as ASD. Neuroinflammation can damage brain tissue through several mechanisms, including plaque formation, abnormal neuron growth, increased tau phosphorylation and proinflammatory citokynes release.[9]

Enviromental toxicant exposure are also very important in the etiology of a wide variety of disorders. Toxicants, such as heavy metals, pesticides and chemicals, can damage cell by converging on similar biochemical pathways to produce adverse effects, such as increasing oxidative stress, depleting glutathione and impairing cellular signaling. Exposures to environmental toxicants were been trusted as causes in certain psychiatric disorders, such as ADHD, depression and schizophrenia as well as ASD.[9]

Furthermore, Oxidative stress is defined as damage to cellular tissue caused by free radicals such as reactive oxygen species. Oxidative stress is also implicated in a large variety of disorders including cardiovascular disease, diabetes and hypertension.

Oxidative stress has also been reported in some individuals with ASD. In fact of the 51 clinical and 7 treatment studies in ASD: the 88% of the studies reported abnormal biomarkers of oxidative stress ,including alterations in glutathione(16 studies), vitamine E (3 studies), tetrahydrobiopterin(4 studies) and antioxidant enzyme levels, such as glutathione peroxidase, paraoxonase, and superoxide dismutase (11 studies). Eight studies reported mutations in antioxidant enzymes or pathways, such as glutathione peroxidase, superoxide dismutase, glutathione-S-trasferase, delta aminolevulenic acid dehydratase, methilenetetrahidrofolate reductase, catechol-O-metiltasferase and reduced folate carrier. Five studies reported a positive correlation between biomarkers of greater oxidative stress and the severity of ASD.[9]

Finally disfuction in mitochondria, distinct cellular organelles that oxidize glucose and fatty acids to generate adenosine triphosphate, the energy carrier in most mammalian cells has been implicated in several psychiatric disorders including schizophrenia, bipolar disorder, depression, dementia as well as ASD. Fifty-three(84%) of these studies reported abnormal biomarkers, which could relate to mithochondrial dysfuction. Commonly abnormal biomarkers included lactic acid(18 studies), pyruvate(11 studies), carnitine(7 studies) and plasma amino acids(4 studies). In addition to energy production, mitochondria are intimately involved in programmed cell death (apoptosis), calcium homeostasasis, synaptic plasticity and neurotrasmitter release. Two studies reported a positive correlation between biomarkers of mitochondrial dysfunction and the severity of ASD.[9]

Consequently these three disorders, Autism, Asperger syndrome (AS) and Pervasive Developmental Disorder not otherwise specified (PDD-nos) are currently conceptualized by most researchers as **a set of disorders with different causes.**

1.3 About Pyrethroids

The Pyrethrins were already known in ancient Mesopotamia and in the ancient world with the name of “Persian powder” .They are natural organic molecule produced by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *C. coccineum*). [10]

Figure-1 *Chrysanthemum cinerariaefolium*

Figure-2 *Chrysanthemum coccineum*

The pyrethrins (natural pyrethroids), obtained from pyrethrum, are very unstable to light, Due to this fact, the use is quite limited. However due to its natural characteristics this substance aroused enormous interest in some organic chemists.

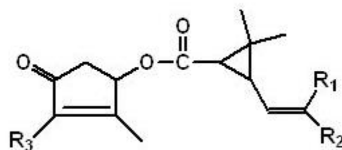


Figure-3 ***Pyrethrum structure***

In mid-year 1910 came the first study about the Pyrethrins by a team of Rothamsted Research scientists, following the elucidation of the structures of pyrethrin I and II by Hermann Staudinger and Leopold Ružička, in 1958 it was completed, and with it, the discovery of six active compounds structures: Pyrethrin I, Pyrethrin II, Cinerin I, II Cinerin, jasmolin I and II jasmolin[11]. In the following period, various pyrethroids were synthesized from pyrethrins. Since that time came to be known as pyrethroids, natural pyrethrins and their synthetic derivatives. It has been shown that the centers of photostable pyrethroids molecular structure could be replaced by alternative units, producing compounds more stable to light, but retaining its insecticidal capacity and continues to be somewhat harmful to mammals, calling up these substances as synthetic pyrethroids [12].

The classification of such insecticides may be made according to when they were created (4 series), or by the presence or absence of the cyano group (type I or type II). The first generation pyrethroids : Allethrin was constituted only by (a synthetic duplicate of cinerin I), the allethrin was first marketed in the year 1949, from this moment start took place an era of very complex synthesis, involving 22 chemical reaction[13]. With new developments appeared to second generation pyrethroids, more effective than the natural pyrethroids since they were photostable and effective for use in agriculture and domestic use. In 1976 it was discovered the first photostable synthetic pyrethroid, Fenvalerate, Permethrin following, already in 1977. These two types of insecticides are also classified as third generation insecticides since their characteristics are listed in stability to sunlight and its low volatility. In the 80's began the fourth generation of pyrethroids, this new step differs from previous ones, because they synthesize from cyclopropane, and because the amount required for the production thereof is much lower than those of previous generation. Examples of fourth generation pyrethroids are cyfluthrin (1980), flumethrin (1982), and fenpropan-fluvalinate (1983), cyhalothrin and bifenthrin (1985) and lastly tefluthrin (1987). Regarding the presence or absence of the cyano group, the pyrethroid can be classified as type I (absence of the cyano group) and type II (presence of cyano group). This classification is also based on the symptoms presented laboratory animals following exposure to various types of pesticides PYRS, since the cyano group influences the mode of action of the insecticide. Of type I pyrethroids are part Bifenthrin and Permethrin, as type II may be considered to fenpropathrin, λ-cyhalothrin, cypermethrin and deltamethrin [14].

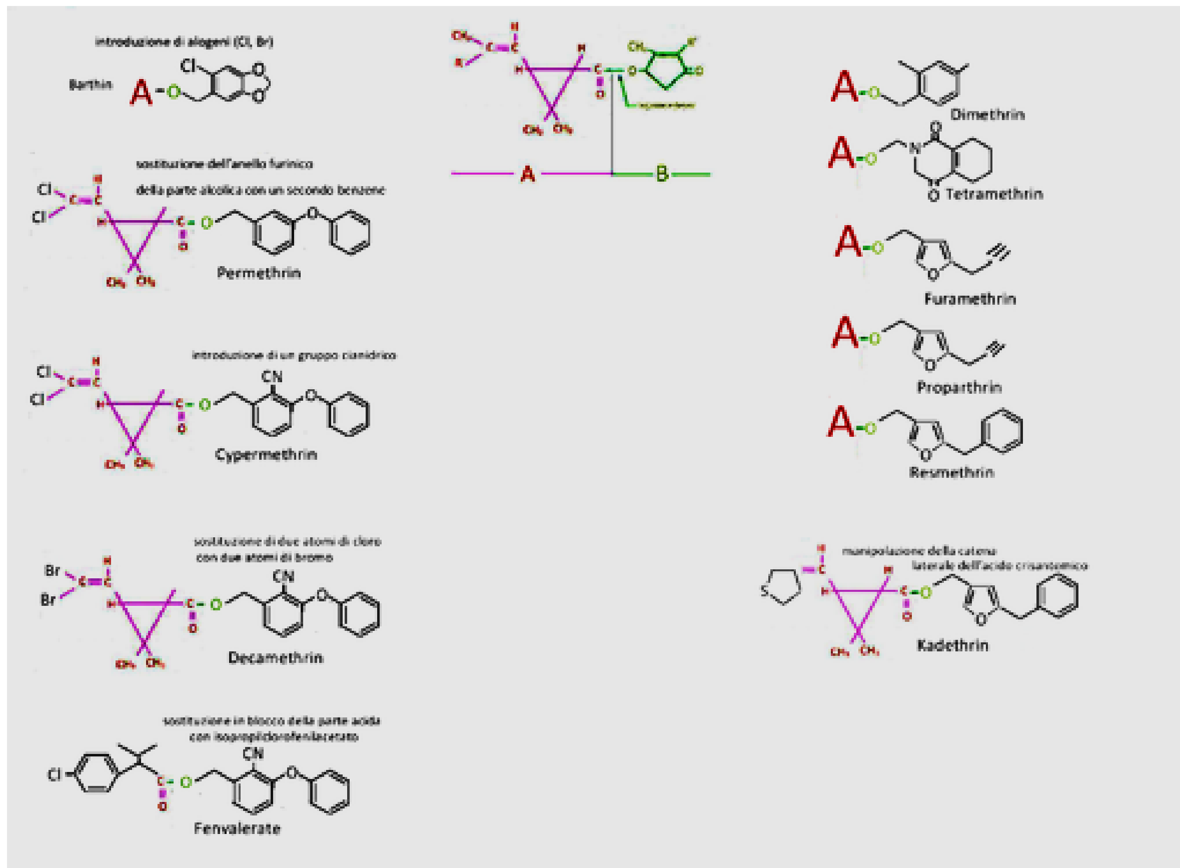


Figure 4-*The four generations of pyrethroids and their structural changes through the years*

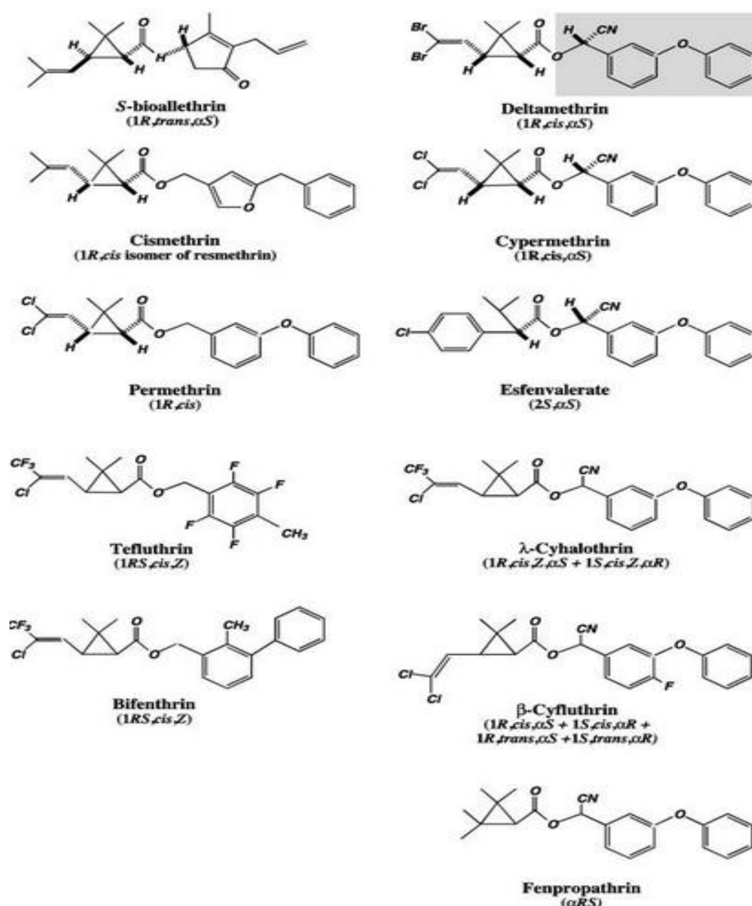


Figure-5 *The two classes of pyrethroid in according with the presence or absence of the cyano group (type I or type II) the insecticidal effectiveness of these compounds is due to their Stereoisomery. Several pyrethroids have different isomeric forms, thus demonstrating toxicities and different intensities.*

Pyrethroids now constitute the majority of commercial household insecticides. In the concentrations used in such products, they may also have insect repellent properties and are generally harmless to human beings in low doses but can harm sensitive individuals.

Metabolism:

The 3-phenoxybenzoic acid (3-PBA) is the most common metabolite of 10 of 18 pyretroids registered in the United States, including permethrin, cypermetrin, deltametrin, esfenvalerate[15]. Both type I and type II pyretroids undergo ester hydrolysis which results in a cyclopropyl acid and either 3-phenoxybenzyl alcohol (type I) or a cyanohydrin (type II) [16]. The aromatic alcohol is further oxidized to 3-PBA while the cyanohydrin is able to spontaneously rearrange to the phenoxybenzylaldehyde in aqueous solution and then undergo hydroxylation to 3-PBA. 3-PBA is a non-specific urinary metabolite, meaning that it is a breakdown product common to most of the pyretroids with the exception of cyfluthrin. It is also the most frequently detected urinary metabolite in humans. 3-PBA has been detected in urine from the general population in the United States (0.32 µg/L), Germany (2.0 µg/L reference value), Italy(0.88 µg/L urban and 0.71 µg/L rural) and Japan (0.29 µg/L). Levels in occupationally exposed population such as pest control workers tend to be higher (6.8 µg/g creatinine)[17][18]. Since 3-PBA it has been used as a marker of pyrethroid.

In Animal feeding studies using rats, goats, cows and hens, pyrethroid was metabolized quickly in the liver. Hydrolysis, hidroxylation, oxidation and conjugation are all involved in

the process of metabolism[17][18]. Permethrin is easily hydrolyzed, and the hydrolysis product is also easily oxidized in rat's tissue in vitro. Permethrin is converted to PBAlc (3-phenoxybenzyl alcohol) and further to PBAcid by mixed function oxidase in rats. Trans-isomers are more rapidly metabolized by hydrolytic (esterase) pathways while cis-isomers are preferentially metabolized by slower oxidative (P450) pathways [19].The trans-isomers of permethrin is mainly catalized by the CES1 family and partly by the CES2 family. The cis-isomers of permethrin, which is metabolized predominantly by P450's in laboratory animals[20], was not detectably metabolized in human liver microsomes in the presence or absence of an NADPH regenerating system[21]. Oxidation of PBAlc and PBAld are mainly mediated by mixed function oxidase, in addition to ADH (alcohol dehydrogenase) and ALDH (Aldhehyde dehydrogenase), which were shown to contribute to the oxidation [Hodson and coworker (2002;2003)]. The alcohol generated from permethrin, PBalc is oxidized to PBAld (3-phenoxybenzylaldehyde), and the aldehyde is further oxidized to 3-PBA. The alcohol and aldehyde moieties of pyrethroids are mainly metabolized by oxidation to the corresponding aldehydes and carboxylic acid by ADH and ALDH in mammalian species [21]. The terminal phase of pyrethroid metabolism is the formation of glucoronide and glycine conjugates. These pathways appear similar between laboratory animals and humans. Common metabolites are found in the urine of both laboratory animals and humans [21][23].

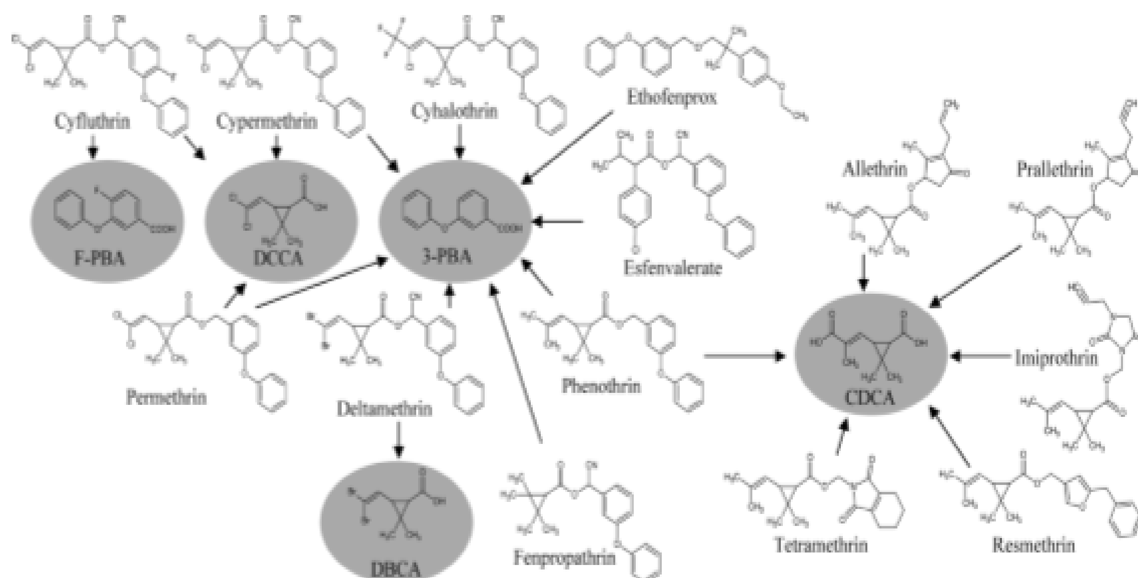


Figure 6. *Scheme representing the pryncipal metabolites of different Pyrethroids classes.*

Table 1. *Pyrethroids and Pyrethrins and their corresponding Metabolite.*

Metabolites	Parent compounds
cDCCA and tDCCA	Cyfluthrin, cypermethrin, permethrin
PBA	Cyhalothrin-λ, cypermethrin, deltamethrin, esfenvalerate, fenpropathrin, flucythrinate, fluvalinate, permethrin, phenothrin
CDCA	Resmethrin, allethrin, phenothrin, prallethrin, tetramethrin, natural pyrethrins (a mix of: cinerin 1&2, jasmolin 1&2 and pyrethrin 1&2)
DBCA	Deltamethrin
FPBA	Cyfluthrin, flumethrin

Excretion of 3-PBA:

Pyrethroids and its metabolites are excreted primarily in the urine, but also in the feces [29],[21]. The 3-PBA excretion was investigated in rats intact and bile duct-cannulated rats. . The major biliary metabolites was 3PBA-glucuronide,but we can have glycine conjugates too, that it was subsequently deconjugated, readsorbed and undegoes further metabolism, principally to the sulfate ester which is excreted in urine[29]. The elimination half-life is about 8 h reported for 3-PBA among workers exposed to cypermethrin[30] suggest that 88% of the metabolite is excreted within the 24 h following exposure.

Toxicity and health effect:

Before 1970, there was little information available on the acute toxicity resulting from exposure to pirethroid insecticides (natural and synthetic) in mammals [31] Studies published in 1970 concluded that there are two syndromes associated with acute exposure to pyrethroids in rats[32]. However, pyrethroids have been the subject of some controversy about the possible toxic effects over time in humans[33][34].Some studies suggest a relationship between exposure to pyrethroids and the appearance of Parkinson's disease .

Pyrethroids are not cholinesterase inhibitors such as organophosphates and carbamates but act as contact or ingestion of toxic affecting the central and peripheral nervous system of insects, even in low doses.[35][36] The activity of pyrethroids is a lipophilic membrane environment and consist of sodium channels excite the sodium-potassium pump, present in the membranes of nerve cells of insects and mammals interfering in permeability to sodium ions, resulting in synaptic repetitive discharges, membrane depolarization and ultimately death. The toxic effects are caused mainly by a repetitive induction of nerve activity mediated by interaction of pyrethroid with the membrane sodium channels. The adverse effects induced by pyrethroids are a consequence of neuronal hyperexcitability[37].

The presence or absence of α-cyano group will be reflected in the duration and effectiveness of the insecticide effect on sodium channels. The absence of α-cyano group (Type I pyrethroid), such as permethrin results in a shorter end.

Pyretroids are lipophilic molecule than they are accumulates in the fat tissues, especially the cerebellum tissue (67% of fat).For example the maximum amounts of permethrin in rats

tissue of cerebellum, hippocampus, caudate putamen, frontal cortex, hypothalamus, and sciatic nerve were about 1.5, 2, 2, 2.7, 4.8, and 7.5 times higher than in plasma, so, respectively, indicating an accumulation of pyretroid by nervous tissue itself [38]

According to their chemical structure and their biodistribution, pyrethroids can cause:

-T-syndrome (tremors, sensivity to sensory stimuli, ataxia, convulsions and paralysis)

-CS-syndrome[39](choreoatheosis, hypersensivity to external stimuli, salivation and, in some cases paralysis)[40]

Usually T-syndrome is caused by pyrethroids non-containing an alpha-cyano group in their molecule and CS-syndrome derives by pyrethroid with an alpha-cyano group. Many pyrethroids, such as permethrin, exihibit both T and CS characteristics and sometimes this two syndromes combine[41]. Pyrethroids interfers with sodium channels and probably with calcium-channels (VSSC) and Chloride channel too[42][43], this interactions disrupt the function of neurons, and causes spasm on muscles, culminating in paralysis and death .[44]

It is demonstrated that Pyrethroids can induce several toxic effects also on:

-*Immune system toxicity*: even small doses of pyretroids can reduce the ability of immune system to respond to foreign proteins. Over 40% of T-lymphocytes and natural killer (NK) cells are inhibited by a dose of pyretroids equivalent to 1/100 of the LD50[45].

-*Lungs*: repeated inhalation may cause asthma and pneumonia, burning, numbness, itching, erythema and hypersensibility .

-*Reproductive system effects:* pyrethroids bind to receptors for androgen[46] and to the peripheral benzodiazepine receptor (which stimulates production of testosterone)[47].Moreover it inhibits the binding of estradiol to the estrogen receptor.[48]

-*Mutagenic effects*: an increase in chromosome aberration, chromosome fragments and DNA lesions[46] may occur after a pyrethroids exposure.

-*Cancer*: According to the World Health Organization pyrethroids increased the frequency of lung tumors in female mice, increasing the expression of a gene involved with the proliferation of cells in mammary gland[48][49]. The risk of prostate cancer in men with a family history of prostate cancer can increase because of Permethrin exposure[50].

Mammals can metabolize in a good way pyrethroids, such as permethrin, before they can affect the nervous system[41]. For example the lethal dose (LD50) for permethrin is variable, ranging from 430 mg/kg body weight to over 4000 mg/kg for rats[45].

But 3-PBA, the most important metabolite of pyrethroids deactivation process can also be an other possible toxicant, in the beginning, the complete metabolization requires more steps than the other two (3-phenoxybenzylalcohol, 3-phenoxybenzylaldehyde); this more articulated deactivation process likely leads to a highest ROS formation [51]. So 3-PBA can exerts harmful effect, mainly in the hydrophilic part, of the external part of bilayer. It can induce carbonyl group formation and this can be explained by the ease where by carboxyl group reacts with protein amino group. When metabolites are in the membrane, they can interact with lipids forming crosslink and with proteins via amino group bond, modifying the

macromolecule dynamics of the bilayer. This means that all the proteins inside the membrane, missing their mobility, will not guarantee the proper cell communication. The 3-PBA is also responsible for highest lipid peroxidation compared with the other two metabolites. This effect could be related with a major production of reactive oxygen species, as sub-products due to an increase in oxidative intracellular processes necessary to metabolize the compound. Moreover, the oxidation of proteins contribute to modify other membrane proteins changing the signal within cells[49].

1.4 SPE (Solid-liquid-extraction)

Solid phase extraction (SPE) is a separation process used in order to separate compounds which are dissolved and suspended in a liquid mixture from other compounds according to their physical and chemical properties. SPE is used by analytical laboratories to concentrate and purify samples for analysis and it can be also used to isolate analytes of interest from a wide variety of matrices, including urine, blood, water, soil, beverages and animal tissue [50]. The affinity of solutes dissolved or suspended in a liquid (mobile phase) for a solid through which the example is passed (stationary phase) is used in order to separate a mixture into desired and not desired components. The portion that passes through the stationary phase can contain desired analytes or undesired impurities. If the portion retained on the stationary phase includes the desired analytes they can then be removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed in appropriate eluent. The stationary phase consists in a packed syringe-shaped cartridge, a 96 well plate or a 47- or 90-mm flat disk, each of which can be mounted on its specific type of extraction manifold. The manifold allows multiple samples to be processed by holding several SPE media in place and allowing for an equal number samples to pass through them simultaneously. A typical cartridge SPE manifold can accommodate up to 24 cartridges. Most SPE manifolds are equipped with a vacuum port. Application of vacuum speeds up the extraction process by pulling the liquid sample through the stationary phase. After that the analytes according to different chemical properties and which are usually based on silica that has been bonded to a specific functional group (hydrocarbon chains for reversed phase SPE, quaternary ammonium or amino groups for anion exchange and sulfonic acid or carboxyl groups for cation exchange)[53]. Sodium chloride can be also added to the sample to the extraction efficiency [53]. SPE cartridges should not be allowed to dry any point during conditioning and sample loading, in order to keep the sorbent ligands active and prevent air from trapping in the cartridges. It would be a good practice to transfer the upper layer (containing fewer solid) of the aqueous solution first and the lower layer (containing more solids) later because there can be a susceptibility to clogging when samples containing suspended solids are to be analyzed. Automation usually reduces or eliminates some variations due to manual extraction and it also shortens time required for extraction process. SPE is normally used for samples that contain matter causing clogging and high back pressure and components that cause high background, misleading peaks or poor sensivity.

Clean-up, trace concentration or purification, sample matrix to a form compatible with chromatographic analyses and concentrates analytes for increased the sensitivity. Moreover can remove interferences and protect the analytical column from contaminants.

- **NORMAL PHASE SPE PROCEDURE**:

In a Normal Phase SPE procedure first of all cartridge have to be equilibrated with a non-polar solvent or slightly polar, in order to wet the surface and penetrate the bonded phase. Then the silica surface is wet with water or buffer of a same concentration as the sample through the column. The sample is then added to the cartridge. When the sample passes through the stationary phase, the analytes in the samples will interact and retain on the sorbent while the solvent, salts, and other impurities pass through the cartridge. After that buffer is used in order to wash the cartridge and remove impurities. Then, the analyte is eluted with a non-polar solvent or a buffer of the appropriate pH. A stationary phase of polar functionally bonded silica with short carbons chains frequently makes up the solid phase. This stationary phase will adsorb polar molecules which can be collected with a more polar solvent [50].

- **REVERSED PHASE SPE**

Analytes based on their polarity are separated with reversed phase SPE. The stationary phase of a reversed phase SPE cartridge is derivatized with hydrocarbon chains, which retain compounds of mid to low polarity due to the hydrophobic effect. The cartridge can be washed with a non-polar solvent, which breaks up the interaction of the analyte and the stationary [50]. A stationary phase of silicon with carbon chains in commonly used. Only non-polar or very weakly polar compounds will adsorb to the surface[50].

- **ION EXCHANGE SPE**

Analytes based on electrostatic interactions are separated from the analyte of interest and the positively charged groups on the stationary phase using ion exchange sorbents. For ion exchange to occur, both the stationary phase and sample must be at a pH where both are charged.

1.5 Gas-Chromatography

Chromatography is an effective method of separation, identification and determination of chemical components that finds application in all areas of science. It began to be used in the early twentieth century by the Russian botanist Mikhail Tswett. The chromatography name comes from the Greek *chroma* meaning color and *graphein* which means write. The name came as a result of using the technique to separate multiple plant pigments such as chlorophyll and xanthophyll. The separate species appear as coloured bands in [51][52] column. The use of chromatography increased exponentially in recent years as a result of the necessity in separation of compounds contained in complex matrices, by identification

comparing to previously existing standards and purification of the compounds, resulting in the development of various types of chromatographic techniques (different possible combinations between the stationary and mobile phases) [51]. In all chromatographic separations, the sample is transported by a mobile phase, which may be a gas, a liquid or a supercritical fluid. The mobile phase is forced via the fixed immiscible stationary phase column and placed on a solid surface. The two phases are chosen such that the sample components are distributed between the mobile and stationary phase to varying degrees. As a consequence the components that are strongly retained on the stationary phase move very slowly in the mobile phase flow. The opposite happens with components that bind weakly to the stationary phase, they move faster.

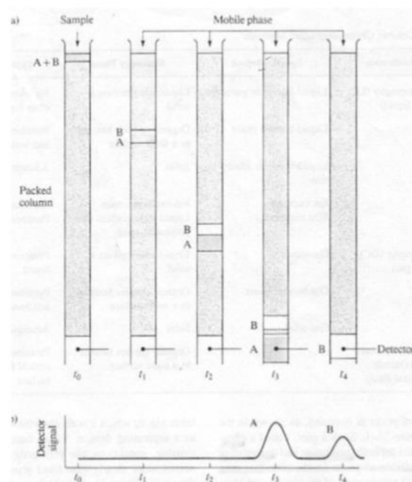


Figure 7. *The image shows schematically how two substances A and B are separated in a column for chromatographic elution (a kind wash through the column by continuous addition of new volumes of solvent) using a liquid mobile phase .*

Gas chromatography (GC or GLC) is a commonly used analytic technique in many research and industrial laboratories for quality control as well as identification and quantification of compounds in a mixture. GC is also a frequently used technique in many environmental and forensic laboratories because is allows for the detection of very small quantities. A broad variety of samples can be analyzed as long as the compounds are sufficiently thermally stable and volatile. A mobile and a stationary phase are required for this technique. The mobile phase (carrier gas) is comprised of an inert gas i. e., helium, argon, or nitrogen. The stationary phase consist of a packed column where the packing or solid support itself acts as stationary phase, or is coated with the liquid stationary phase (a high boiling polymer). Most analytical gas chromatographs use capillary column, where the stationary phase coats the walls of a small-diameter tube directly (i.e. 0.25μm film in a 0.32 mm tube). The separation of compounds is based on the different strengths of the interactions of the compound interact with the stationary phase and more time it takes to migrate through the column (=longer retention time).

The factors which influence the separation of the components are:

Boiling Point:

The boiling point of a compound is often related to its polarity. The lower the boiling point is, the shorter is the retention time because the compound will spent more time in the gas phase. The temperature of the column does not have to be above the boiling point because every compound has a non-zero vapor pressure at any given temperature, even solids.

The polarity of the component versus the polarity of stationary phase:

If the polarity of the stationary phase and compound are similar, the retention time increase because the compound are similar, it's increase because the compound interact stronger with the stationary phase. As a result, polar compounds have long retention times on polar stationary phases and shorter retention times on non-polar columns using the same temperature.

Column temperature:

An excessively high column temperature can result in a very short retention time but also in a very poor separation because all the component mainly stays in the gas phase. If the compound does not interact with the stationary phase, the retention time will decrease. At the same time, the separation quality deteriorates, because the differences in retention times becomes not as pronounced anymore.

Carrier gas flow rate:

A high flow rate reduces retention times, but a poor separation would be observed as well. Like above, the components have very little time to interact with the stationary phase and are just being pushed through the column.

Column length:

A longer column generally improves the separation. The trade-off is that the retention time increases proportionally to the column length and a significant peak broadening will be observed as well because of increased longitudinal diffusion inside the column. Amount of material injected: Ideally, the peaks in the chromatogram display show a symmetric shape. If too much of the sample is injected, the peak shows a significant tailing, which causes a poorer separation. Most detectors are relatively sensitive and do not need a lot of material in order to produce a detectable signal.

Microsyringes:

Syringes are used to inject liquid samples through a rubber diaphragm or septum. The sample is heated and maintained at a temperature above the boiling point of the least volatile substance of the compound to the entry in the column. The amount of sample to be analyzed may range from a few tenths of a microliter up to 20 µL . A "splitter" is often necessary since it allows to have knowledge only a fraction of the injected sample, the remaining sample being eliminated.

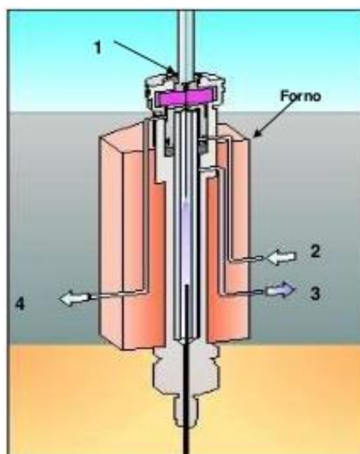


Figure-8 *The sample (1-2 uL) is introduced from above through a syringe(1) arrives in a cylindrical chamber called "liner" where with the effect of the high temperature (200-300 °C) all components evaporate . The resulting gaseous mixture is transferred into the column by the gas flow in transportation.*
Splitless Mode: path 3 is closed and the entire sample is transferred by the gas in (2) to the column. The path 4 is the carrier gas out.
Split Mode: path 3 is open and part of the sample injected is carrying by the gas in (2) to escape through the path 3. Not the entire sample is transferred to the column.

Detectors in Gas Chromatography:

Various detectors can be utilized using GC. The characteristics that should provide an ideal detector are adequate sensitivity, and typically ranging from 10^{-8} to 10^{-15} g solute, good sensitivity and reproducibility, linear response to various solutes, temperature of at least 400 ° C , a short response time; does not destroy the sample. But the perfect detector doesn't exist. In many currently available detectors for gas chromatography, one of the most effective is the mass spectrometer.

1.6 Mass-Spectrometry

Gas-chromatography mass spectrometry (GC/MS) is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample.
In order, a compound to be analyse by GC/MS it must be sufficiently volatile and thermally stable and some of them require a chemical modification (derivatization), prior to analysis, to eliminate unwished absorption effects that, would otherwise affect the quality of the data

obtained. Carry by the Helium, the molecules are retained and then elute by the column at different retention times. In that way mass spectrometer can detect the ionized molecules separately. The samples eluting from the column in the gaseous state, are bombarded and fragmented by an electron source, at this stage the chemical bonds of the sample molecules are broken down and converted into molecular ions , radical and molecules non-ionized which will later be separated from the difference between mass / charge (Ratio). The detector determines the load mass molecular weight in relation to the ions produced in the sample (m/z), the output is amplified to boost the signal. The signal is send to a computer that record all the data produced, converts the electrical impulses into visual displays and hard copy displays. In addition, the computer also controls the operation of the mass spectrometer.

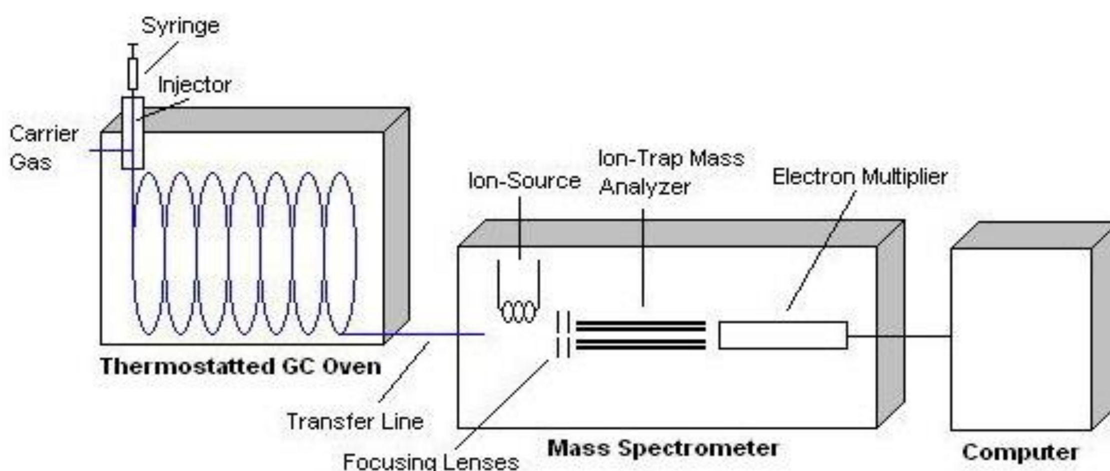


Figure-8 *General scheme of a GC-MS*

The mass analyzer separates the ionized species according to the ratio (m / z). This separation is achieved through the use of magnetic or electric fields sectors. There are a large number of ion separation methods, but the most common are the ion trap and Quadrupole. In this work we used the method ion trap.

Quadrupole Mass Filter

The quadrupole analyzer consists of four parallel metal electrodes arranged in two opposite pairs. A direct current (DC) and Alternated current (AC) are applied to each of the ion pairs. The use of this combination changes the central trajectory of ions. With the change of the potential, only ions with a given ratio m/z will pass through the center of the quadrupole, while others are diverted from the central trajectory. In a particular field, the ions rightly (m/z) set can pass through the quadrupole oscillating following the route, being called as resonant ions, which are those that reach the detector. All non-resonant ions are stopped by quadrupole. If the objective is to obtain a spectrum with all the ratios (m / z), the potential of the direct current and the amplitude of the alternating current are increased and all ions become sequentially resonant thus reaching the detector. The quadrupole analyzers are characterized by easy handling, its robustness and Hit ratios (m / z) values up to 4000 [56][57]. The ion trap is formed by a central electrode (annular shape), and two terminal electrodes, and in the center of the ion trap ions are captured. For greater effectiveness ions must be

located right in the center, an RF potential is being applied to the central electrode, causing ions in a stable trajectory. There are two types of ion trap: three-dimensional quadrupole (dynamic capture) and ion cyclotron resonance (still capture). Both work accumulating ions inside, through the manipulation of alternating current and radio frequency simultaneously. The capture allows a controlled ions release, which permit their separation and control with a high resolution [53].

Ion trap

The ion trap has function and look very similar to the quadrupole, and this is sometimes considered a variant of this , however , it is potentially much more versatile and has undoubtedly a greater potential for development. Once it had a bad reputation because of the very first versions provided inferior results compared to quadrupoles. These results were often dependent on the concentration; samples rather concentrated usually produced several peaks with mass equal to [ion + 1] , making the spectrum unusable for research on commercial archives . Today, the problems have been overcome and the ion trap was found to be more sensitive than quadrupole and able to perform analysis tandem without use of further experimentation.
The designation "ion trap" is considered appropriate because unlike the quadrupole which acts only as a mass filter, the spectrometer under examination can trap ions for a period of time relatively long, with important consequences.

This apparatus consists, generally, of three electrodes: a ring electrode with a surface shaped as a loop with an inner surface hyperbolic, and two cap-shaped electrodes at the two ends (Figures 9).

The ring electrode works with a sinusoidal radiofrequency field (RF), while the electrodes in the cap operate in one of the following three ways: A Potential mass, by means of a DC voltage, or AC.

The mathematics that describes the motion of the ions within the trap is provided by the equation of Mathieu. The great advantage of the ion trap is that by controlling the three parameters of the RF voltages, AC and DC, you can very easily conduct a variety of experiments (for details, see March and Hughes 1989)[53][54].

There are three basic methods by which the ion trap can work. First, when the trap is operated with a constant voltage Rf and no DC bias between the electrodes in the cap and the ring, can expect trapped all ions above a certain threshold value of the ratio m / z. Increasing the tension Rf, the threshold value m / z is incremented in a controlled manner and the ions expelled in sequence and revealed. The result is the classic mass spectrum and this procedure is called mode to "mass-selective instability". The maximum potential Rf which can be applied between the electrodes limits the upper extreme of the range of mass for this mode. The ions of mass over this upper limit are removed after the voltage Rf is reset to zero.

The second mode operation using a DC voltage between the caps; the overall result is that the ions exist for both a threshold value m/z less, both a higher value. The experimental potential of this mode of operation are huge and most of the transactions with ion trap using this mode. It is possible to select even a single mass ion. Control selective ion (SIM) is an important application of this mode of use. There is no practical limit to the number of ion masses that can be selected.

The third mode of operation is similar to the second, with the addition of an auxiliary oscillating field between the electrodes to the cap, so as to increase selectively the kinetic energy of an ion in particular. With an auxiliary field of small amplitude ions selected get kinetic energy slowly and, during this period, generally undergo collisions that produce fragmentation; the result can be an efficient MS-MS close to 100%. If you consider the intrinsic sensitivity of the ion trap MS-MS tandem jointly efficiency close to 100%, the use of the ion trap experiments tandem mass far exceeds the so-called triple quad.

Detector

In most detectors ions are detected after the collision with the surface, resulting in the emission of electrons, photons and other ions that can be measured by load detectors and radiation. Examples of some detectors are Faraday cup, electron multiplier, ion cyclotron resonance, or a multichannel plate (MCP).

1.7 Derivatization

The derivatization is a fundamental step for the analysis of samples to GC, especially for compounds that are poorly volatile or, to eliminate undesirable absorption effects that could affect the quality of data obtained. In particular, in this analysis we used a process that aims to form derivatives of carboxylic acids with good chromatographic properties using an esterification on 3-PBA carboxylic group catalized by an immide.

The procedure is based on two reagents N,N diisopropylcarbodiimide (DIC) and 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP).

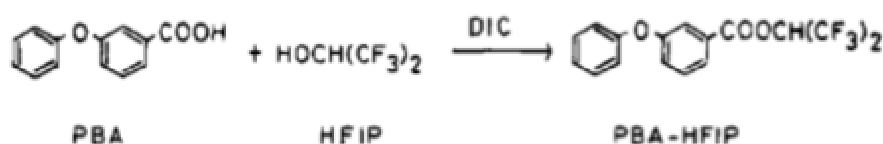


Figure-9 *Derivatization chemical proces : Esterification on 3-PBA carboxylic group catalized by an immide (DIC).*

2. Material and methods

The experimental part of this study includes different phases:

1. Adaptation and Develop a method to extract the compound of our interest (SPE).
2. Optimisation of derivatization procedure.
3. Quantification of 3-PBA level with GC-MS.

The Work started with a bibliographic research to adapt the method at our analisys. After that, was identified the ions ratios m/z and the retention time used to identification the compound in the samples. These were followed by the determination of the calibration curve realized in according with the bibliographic references, to build a measure scale to quantify the amount of the 3-PBA in the samples.

2.1 Reagents

Always in this work was used deionized water (18.2 MΩ/cm) product by Elix apparatus (Millipore, Molsheim, France). The pyrethroid metabolite selected was the 3-Phenoxybenzoic-acid (3-PBA) assay 98%, the 2-phenoxybenzoic acid (2-PBA) assay 98% was used how internal standard, both compound comes from Sigma Aldrich, Madrid®. Derivatization agents: 1,1,1,3,3,3 - Hexafluoroisopropanol (HFIP) assay 99%, N,N diisopropilcarbodiimmide (DIC) assay 99%, both from Sigma Aldrich.

Solvents used in SPE were: Chloride Acid (HCl) assay 37% from Carla Erba Reagents. Rodano Italy; Etil acetate (EtOAc) assay ›99,5% from Sigma Aldrich, France; Ammonium Hydroxide (NH4OH); methanol (CH3OH) from Valente Ribeiro, Belas Portugal e Sodium Hydrogen Carbonate($NaHCO_3$) assay 99,7% product by Riedel-Haen. Other substances were used in this work: Acetonitrile(ACN) by Merck, Darmstadt Germany; Potassium Hydroxide (KOH) assay 87,5 % by José M. Vaz Pereira, Lisboa Portugal 99,7% product by Riedel-Haen and n-hexane by Sigma Aldrich, Germany.

The SPE cartridge used in our optimized extraction method was **Oasis Strata-XC.** It's a stationary phase with area of 800 m^2/g commonly used for biological matrix containing bases of Pka 2-10, so weak bases. It's a mixed-mode, reversed-phase/strong cation exchange, water-wettable polymer. It's formed by a silica base where it was bound a long-carbon chain (Hydrophobic group) and an alkyl or aromatic sulfonic acid (Strong cation exchanger), creating "a double selectivity effect".

2.2 Equipment

The GC analysis were performed on a Thermo Trace Ultra equipment coupled to the detector mass Thermo Polaris working in electron impact mode (EI) at 70 eV, being connected to an acquisition system and the integration of Xcalibur data.

Figure-10 *Gas Chromatography Equipment Trace GC Ultra (1) with detection by tandem mass spectrometer (MS / MS9 with ion- trap MS Thermo Polaris Q (2) coupled to the autosampler AS - I 3000* [57][58].

2.3 Collection and storage of samples

The urine collection was from the morning (the families brought them to the hospital in occasion of blood sampling). An agent took them to the laboratory where the urines were centrifuged at 2500 rmp for 10 min. Subsequently were eliminated any precipitate and the supernatant was aliquoted and frozen at -80°C .

2.4 Analytical Method

The analysis in urine cannot be performed without a prior sample preparation. On one hand, the urine is a complex matrix , and on the other, the metabolite was found in very low concentrations to analytical techniques commonly used. For that, it was necessary to extract the compound from the medium in which they are, in this case urine, concentrating them and removing other compounds that may interfere with the analysis.
The recommended methods for the extraction and concentration of pyrethroids in urine are the solid phase extraction (SPE)[59][60] and liquid-liquid extraction (LLE)[58][53] .

2.4.1 Deconjugation of metabolites

It was added 1 ml of potassium hydroxide to the samples (V= 5 ml, ≈1 mL urine and water ≈4 mL) and then heated in a bath at 70 °C for 15 minutes.[56]

Figure-11 *Heating the sample to deconjugate the metabolites*

2.4.2 Solid Phase Extraction

In the solid phase extraction of metabolites 3- PBA and internal standard, 2- PBA, the procedure consists of three phases: conditioning, the sample passage, washing and elution. On the conditioning phase were used 5 mL of ethyl acetate (EtOAc), 5 mL of methanol (MeOH), 5 ml deionized water and 5 ml 1N HCl.
After conditioning the sample was passed through the cartridge without letting air. After in the washing phase were added 5 ml of 0.1N (HCl) hydrochloric acid, 5 mL of ammonium hydroxide (NH_4OH), 5mL MeOH and 5mL EtOAc. Subsequently was left the vacuum pump on, for about 1 hour to ensure the drying of all the cartridges. Finally, extraction was performed with 5 mL of 5 % MeOH in EtOAc [56] (Figure 12).

Figure-12 *SPE system used with cartridges Strata X-C*

2.4.3 Derivatization

Derivatization is a critical step for determination of pyrethroid metabolites in urine. After the addition of the derivatizing reagent HFIP and DIC , derivatives of carboxylic acids are formed with good chromatographic properties which allow easily be detected in GC-MS (increased sensitivity), while the interfering substances are eliminated[59]. It was did adding 20 µL and 30 µL of DIC and HFIP at the eluate obtained from the SPE , followed by a vortex mixing for 10 minutes.

2.4.4 Liquid Liquid Extraction

In the final phase of the procedure was made a liquid-liquid extraction (LLE) with the goal to obtain a pure non-polar phase of metabolites , which also has the function of neutralizing the excess of derivatizing reagent [59]. Subsequently It was added 1 mL of Natrium hydrogencarbonate in the same vial (150 µL of n-hexane and 50 µL of derivatizing reagent), followed by a vortex mixing during 10 minutes. Using a micropipette were withdrawn 50mL of supernatant (n-hexane) where the metabolites were concentrated, this quantity was transferred in an "insert" placed in a vial. The step is repeated adding a second time other 150 µl of n-Hexane and vortex 10 min.

2.4.5 Chromatography

The table 2 show the GC-MS used parameters.

Table 2- *The Table summarize the most relevant data on the separation and detection of pyrethroid metabolites in urine by GC-MS. In this table are specified type and size of the column, the carrier gas and the detector used, the volume injected , the temperatures of the oven, injector , and detector.*

Column Type	ZLB-XLB
Column Measure	30mm x 0.25 mm (internal diameter) Phenomenex
Column Program	40-260°C (15°C/min)
Temperature Liner	250°C
Carrier Gas	Helium; 1,3 mL/min
Volume Injected	1µL
Detector	Electron Impact, Polaris Q , MS.
Elaboration Program	Xcalibur from Thermo Finigan

3. Results and Discussion

3.1 Population characterization

In this study were analyze urine's samples came from 46 children of ages between 5 and 12 years. This population including 24 autistic children with different levels of CARS (Childhood Autism Rating Scale) and different sex (20 male and 4 female); compared with 12 controls (6 male and 6 female) (Fig. 13).

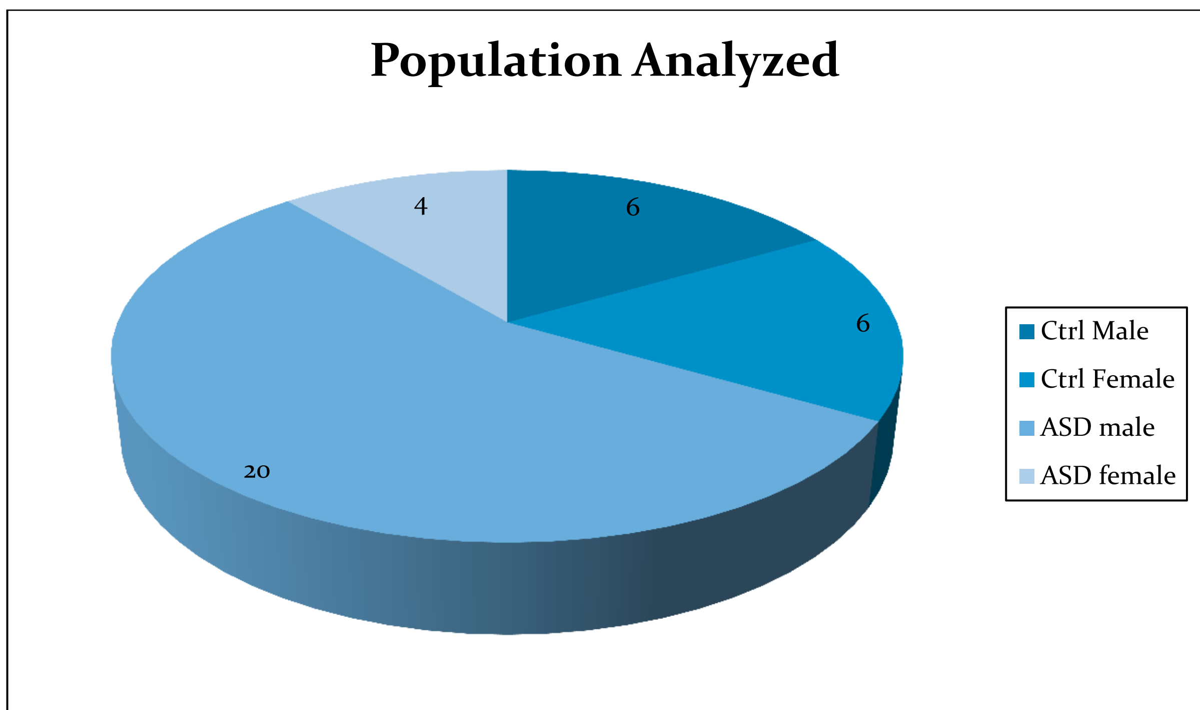


Figure 13- *In this Pie chart is represented the population of children studied divided by gender, control and ASD affected.*

3.2 GC-MS analysis

Initially the analysis is operated in total ion scan mode ("Full scan") m/z, [50-650], a qualitative analysis performed by GC-MS used to identify the spectrum of our compound in interest(3-PBA) and the internal standard (2-PBA).

The molecules of 2-PBA and the 3-PBA are chemically very similar and so the ions selected for the identification of metabolites 2-PBA (Fig-14) and the 3-PBA (Fig-15) are also related. The molecular ion 364 is common to both, and is also the most abundant ion in the 3-PBA compound. The identification of 3-PBA related to the 2-PBA becomes more precise with the identification of ions m/z 134; 140; 169 ; 195; 197.

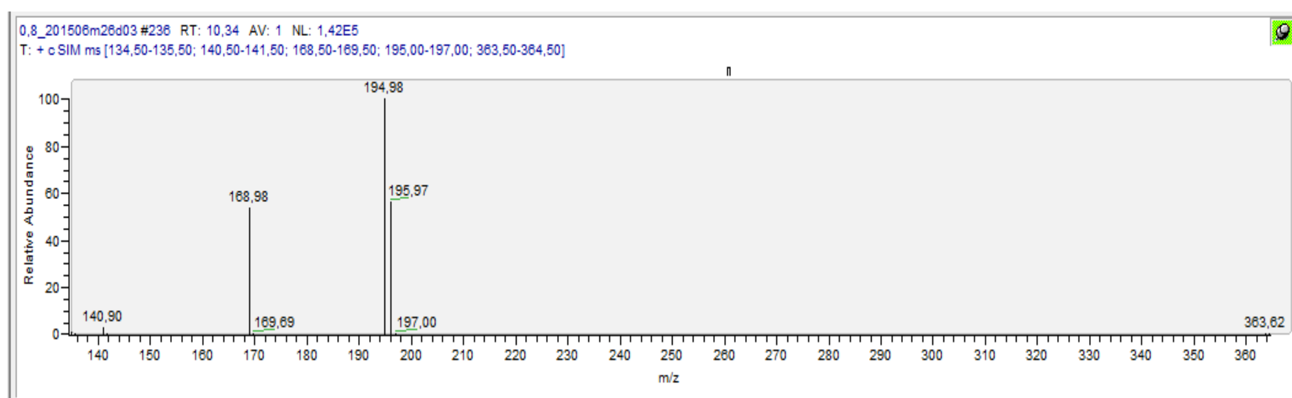


Figure 14-*Mass fragmentation spectrum for 2-PBA (internal standard)*

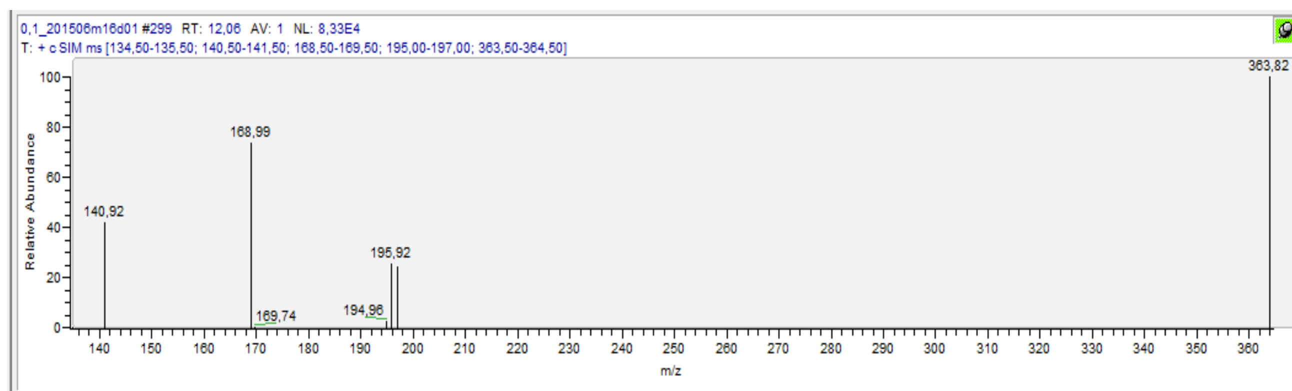


Figure 15-*Mass fragmentation spectrum for 3-PBA*

Subsequently the identification of the mass fragmentation spectrum and the retention time of our compounds, the spectrometer is set in selective ion monitoring mode (SIM) method really useful for quantitative analysis with different concentrations of analytes, so the analysys is displacing in the Peaks Areas interpretation and relative software Xcalibur®.

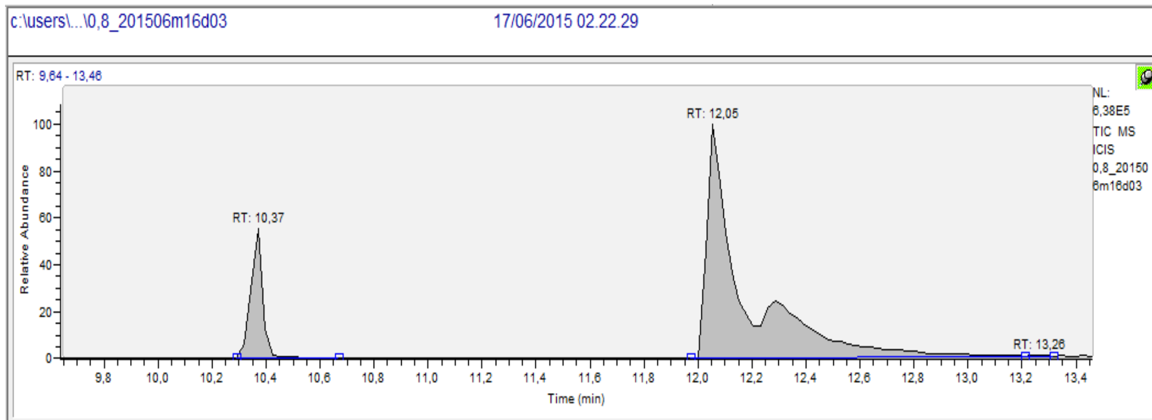


Figure 16- Chromatogram showing at Rt 10.37 min: the 2-PBA peak and at Rt 12.05 min: 3- PBA.

Here the relative intensities of the detected ions are compared with the calibration curve. Technical parameters such as injection conditions, flow and temperature gradients were continuously monitored in order to obtain a better resolution of the chromatographic peaks.

3.3 Calibration curve

This step aims to see the linearity of the method used and also permitted us to give an appropriate valor at the Peaks Areas comes from the samples data analysis. The linear direct calibration is performed with the compounds 2- PBA and 3- PBA. The 2-PBA has internal standard function, the concentration chosen was: 50 µg / L, for a quantity of 200 µL in every sample contamination.
The concentrations chosen for the direct calibration linear with 3-PBA were: 0.1 ; 0.2; 0.5; 0.8; 1.25; 1.5 µg / mL.

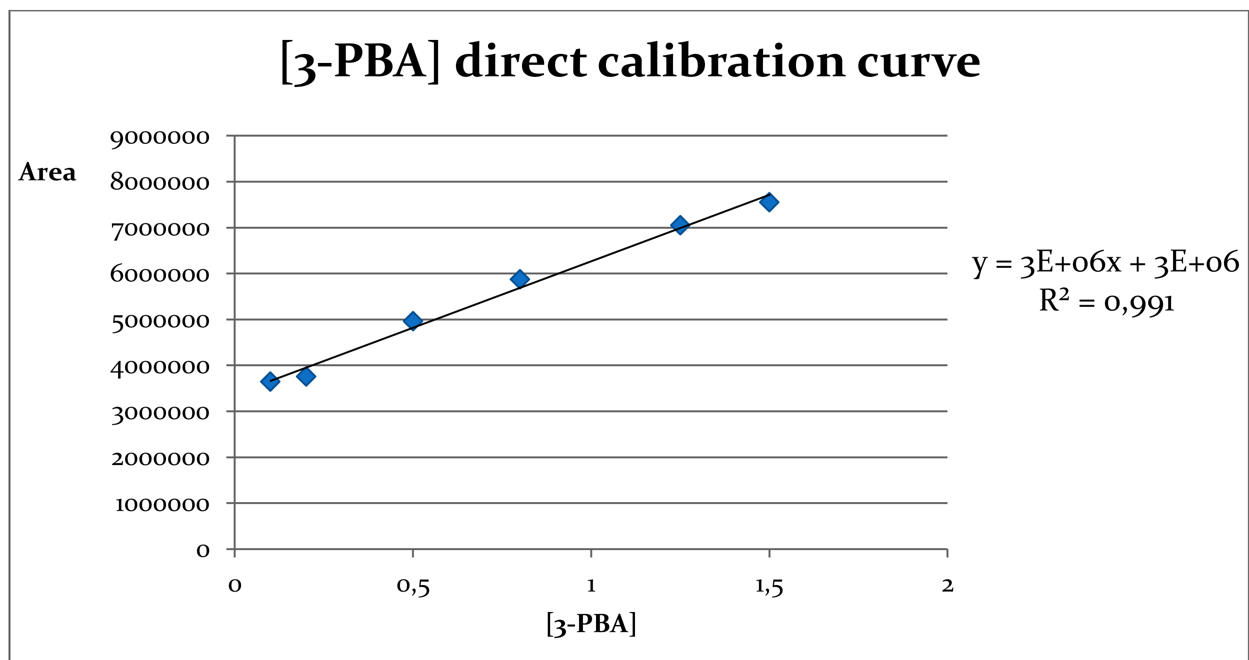


Figure 17- *Direct Calibration Curve.*

3.4 Calibration curve in matrix

After the direct calibration curve, a calibration curve was made "in matrix" consisting in a contamination in the same sample composition, in this case urine. A calibration curve was carried out with 6 points: 0.1 ; 0.2; 0.5; 0.8; 1; 2 µg /mL.
After injection in the GC-MS, was obtained an: $R^2 = 0.9934$

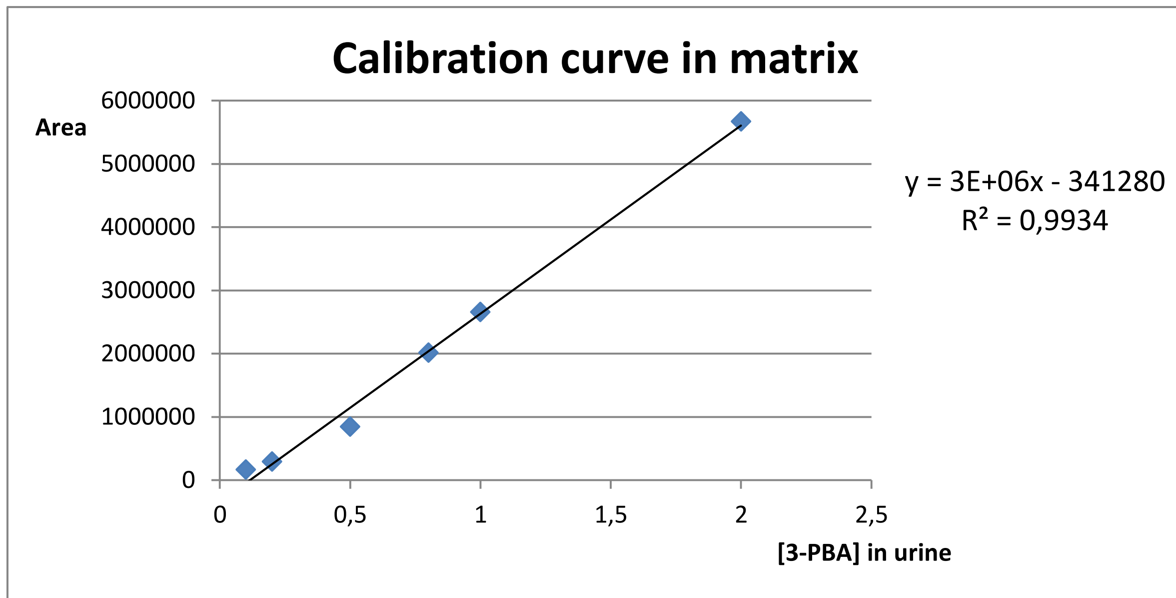


Figure 18- Calibration curve in matrix.

It was observed a Peaks Area variation in the internal standard. To validate our results, was built a linear recovery using the ratio between the peak areas of the 3 -PBA /2-PBA and then compared with the data from urine samples.

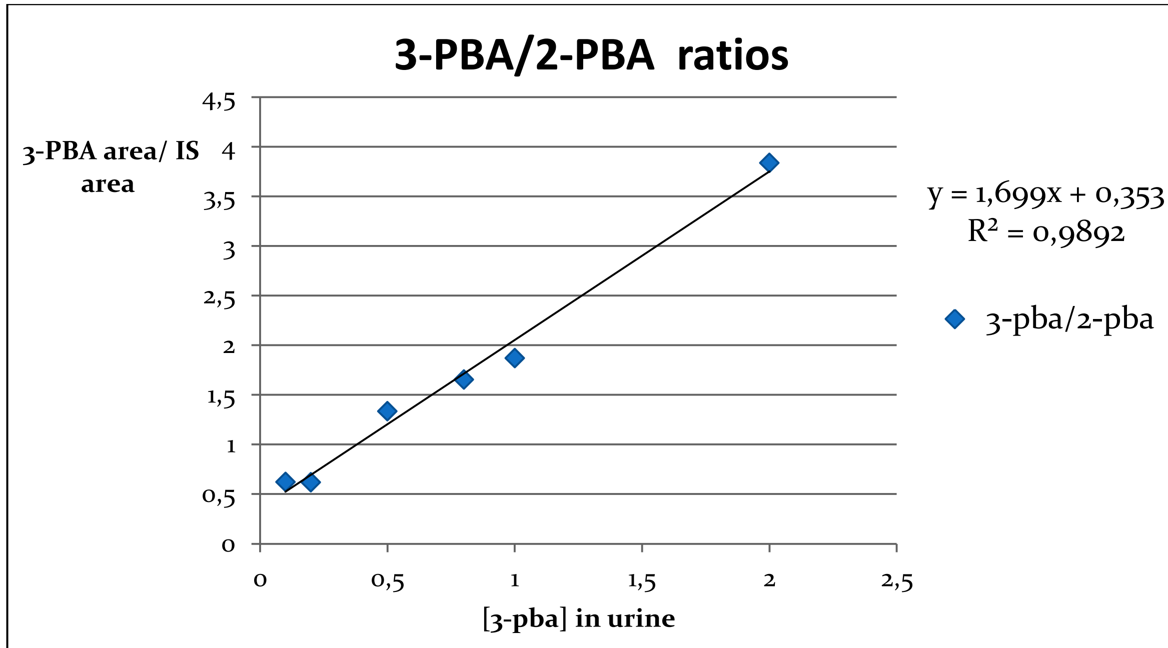


Figure 19- Recta Ratios 3-pba/2pba

The accuracy of the methods developed was assessed by repeatability of parameters for a number of N successive injections, under the same operating conditions, with the calculation of **Relative Deviation Standard (RSD)** expressed as a percentage defined by the following expression (*Equation 1*) :

$$RSD\% = 2^{[1 - 0,5 \log(C)]}$$

Equation 1- (Horwitz Eq.)

During this work we used whenever the average of at least three injections for quantifying a metabolite, eliminating the amount corresponding to one injection when the RSD was greater than 10 %.

The Limit Of Detection (LOD) of the chromatographic method was calculated from the statistical parameters of calibration curves for the expression (*Equation 2*) :

$$LOD = \frac{3 \times (conc.chosen)}{sn} \qquad (2)$$

where sn is the signal / noise corresponding to the concentration chosen in the calibration line.

In this analysis was calculated: **LOD=0.0038 µg/ml**

The Limit Of Quantification (LOQ) in the chromatographic method were calculated from the expression (*Equation 3*):

$$LOQ = \frac{10\times(conc.chosen)}{sn} \qquad (3)$$

where sn is the signal / noise corresponding to the concentration chosen in the calibration curve.

In this analysis was calculated: **LOQ=0.0127 µg/ml**

3.5 Values of 3-PBA in studied population

After calculating the values of LOD and LOQ achieving 0.0038 µg / ml and 0.00127 µg / ml respectively the comparison with the data obtained were performed. 6 samples (3 Autistic and 3 controls) corresponding to 13.04% of our samples had a concentration of 3- PBA in urine < LOD; 11 (5 and 6 Autistic Controls) LOD <[3 - PBA]< 0.5µg /ml corresponding to 23.91 %; the 19.56 % 10 (2 Autistic and 8 controls) had values including 0.5µg/ml <[3 - PBA]< 1 µg / ml; the 36.95 % 17 (12 Autistic and 5 controls) had values of 1 µg /ml <[3 - PBA]< 3 µg / ml; the remaining samples (2 Autistic) had 4.34% [3 - PBA]> 3µg / ml (Figure 20).

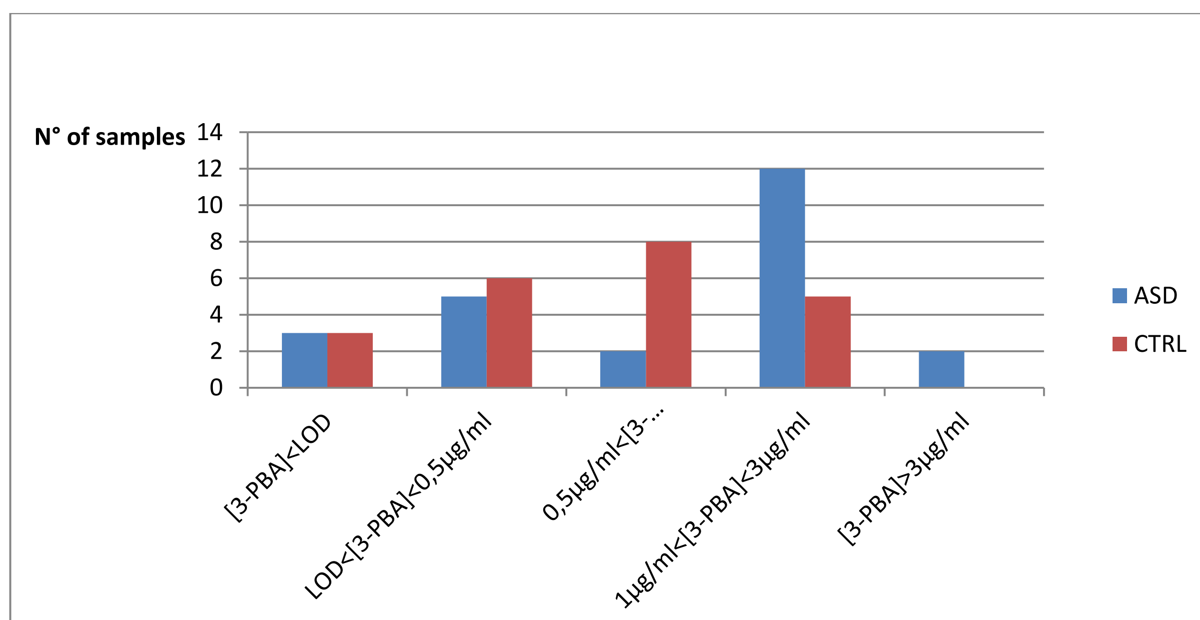
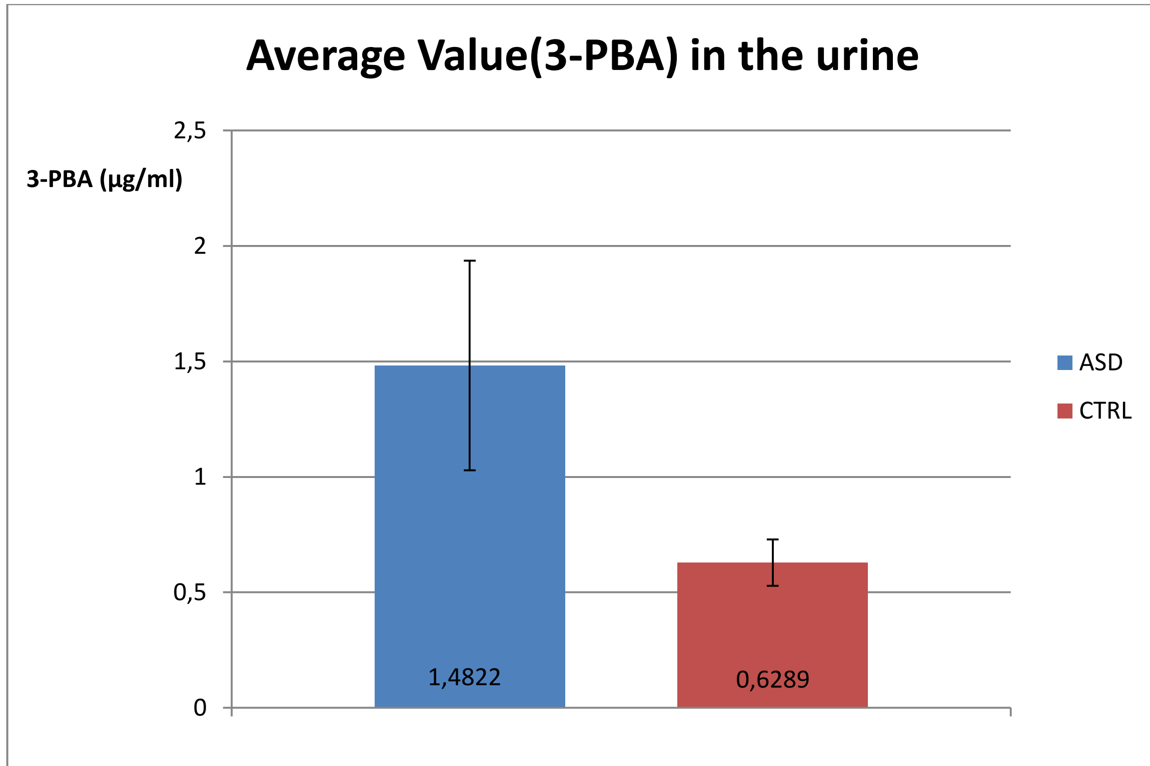


Figure 20- *The picture show the population of samples divided through Autistic and Controls in front of their [3-PBA] in urine. We can notice an important decrease in the number of controls along the increase of the [3-PBA] concentrations*



Picture 21- *Here is summarize the difference through the two populations (ASD/CTRL) through their average [3-PBA]µg/ml concentrations in urine.*

An F-Test Two-Sample for Variances was performed in Excel to the values of the samples (Table 3)

Table-3 *F-Test Two-Sample for Variances*

F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	**1,4822625**	**0,628918182**
Variance	4,**946751486**	**0,221566995**
Observations	24	22
df	23	21
F	22,32621103	
P(F<=f) one-tail	4,2733E-10	
F Critical one-tail	2,063280363	

Taking account the F critical it can be assumed that control and autistic samples has different variances.

A t-Test: Two-Sample Assuming Unequal Variances was executed to understand if controls and autistic have different average values of 3-PBA. (Table 4)

Table 4- *t- Test: Two-Sample Assuming Unequal Variances*

t- Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	1,4822625	0,628918182
Variance	4,946751486	0,221566995
Observations	24	22
Hypothesized Mean Difference	0	
df	25	
t Stat	1,835315137	
P(T<=t) one-tail	**0,039192008**	
t Critical one-tail	1,708140761	
P(T<=t) two-tail	0,078384016	
t Critical two-tail	2,059538553	

Conclusions

The derivatized 3-PBA compound achieved linearity between the concentration and the area of the peak.
The levels of 3-PBA in 36 urine samples were achieved using the matrix calibration curve.
Having thus found a statistical difference between the variances in the two populations and the majority report confirmed by the one-tail t-Test, we can confirm an higher average concentration of 3-PBA in the urine of autistic children compared to controls.

Future Work

In consequence of this results, we should encourage a persistence in the study of this correlation on a more extensive population, involving more parameters to better classify the phenomenon, like: the place of birth, residence, the diet habits, the withdrawal period (Winter / Summer) and any anthelmintic treatments.
In fact, most of these products are insecticides, pesticides and shampoos for pets, skin treatments, head lice and mosquito repellents. All products commonly used in the houses where the children playing habits, the shorter height, and the incomplete metabolic development, especially in the early ages may have a role in the exposition increasing, and the consequently toxicant actions of this compounds.
For that reason it is our duty to know more in depth the toxicity mechanisms about this family of compounds, in order to better protect public health and especially the one of our children.

REFERENCES

1. Organisation. The ICD-10 Classification of Mental and Behavioural Disorders. Clinical Descriptions and Diagnostic Guidelines. World Health Organisation: Geneva, 1992.
2. Kanner L. Autistic disturbance of affective contact. Nervous Child 1943; 2: 217–250.
3. Asperger H. Die 'Autistischen Psychopathen' im Kindesalter. Arch Psychiat Nerven 1944; 117: 73–136.
4. Rutter M. Concepts of autism: a review of research. J Child Psychol Psychiatry 1968; 9: 1–25. | Article | PubMed | ISI | ChemPort |
5. Wing L. Asperger's syndrome: a clinical account. Psychol Med 1981; 11: 115–129. | PubMed | ChemPort |
6. Percy AK, Lane JB. Rett syndrome: clinical and molecular update. Curr Opin Pediatr 2004; 16: 670–677. | Article |
7. Fombonne E. Epidemiological surveys of autism and other pervasive developmental disorders: an update. J Autism Dev Disord 2003; 33: 365–382. | Article | PubMed | ISI |
8. Fombonne E. Epidemiological surveys of autism and other pervasive developmental disorders: an update. J Autism Dev Disord 2003; 33: 365–382. | Article | PubMed | ISI |
9. *"A review of research trends in physiological abnormalities in autism spectrum disorders: immune dysregulation, inflammation, oxidative stress, Mitochondrial dysfunction and environm-ental toxicant exposures"* Rossignol(international child development resource center, Melbourne,Fl,USA and RE Fry Molecular Psichiatry (2012)-17
10. Tomlin, C.D.S. The Pesticide Manual: A World Compendium, 14th ed.;British Crop Production Council: Alton,England,2006;pp 813-814
11. Ferreira MC. Pragas de viveiros florestais indentificação e control. Edição, editor: Editora plàtano edições tecnicas 1999.
12. Leng G. Gries W. Simulataneous determination of pyrethroids and pyrethrins metabolites in human urine by gas-chromatography-high resolution mass spectrometry. J chromatogr B. [article]. 2005 Jan; 814(2): 285-94.
13. Henriques ABOM. Guia de produtos Fitofarmaceuticos com venda autorizada in Portugal. Lisboa 2011
14. Draper WM. Biological Monitoring: exquisite research probes, risk assessment, and routine exposure measurement. Anal chem. (review). 2001 Dec; 45(8):1181-200
15. Chen ZM, Wang YH. Chromatographic methods for the determination of pyrethrin and pyrethroid pesticide residues in crops, foods and environmental samples. J chromatograph A [review]. 1996 Nov; 754 (1-2): 367-95
16. Veja ELS. High-performance liquid chromatography. Horvarth C.,editor: New York: Academic Press; 1980
17. Registration2. Elegibility Decision (RED) for Permethrin; U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substance, Office of Pesticide Programs; U.S. Government Printing Office: Washington, DC, 2007
18. Krieger,R., Handbook of Pesticide Toxycology-Agents, 2nd ed.; Academic Press: San Diego, 2001; Vol.2, pp 1279-1280
19. Anadón A1, Martinez-Larrañaga MR, Diaz MJ, Bringas P. Toxicokinetics of permethrin in the rat. Toxicol Appl Pharmacol. 1991 Aug;110(1):1-8 Toxicokinetics of permethrin in the rat. Anadón A1, Martinez-Larrañaga MR, Diaz MJ, Bringas P.

20. Tordoir at al., 1994 Levels of organochlorine pesticides in the blood serum of agricultural workers from Rio de Janeiro State,Brazil
21. Choi et al. 2002, Human metabolism and metabolic interactions of deployment-related chemicals
22. Casida, 1983 and Crawford et al.,1981 Metabolic Pathways of Agrochemicals: Insecticides and fungicides
23. FAO. Pesticides residue in Food, Toxicological Evalutions; Food and Agriculture Organization Of the United Nations and World Health Organization: Rome, 1999
24. Chen, H., Xiao, G. Hu, J. Zhoun, H. Xiao, and X. Wang. 2002. Journal of Toxicology and environmental health, Part A 65 (19): 1419-1435
25. Hardt and Angerer, 2003 Urinary concentration of metabolites of Pyrethroid insecticides in the general U.S. population: National Health and Nutrition Examination Survey 1999-2002Van der Rhee, H.J.;Farquhar, J.A.; Vermeulen, N.P.E Efficacy and Transdermal Absorption of Permethrin in Scabies Patients. Acta Derm. Venereol. 1989, 69 (2), 170-173
26. Baker, S.E: Olsson, A.O.; Barr, D.B. Isotope dilution high-performance liquid chromatography-tandem mass spectrometry method for quantifying urinary metabolites of synthetic insectisides. Arch. Environ. Contam. Toxicol. 2004, 46,281-288
27. Krieger,R., Handbook of Pesticide Toxycology-Agents, 2nd ed.; Academic Press: San Diego, 2001; Vol.2, pp 1279-1280
28. Huckle KR, Chipman JK, Hutson DH, Millburn P Metabolism of 3-phenobenzoic acid and the enterohepatorenal disposition of its metabolites in the rat.Drug metab dispos. 1981 jul-Aug; 9(4):360-8
29. KÜhn, K.; Wieseler; B.;Leng, G. ; Idel, H. Toxicokinetics of pyrethroids in humans: Consequences for biological monitoring. Bull. Environ. Contam. Toxicol. 1999, 62, 101-108
30. Eil,C. and B.C. Nisula. 1990. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. Journal of Steroid Biochemistry 35:409-414.
31. World Health Organization(WHO 1990.Permethrin.Enviromental Health Criteria 94: 25-26.
32. Toxicol Appl Pharmacol. 2004 Apr 15;196(2):303-18.Effects of pyrethroids on voltage-sensitive calcium channels: a critical evaluation of strengths, weaknesses, data needs, and relationship to assessment of cumulative neurotoxicity. Shafer TJ1, Meyer DA.
33. Douglas A. Skoog, Donald M. West, F. James Holler, Couc SR. Fundamentals of analytical Chemistry. 8 ed: David Harris; 2004
34. Tsumura Y, Wada I, Fujiwara Y, Nakamura Y, Tonogai Y, Ito Y. Simultaneus determination of 13 Synthetic Pyrethroids and their metabolite, 3-phenoxybenzoic Acid, in Tea by Gas-chromatorgraphy. J Agric Food Chem. 1994;42(12):2922-5
35. Leng G, Leng A, Kuhn KH, Leawalter J, Pauluhn J. Human dose-extraction studies with the pyrethroid insecticide cyfluthrin: urinary metabolite profile following inhalation. Xenobiotica. [Article]. 1997 Dec; 27(12):1273-83
36. PAN pesticides Database-Chemicals.

37. Leng G, Leng A, Kuhn KH, Leawalter J, Pauluhn J. Human dose-extraction studies with the pyrethroid insecticide cyfluthrin: urinary metabolite profile following inhalation. Xenobiotica. [Article]. 1997 Dec; 27(12):1273-83
38. Henriques ABOM. Guia do Produtos Fitofarmacêuticos. Lista con Produtos com venda autorizada. Lisboa 2010.
39. [M.J. Hengel, C.R. Mourer, T. Shibamoto. Bull. Environ. Contam. Toxicol.59(1997) 171
40. Blayblock; R. L.,M. Abdel-Nasser, S. M. McCarty, J. A. Knesel, K.M. Tolson, P. W. Ferguson, and H. M. Mehendale. 1995. Suppresion of cellular immune responses in BALBC/c mice following oral exposure to permethrin. Bulletin of Environmental Contamination and Toxicology 54(5):768-77
41. Toxicol Appl Pharmacol. 2004 Apr 15;196(2):303-18.Effects of pyrethroids on voltage-sensitive calcium channels: a critical evaluation of strengths, weaknesses, data needs, and relationship to assessment of cumulative neurotoxicity.Shafer TJ1, Meyer DA.
42. Kasat K., V. Go, and B. G. Pogo. 2002. Effects of pyrethroid insecticides and estrogen on WNT10B proto-oncogene expression. Environment International 28(5): 429-32.
43. BradBerry, S. M.; Cage, S. A. ; Proudfoot, A. T.; Vale, J. A. Poisoning due to Pyrethroids. Toxicol. Rev. 2005, 24 (2), 93-106
44. Satoh and Hokosawa, 1998 The mammalian carboxylesterases: from molecules to functions
45. World Health Organization(WHO 1990.Permethrin.Enviromental Health Criteria 94: 25-26.
46. Eil,C. and B.C. Nisula. 1990. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. Journal of Steroid Biochemistry 35:409-414.
47. KÜhn, K.; Wieseler; B.;Leng, G.; Idel, H. Toxicokinetics of pyrethroids in humans: Consequences for biological monitoring. Bull. Environ. Contam. Toxicol. 1999, 62, 101-108
48. BradBerry, S. M.; Cage, S. A. ; Proudfoot, A. T.; Vale, J. A. Poisoning due to Pyrethroids. Toxicol. Rev. 2005, 24 (2), 93-106
49. Cole et al. 1982; Ruzo et al. 1978 Mechanism of Pyrethroid neurotoxicity: implications for cumulative risk assessment
50. Arch Toxicol. 2012 Feb;86(2):165-81. doi: 10.1007/s00204-011-0726-x. Epub 2011 Jun 28.Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances.
51. Supelco(1998),Guide to Solid Phase Extraction (http://www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4538.pdf) pp.9
52. Veja ELS. High-Performance liquid Chromatography: New York: Academic press 1980.
53. Leng G, Kuhn KH, Idel H. Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: Applications and limitations. Sci Total Environ. 1997 Jun;199(1-2):173-81.
54. Silverstein R. M. WFJ, Kiemle D. J. Spectrometric Identification of organic Compounds. 7 ed: John Wiley e Sons 2005.

55. Susan R. Mikkelsen ECn. BIOANALYTICAL CHEMISTRY. New Jersey: A JOHN WILEY & SONS, 2004.

56. Barr D, Leng G, Berger-Preiß E, Hoppe H-W, Weerasekera G, Gries W, et al. Cross validation of multiple methods for measuring pyrethroid and pyrethrum insecticide metabolites in human urine. Analytical and Bioanalytical Chemistry. 2007;389(3):811

57. Cientific T. GC ultra MS polaris Q. [cited; Available from: http://www.thermoscientific.com/ecomm/servlet/productsdetail?productId=11962236&groupType=PRODUCT&searchType=0&storeId=11152&from=search

58. Leng G, Gries W. Simultaneous determination of pyrethroid and pyrethrins metabolite in human urine by gas-chromagraphy-high resolution mass spectrometry. J. Chromatogr B. [article]. 2005 Jan;814[2]:285-94.

59. Arrebola FJ, Martinez-Vidal JL, Fernandez-Gutierrez A, Akhtar MH. Monitoring of pyrethroid metabolites in human urine using solid-phase extraction followed by gas chromatography-tandem mass spectrometry. Anal Chim Acta. 1999 Nov; 401(1-2):45-54.

60. Colume AC, S; Gallego, M ; Valcarcel, M A solid phase extraction method for the screening and determination of pyrethroid metabolites and organochlorine pesticides in human urine RAPID COMMUNICATIONS IN MASS SPECTROMETRY. 2001; 15(21):2007-13.