INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO
MESTRADO EM ENGENHARIA QUÍMICA




Determination of permethrin and its metabolite in brains and urines of rats by GC-ECD and GC-MS

MARIA CAVALIERE
Julho de 2014

POLITÉCNICO
DO PORTO

Master in Pharmacy

Biochemistry/ Molecular Biology

Determination of permethrin and its metabolite in brains and urines of rats by GC-ECD and GC-MS

Maria Cavaliere

(1131609)

Supervisor (University of Camerino): Rosita Gabbianelli

Supervisor ISEP: Valentina Domingues

Co-supervisors: Luìsa Correia-Sá

2013/2014

Acknowledgement

My first thanks goes to Professor Valentina F. Domingues for her constant availability and kindness, moreover I want to thanks all the staff of the laboratory but in a particular way Luisa Correia-Sá and Paula Paiga.

Abstract:

Synthetic pyrethroids are pesticides derived from naturally occurring pyrethrins, taken from pyrethrum of dried Chrysanthemum flowers. Pyrethroid insecticides are one of the most commonly used residential and agricultural insecticides. Many pyrethroids can significantly harm the nervous system. Permethrin could be one of the factors involved in the onset of neurodegenerative diseases. The present study aims to evaluate in brain, the effect that can induce the exposure to permethrin, during early life of female rats (from 6 to 21 days of life). Therefore, have been examined the concentrations of permethrin and its main metabolite (3-PBA) in the brain and urine in female rats sacrificed the day after and 14 days after treating. The different concentrations of permethrin and 3-PBA (after 24h and after 14 days in the end of treatment) were obtained using two different methods. The evaluation of permethrin by liquid-liquid extraction and GC-ECD was performed. The levels of the 3-PBA (in urine and brains) were obtained by SPE procedure and GC-MS using the 2-PBA as internal standard.

Keywords: pesticides, pyrethroids, permethrin, brain toxicity, 3-PBA (3-phenoxybenzoic acid), 2-PBA (2-phenoxybenzoic acid), SPE, GC-ECD, GC-MS

INDEX

INDEX FIGURES

List of abbreviation, acronyms and symbols

FDA: Food and Drugs Administration

CNS: Central Nervous System

WHO: World Health Organization

VGSC: Voltage-gated sodium channel

DA: Dopamine

NO: Nitric oxide

SOD: Superoxide dismutase

NMDA: N-methyl-D- aspartate receptor

3-PBA: 3- phenoxibenzoic acid

ADH: Alcohol dehydrogenase

ALDH: Aldehyde dehydrogenase

SPE: Solid phase extraction

GC: Gas chromatography

1. Pyrethroids and Permethrin

Synthetic pyrethroids such as, cypermethrin, deltamethrin, permethrin and cyfluthrin originate from the botanical insecticide pyrethrum, an extract obtained from the flowers of *Chrysanthemum cinerariaefolium*. Pyrethrins as one of the natural esters of pyrethrum and the synthetic pyrethroids are among the insecticides most often used worldwide (1). Pyrethrins, a single pesticide active ingredient, contain six components that have insecticidal activity: pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I and jasmolin II (Figure 1).

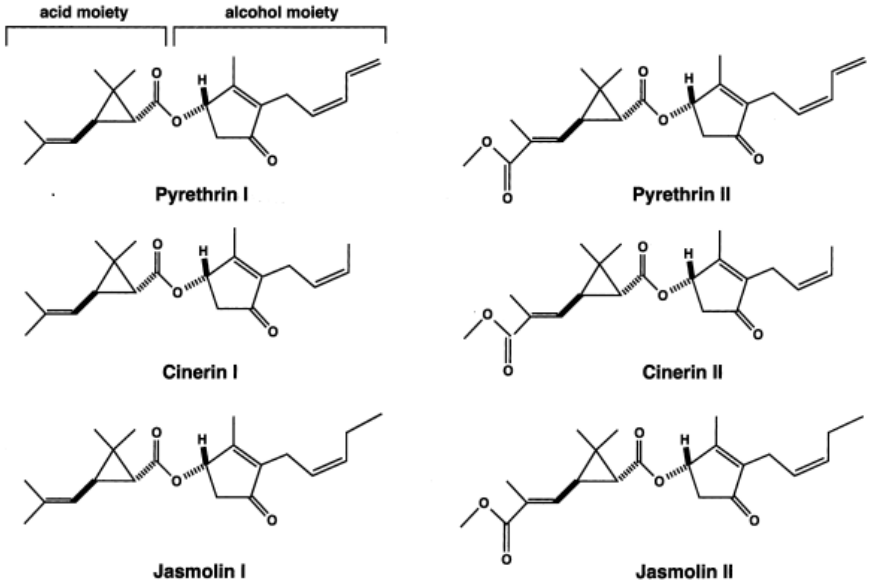


Figure 1: Structures of the six natural pyrethrins.

The insecticidal properties of pyrethrins are derived from ketoalcoholic esters of chrysanthemic and pyrethroic acids. These acids are strongly lipophilic and rapidly penetrate many insects and paralyze their nervous system (2). Both pyrethrins and synthetic pyrethroids are sold as commercial pesticides used to control pest insects in agriculture, homes, communities, restaurants, hospitals, schools, and as a topical head lice treatment. Various formulations of these pesticides are often combined with other chemicals, known as synergists, to increase potency and persistence in the environment. Formulations that are commercially available include aerosols,

dips, emulsifiable concentrates, wettable powders, granules, and concentrates for ultra-low volume applications targeting mosquitoes. While chemically and toxicologically similar, pyrethrins are extremely sensitive to light, heat and moisture. In direct sunlight, half-lives that can be measured in hours. However, the pyrethroids, the synthetic analogues of naturally occurring pesticides, were developed to capture the effective insecticidal activity of this botanical insecticide, with increased stability in light, yielding longer residence times (3). Pyrethroids have replaced organophosphorus and carbamate insecticides because of their low toxicity toward mammals and high insecticidal activity.

Permethrin was the first synthetic pyrethroids photostable enough to be used in agriculture and the most used pyrethroids in USA. Permethrin is an insecticide in the pyrethroids chemical family (Figure 2).

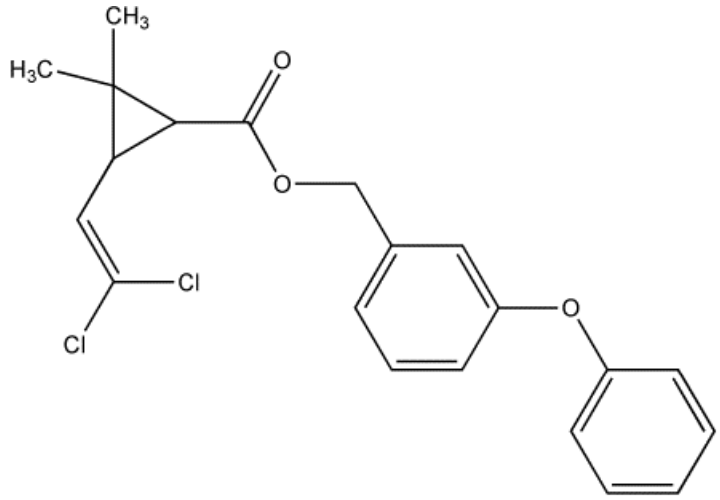


Figure 2- Permethrin structure

Permethrin was synthesized by replacing the methyl groups with chlorine atoms in the acid side-chain, which block photochemical degradation on the adjacent double bond. Permethrin has 4 isomers; the trans-isomer degrades more rapidly than the toxic cis-isomer and they have a lower toxicity in rats than do the corresponding cis-isomers (Figure 3).

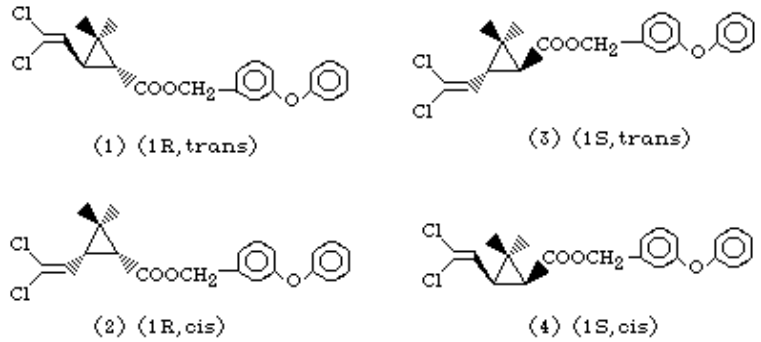


Figure 3: Isomers of permethrin

Permethrin is used as an insecticide or as an insect repellent or insect screen

- in agriculture, to protect crops
- in agriculture, to kill livestock parasites
- for industrial/domestic insect control
- in timber treatment
- as a personal protective measure (cloth impregnant, used primarily for US military uniforms and mosquito nets)
- in pet flea preventative collars or treatment

Formulations of Permethrin are considered pharmaceuticals, which are regulated by the United States Food and Drug Administration (FDA) (4). Permethrin comes in many forms: sprays, dusts, fogs, emulsifiable concentrates and creams. Like all the pyrethroids, the permethrin belong to a group of chemicals that are neurotoxic and share a similar mode of action that is distinctive from other classes of insecticides. There are several ways that the permethrin can enter the body of an organism to exert their effects. The first mode is non-stereospecific with rapid penetration through the epidermis, followed by uptake by the blood or hemolymph carrier proteins and subsequent distribution throughout the body. Pyrethroid diffusion along the epidermis cells is the main route of distribution to the central nervous

system (CNS) after penetration (5). Pyrethroids also can enter the CNS directly via contact with sensory organs of the peripheral nervous system. The sensory structures of both invertebrates and vertebrates are sensitive to pyrethroids (6). Pyrethroids can also enter the body through the airway in the vapour phase, but such penetration represents only a small contribution due to the low vapour pressure of pyrethroids. Pyrethroids can also be ingested, and penetration into the blood-hemolymph through the alimentary canal can play an important role in toxicity. Because these insecticides are relatively non-volatile, the primary source of exposure is believed to be through diet. Additional exposure via ingestion of contaminated household dust may occur after the indoor application of pesticides such as Permethrin that is the main Pyrethroids used in the home for indoor pest control, in pet shampoos and treatment for wood furniture (7).

1.1. Mode of action of pyrethroids:

Pyrethroids have been classified toxicologically into two subclasses based on the induction of either whole body tremors (T syndrome) or a coarse whole body tremor progressing to sinuous writhing (choreoathetosis) with salivation (CS syndrome) following near-lethal dose levels in both rats (*Rattus norvegicus*) and mice (*Mus musculus*), and closely follows the chemical structure of the two types of pyrethroids (8,9). Type I pyrethroids (Pyrethroids lack a cyano group at the “carbon of the 3-phenoxybenzyl alcohol moiety”) are characterized by the T -syndrome which consists of aggressive sparring, sensitivity to external stimuli, fine tremors progressing to whole body tremors and prostration. Type I pyrethroids also elevate core body temperature, which is attributed to the excessive muscular activity associated with tremors. Type II pyrethroids (pyrethroids have a cyano group at the “carbon of the 3-phenoxybenzyl alcohol moiety”) are characterized by the CS syndrome which is comprised initially of pawing and burrowing behaviour followed

by profuse salivation, choreoathetosis, increased startle response, and terminal chronic seizures. Type II pyrethroids decrease core body temperature, which is attributed to excessive salivation and wetting of the ventral body surface. There are a few pyrethroids that produce mixed signs, including both tremors and salivation, and these are classified accordingly as type I/II (10).

Multiple lines of evidence show that pyrethroids, as a class, do not act in a similar fashion on the voltage-gated sodium channels, and the classifications of toxicology are not absolute for either invertebrates or vertebrates (11, 12). Increasing the dose levels of pyrethrins and pyrethroids results in a proportional increase in motor activity, which is the classic dose-response effect with respect to neurotoxic substances. Pyrethroids and permethrin act very quickly to produce symptoms of lost coordination and paralysis which are known as "the knockdown effect", and which are often accompanied by spasms and tremors that induce intense repetitive activation in sense organs and in myelinated nerve fibres. The spasms can be violent and can cause the loss of extremities, such as legs and wings in insects. Physiological and biochemical studies, of pyrethroids show that in both vertebrates and invertebrates the primary mode of action is the binding of the voltage-gated sodium channel (6, 12-13). Mammals, unlike insects, however, have multiple iso-forms of the sodium channel that vary by tissue type, as well as biophysical and (14) pharmacological properties. Type I pyrethroids modify the sodium channels such that there is a slight prolongation of the open time, which results in multiple long action potentials. Type II pyrethroids significantly prolong channel open time, resulting in an increased resting membrane potential and often inducing a depolarization-dependent block of action potentials. Type I pyrethroids cause multiple spike discharges, while type II pyrethroids cause a stimulus-dependent depolarization of the membrane potential which reduces the amplitude of the action potential, and a loss of electrical excitability in both vertebrates and

invertebrates (6, 15). The toxic action is exerted by preventing the deactivation or closing of the gate after activation and membrane depolarization. This results in destabilizing the negative after potential of the nerve due to the leakage of Na^+ ions through the nerve membrane. This causes hyperactivity by delaying the closing sodium channels which allows a persistent inward current to flow after the action potential, causing repetitive discharges that can occur either spontaneously or after a single stimulus. The differences between type I and II pyrethroids are expressed in the motor nerve terminals, where type I cause presynaptic repetitive discharges, and type II cause a tonic release of transmitter indicative of membrane depolarization (16, 17). Type II pyrethroids are a more potent toxicant than type I in depolarizing the nerves (17). Type II pyrethroids are associated with faster activation-deactivation kinetics on the Na^+ sodium channels than type I pyrethroids in vertebrates (12). The higher toxicity of type II pyrethroids is mostly attributed to the hyper-excitatory effect on the axons which results from their stronger membrane depolarizing action. Type I pyrethroids modify the sodium channels in the closed state, while type II pyrethroids modify the open but not inactivated sodium channels (18). However, this relationship does not always hold true; for example the cis-permethrin interact with both closed and open sodium channels, but bind with greater affinity to the open state (19-21) (Figure 4)

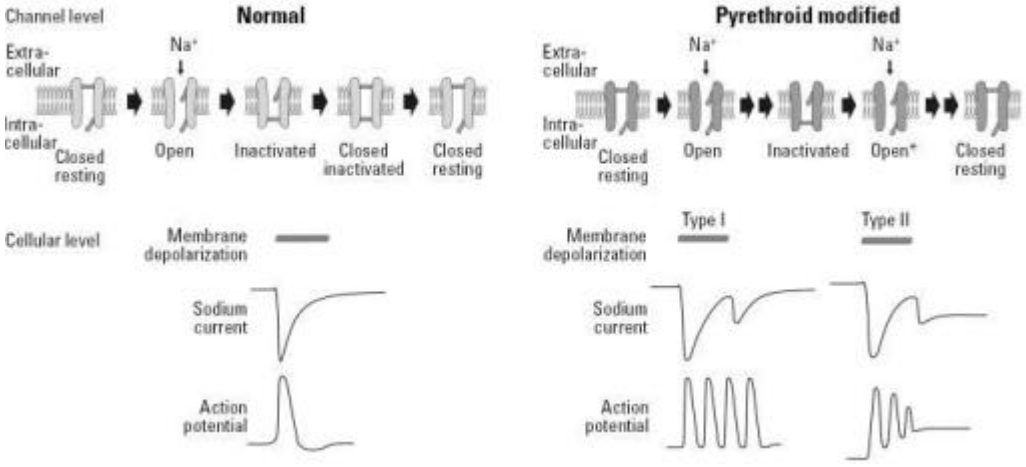


Figure 4- Pyrethroid effects on neuronal excitability.

Pyrethroids affect the voltage-sensitive calcium channels, y-aminobutyric acid (GABA) receptors and GABA-activated channels, and voltage-sensitive chloride channel (22, 23). Type II pyrethroids are more potent enhancers of Ca^{2+} influx and glutamate release under depolarizing conditions than type I pyrethroids (11, 19). The GABA receptor-chloride ionophore complex is also a target of type II pyrethroids. GABA is an inhibitory transmitter in the synapse of the CNS of both vertebrates and invertebrates. Pyrethroids inhibit the Ca-ATPase, Ca-Mg ATPase neurotransmitters and the peripheral benzodiazepine receptors, (24) but their action on these sites is minor compared with the voltage-gated sodium channels. The effects on these (22) sites could, however, enhance the uncontrolled convulsions and tremors. These changes in synaptic transmission may alter neuronal function and may contribute to toxicity and neurodegenerative pathology (24).

1.2. Health effect and toxicity of permethrin

The environmental presence of this pesticide as well as of other insecticides and fungicides has been correlated with neurological disorders such as Alzheimer's disease, Parkinson's disease or dementia in old age (25). Furthermore previous studies showed that Permethrin treatment in rats induced oxidative stress, immune system imbalance, cardiac and neuronal damage and cognitive deficits (16). The LD50 (the lethal dose that kills 50% of a population of test animals) for Permethrin is variable, ranging from 430 mg/kg body weight to over 4000 mg/kg for rats (26). Permethrin induces neurotoxicity, mutagenicity, cancer, immune system effects, cardiac toxicity and other effects. At relatively high doses, neurotoxic symptoms in mammals include tremors, loss of coordination, hyperactivity and an increase in body temperature (26). Permethrin leads to neuronal cell death in various parts of the brain, which could lead to motor deficits, learning and memory dysfunction. Ingestion of even small doses of Permethrin reduces the ability of immune system

cells to recognize and respond to foreign proteins. Doses equivalent to 1/100 of the LD50 inhibited T-Lymphocytes and Natural Killer (NK) cells by over 40% (27). Permethrin affects both male and female reproductive systems too; it binds to receptors for androgen (28). Permethrin was found to have mutagenic effects; in the human cell cultures, and caused an increase in chromosome aberrations, chromosome fragments and DNA lesions (26). Moreover, the World Health Organization (WHO) reported that permethrin increased the frequency of lung tumors in female mice, increasing the expression of a gene involved with the proliferation of cells in mammary gland (29). Permethrin was shown to increase the risk of prostate cancer in men with a family history of prostate cancer (30).

1.3. Brain toxicity:

Epidemiologic evidence suggests that environmental factors are an important cause of neurodegenerative diseases and psychiatric disorders (31). Contaminants accumulated through the food chain and environmental exposure can modify gene expression changing protein synthesis in different tissues (32). Pesticides are identified as one of the risk factors involved, through epigenetic mechanisms, in the regulatory processes controlling gene expression leading to the initiation and progression of age-related diseases (32). Even low levels of pesticides may negatively affect the brain inducing loss of neurons that leads to cognitive decline, impaired memory and attention, and motor function (31).These neuro behavioral disturbances may be associated with neurological disorders such as Alzheimer's disease, Parkinson's disease or dementia in old age (31). Permethrin is able to induce impairment of striatal mitochondrial function, changes in the immune system and oxidative stress in adult rats treated sub-chronically with a low dose of this pesticide (33). This pesticide could be one of the factors involved in the onset of neurodegenerative diseases, the results are discussed considering the variations in

the levels of neurodegenerative markers in cerebral areas, in plasma and in leukocytes. A developing brain is much more susceptible to the toxic effects of chemicals than an adult brain. This vulnerability period extends from fetal development through infancy, childhood and adolescence. The biological effects of pyrethroids are in part caused by their ability to alter neuronal activity since they interact with specific binding sites of voltage-gated sodium channel (VGSC) (VGSCs are the primary molecular targets for pyrethroids insecticidal activity and their acute neurotoxicity in vertebrates) (34) slowing the rate of VGSC closing, prolonging the inward sodium conductance and then shifting the membrane to more polarized potentials (35). A secondary consequence to cell membrane depolarization is an increased Ca^{2+} influx into the neurons through voltage-gated calcium channel (VGCC) that contributes to impact neuronal synaptic plasticity of neurons. Lower Dopamine (DA) levels and accelerated DA turnover following early life permethrin treatment was also observed (35). Midbrain dopamine (DA) neurons are formed in the ventral tegmentum during development. DA cells have been extensively studied, mostly because of their critical involvement in disorders such as Parkinson's disease and schizophrenia. The pesticides may negatively affect the brain inducing loss of neurons that leads to cognitive decline, impaired memory and attention, and motor function (35). Recently, showed that when permethrin was administered during early life (from 6th to 21st day), alterations in striatum (36), in heart and in plasma (37), were observed during adult age. The dopamine turnover and oxidative stress measured in adult rats treated with a low dose of permethrin during early life (1/50 LD50, from 6th to 21st day of life) (35), can be associated with other signs characteristic of neurological disorders. With this aim, the levels of Nurr1, a transcription factor essential for the maintenance of dopaminergic neurons, and other markers correlated with neurodegeneration such as glutamate, calcium, nitric oxide (NO), superoxide dismutase (SOD) were evaluated. Some results show that

early life permethrin treatment reduces Nurr1 mRNA and protein expression as well as calcium and NO levels in striatum from adult rats. An imbalance in glutamate, calcium and NO levels was measured in hippocampus from treated rats, while no changes in prefrontal cortex were detected. Calcium levels measured in leukocytes from treated rats resulted decreased, as well as calcium and SOD in plasma, in which increased NO levels were found. The hypothesize is that the intake of this pesticide could be one of the factors involved in the onset of neurodegenerative diseases, the results are discussed considering the variations in the levels of neurodegenerative markers in cerebral areas, in plasma and in leukocytes. Nurr1 was chosen because it is a nuclear transcription factor regulating the development and maintenance of dopaminergic neurons, in limbic areas, in the ventral DA neurons, and in cortical areas, where its distribution overlaps with DA containing neurons (38). It is important for neuronal plasticity and has been reported decreased in PD as well as modified in other neuronal disorders (39). Nurr1 can modulate the NF-kB ("nuclear factor kappa-light-chain-enhancer of activated B cells") activity in brain microglia, and since the effect of permethrin treatment can trigger an oxidative stress imbalance on redox systems (40), NF-kB and Nrf2(NF-E2-related factor 2), two of the main transcription factors regulating the genes involved in pro-inflammatory and anti-inflammatory responses respectively, were studied. Moreover NF-kB is even modulated by calcium; in fact this element is a common second messenger whose concentration within a cell is finely modulated to promote many physiological functions as hormonal release, muscular contraction, and gene expression (41). Calcium is linked with membrane polarization and therefore related with neuronal homeostasis and brain senescence (42). In neurons the calcium channels function is depending on their position, at presynaptic level, they modulate the neurotransmitters release, while at post-synaptic level they can modulate learning and memory processes (43), in fact an increase in the luminal level of calcium is

correlated with the learning and memory deficits that occur during the onset of Alzheimer disease (43). Moreover, together with the up regulation of dopamine synthesis and the presence of α-synuclein aggregates, high cytoplasmic Ca^{2+} levels led to selective death of substantia nigra dopaminergic neurons, causing the typical motor deficits of Parkinson's disease. Neurotransmitters as glutamate induce an influx of calcium by receptor-operated channels such as NMDA receptors (N-methyl-D-aspartate receptor) at post-synaptic sites (43) where an abnormal release of glutamate causes their over-activation that occurs in excessive accumulation of intracellular calcium. NMDA receptor anchored to NO synthase modulate the production of nitric oxide (NO), a neural modulator involved in neurotransmitters release, neuronal excitability, learning and memory (44). Therefore nitric oxide overproduction due to glutamate triggered toxicity, together with the NO released by astrocytes and microglia with response to inflammatory stimuli, contribute to the oxidative damage reported in post mortem studies, in vitro and in vivo Parkinson's disease models (45) and also in the neuropathology of Alzheimer disease (46).

2. Permethrin kinetics

2.1. Absorption:

Absorption of pyrethroids through the gastrointestinal tract and the skin is variable and depends on the vehicle of administration. For the general population the main route of exposure to agricultural pesticides is through residues in food, studies carried out on workers suggest that, similarly to any other pesticide, dermal exposure is the most significant route of absorption for agricultural applicators and sprayers in fact minimally it is absorbed through intact skin (studies involving skin applications of Permethrin indicated that about 0.5% of the applied dose was absorbed dermally) (47). However the penetration of pyrethroids into the skin is slow and may cause a typical local paraesthesia (tingling and burning sensations),

which may persist for several hours. Can be absorbed even from the gastrointestinal tract and by inhalation of dust and spray mist. Animal studies showed quick and substantial absorption of Permethrin upon ingestion (48). In one study of rats, about 60% of the orally administered dose was absorbed with an absorption half-life of less than one hour (49).

2.2 Distribution:

After absorption, pyrethroids are rapidly distributed throughout the body, mainly in the adipose tissue, stomach, intestine, liver and kidneys and the nervous system. Peak concentrations measured in plasma, nerve tissue, liver, and kidneys occurred around four hours after ingestion. Levels found in nervous tissues were generally higher compared with in plasma (50). A human case study involving an intentional ingestion of Permethrin showed a similar distribution pattern with a peak Permethrin concentration found in blood three to four hours after ingestion (51).

2.3. Metabolism:

Several studies done using rats, goats, cows, and hens, have demonstrated that the Permethrin were metabolized quickly in the liver. Hydrolysis, hydroxylation, oxidation, and conjugation are all involved in the process of metabolism (48) (52). Permethrin itself is considered the only compound of toxicological significance (48) (53) even if were identified several metabolites of permethrin. Permethrin is easily hydrolysed, and the hydrolysis product is also easily oxidized in rats in vitro. Permethrin is converted to PBAlc (3-Phenoxybenzyl alcohol) and further to PBAld (3-phenoxybenzylaldehyde) and 3-phenoxybenzoic acid (3-PBA) 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 (50). The cis-isomer of permethrin, which is metabolized

predominantly by P450's in laboratory animals (50), was not detectably metabolized in human liver microsomes in the presence or absence of an NADPH regenerating system (52). Oxidations of PBAlc and PBAld are mainly mediated by mixed function oxidase, in addition to ADH (alcohol dehydrogenase) and ALDH (aldehyde dehydrogenase), which were shown to contribute to the oxidation by Hodson and co-workers (2002; 2003). The alcohol generated from permethrin, PBAlc is oxidized to PBAld, and the aldehyde is further oxidized to 3-PBA by rat liver microsomes. The alcohol and aldehyde moieties of pyrethroids are mainly metabolized by oxidation to the corresponding aldehydes and carboxylic acids by ADH and ALDH in mammalian species (52). The terminal phase of pyrethroids metabolism is the formation of glucuronide and glycine conjugates (54). These pathways appear similar between laboratory animals and humans. Common metabolites are found in the urine of both laboratory animals and humans (53, 54). The 3-PBA metabolite is common to 10 of the 18 pyrethroids registered in the United States, including permethrin, cypermethrin, deltamethrin, esfenvalerate (55). Both type I and type II pyrethroids undergo ester hydrolysis which results in a cyclopropyl acid and either 3-phenoxybenzyl alcohol (type I) or a cyanohydrin (type II) (56). The aromatic alcohol is further oxidized to 3-PBA while the cyanohydrin is able to spontaneously rearrange to the phenoxybenzaldehyde in aqueous solutions and then undergo hydroxylation to 3-PBA. 3-PBA is a nonspecific urinary metabolite, meaning that it is a breakdown product common to most of the pyrethroids with the exception of cyfluthrin. It is also the most frequently detected urinary metabolite in humans (Figure 5). Since 3-PBA is a common metabolite for most pyrethroids pesticides, it has been used as a marker of pyrethroids exposure by the Center for Disease Control and Prevention in the National Health and Nutrition Examination Survey study (57). The elimination half-life of about 8 h

reported for 3-PBA among workers exposed to cypermethrin (58) suggests that 88% of the metabolite is excreted within the first 24 h following exposure.

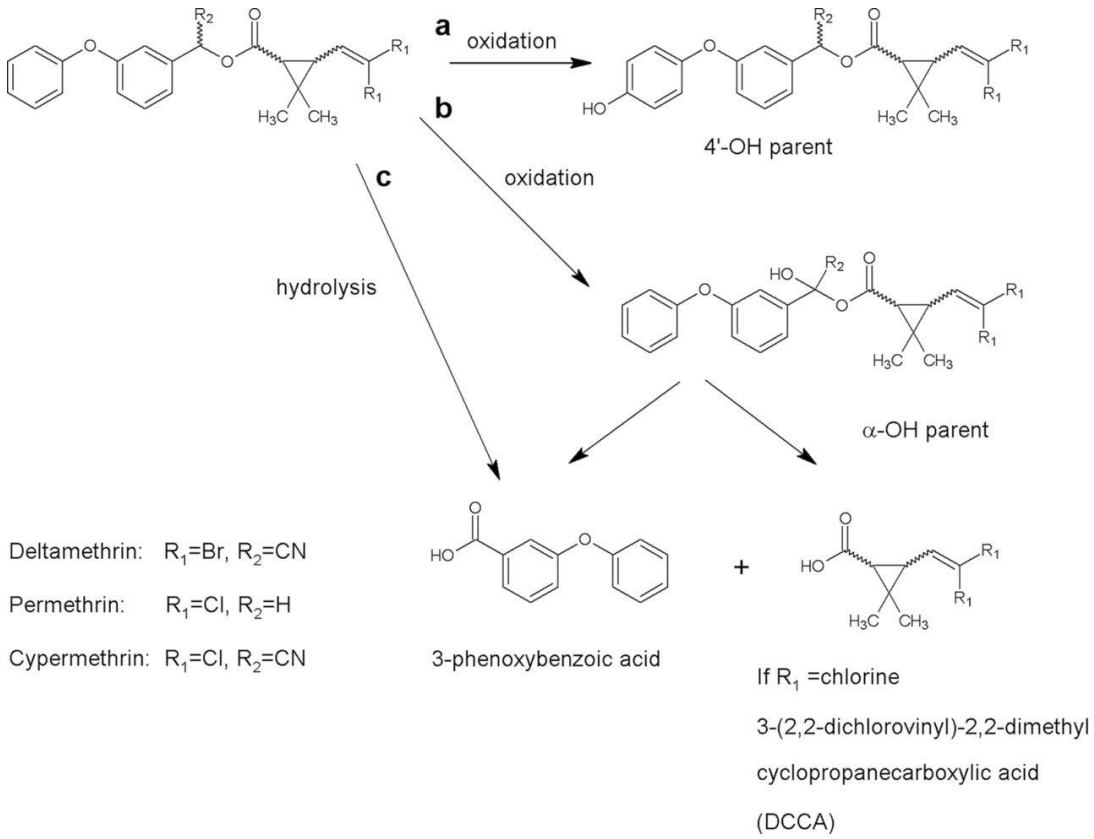


Figure 5: Metabolic clearance of permethrin [1RS, 3RS:1RS, 3RS (*cis: trans*)], cypermethrin [*RS*- α-cyano; 1*RS*, 3*RS*; 1*RS*, 3*SR* (*cis: trans*)], or deltamethrin-[1R; *cis*; alpha S]. Oxidation via cytochrome P450 yields hydroxyl derivatives (a) of the parent compound (e.g., 4′-hydroxy permethrin). Hydrolysis (c), via carboxylesterases, yields a dichlorovinyl acid (permethrin, cypermethrin) or dibromovinyl acid (deltamethrin) and phenoxybenzyl alcohol. An important pathway for urinary metabolite of *cis* isomers oxidation leads to ester cleavage products following hydroxylation at the carbon proximal to the ester (b).

2.4. Excretion:

Permethrin and its metabolites are excreted primarily in the urine, but also in the feces (48, 59). In rats given oral doses of permethrin, the excretion half-life was measured at 12.3 hours for plasma and from 9 to 23 hours for brain and nervous

tissue, including the medulla oblongata (60). 3-PBA is the main metabolite of the permethrin and other pyrethroids resulting from the oxidation of the 3-phenoxybenzyl alcohol. Its concentration in the urine is the most representative marker for pyrethroids exposure in rodents (61) and humans (62).

3. Materials and methods

3.1. SPE (Solid-phase extraction) Method Development

Extraction methods:

Solid-phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution. It is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s) in the sample. It is a fast method, easy to handle for analysis in a variety of domains:

The common SPE applications are:

- Pharmaceutical compounds and metabolites in biological fluids;
- Drugs of abuse in biological fluids;
- Environmental pollutants in drinking and waste water;
- Pesticides and antibiotics in food/agricultural matrices;
- Desalting of proteins and peptides;
- Fractionation of lipids;
- Water and fat soluble vitamins

The SPE process basically consists in four different steps: conditioning, sample addition, washing and elution (63) (Figure 6).

In general, SPE can be used for three important purposes in up-to-date analyses:

- Concentration of the analyte;
- Removal of interfering substances;
- Changing the matrix of the analyte as needed for subsequent analyses

In most cases these three effects occur together.

SPE uses the affinity of solutes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase. The portion that passes through the stationary phase is collected or discarded, depending on whether it contains the 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 with an appropriate eluent.

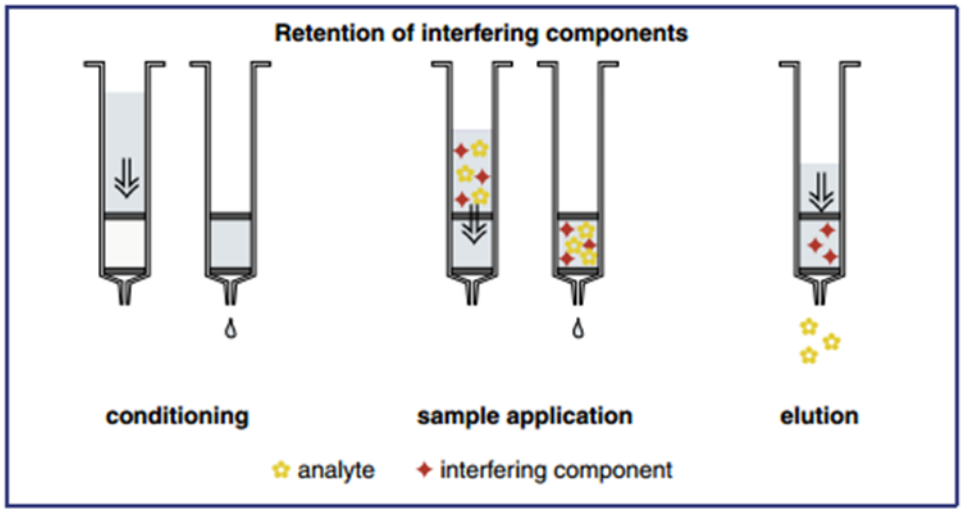


Figure 6: Representation of SPE clean-up procedure

The stationary phase comes normally in the form of a packed syringe-shaped cartridge 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 of samples to pass through them simultaneously. 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. The analytes are collected in sample tubes inside or below the

manifold after they pass through the stationary phase. Solid phase extraction cartridges and disks are available with a variety of stationary phases, each of which can separate analytes according to different chemical properties. Most stationary phases are based on silica that has been bonded to a specific functional group. Sorbent selection requires consideration of sample volume, the nature of the analyte, analyte concentration and the inherent properties of the sorbent itself. Silica and polymers are the most popular sorbents used for SPE. Polymer loading capacities are higher than silica sorbents; however, silica sorbents exhibit greater selectivity. During the drying step, the risk of oxidation of the analytes could be minimized by the addition of anti-oxidant agents, such as ascorbic acid (64). In addition, sodium chloride (NaCl) can be added to the sample to improve the extraction efficiency (65). SPE cartridges should not be allowed to dry at any point during conditioning and sample loading, in order to keep the sorbent ligands active and prevent air from trapping in the cartridges. A comparison between automated SPE-GC-ECD with non-automated SPE-GC-ECD in the extraction of some pyrethroids from surface water showed that automated SPE could be used successfully for extraction and pre-concentration of pesticides (66).

3.1.1. Normal Phase SPE procedure (Non-polar Sample Matrix)

A typical solid phase extraction involves four basic steps. First, the cartridge is equilibrated with a non-polar solvent or slightly polar, which wets the surface and penetrates the bonded phase. Then water, or buffer of the same composition as the sample, is typically washed through the column to wet the silica surface. The sample is then added to the cartridge. As the sample passes through the stationary phase, the analytes in the sample will interact and retain on the sorbent while the solvent, salts, and other impurities pass through the cartridge. After the sample is loaded, the cartridge is washed with buffer or solvent ton remove further

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 silicas 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 (67).

3.1.2. Reversed phase SPE (Aqueous Sample Matrix)

Reversed phase SPE separates analytes based on their polarity. 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 analyte can be eluted by washing the cartridge with a non-polar solvent, which disrupts the interaction of the analyte and the stationary phase (67). A stationary phase of silicon with carbon chains is commonly used. Relying on mainly non-polar, hydrophobic interactions, only non-polar or very weakly polar compounds will adsorb to the surface (67).

3.1.3. Cation Exchange

Cation exchange sorbents are derivatized with functional groups that interact and retain positively charged cations, such as bases. Strong cation exchange sorbents contain aliphatic sulfonic acid groups that are always negatively charged in aqueous solution, and weak cation exchange sorbents contain aliphatic carboxylic acids, which are charged when the pH is above about 5. Strong cation exchange sorbents are useful because any strongly basic impurities in the sample will bind to the sorbent and usually will not be eluted with the analyte of interest; to recover a strong base a weak cation exchange cartridge should be used. To elute the analyte from either the strong or weak sorbent, the stationary phase is washed with a solvent that neutralizes ionic interaction between the analyte and the stationary phase (67).

For this study was used:

Selectivity: strata™-X-C Strong Cation Exchange and Reversed Phase (Figure 7)

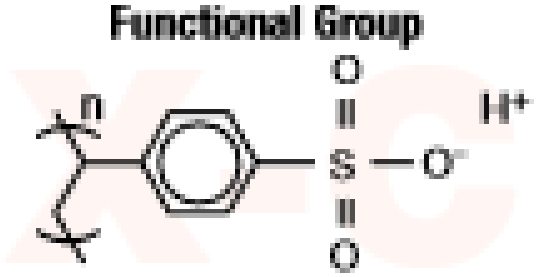


Figure 7: Functional group of cartridge

- Particle size (µm) 33
- Pore size (Å) 85
- Surface area (m2 /g) 800
- pH stability 1-14
- Ionic capacity 1 meq/g

Cleaner Reproducible Extractions:

Unlike traditional silica-based mixed mode sorbents that are blended, strata-X-C has a strong cation exchange group uniformly bonded on the polymeric surface completely eliminating recovery or reproducibility problems. The strong cation exchange mechanism gives consistent and extremely clean extracts from biological matrices such as plasma and urine since hydrophobic contaminants can be completely removed by using 100 % organic wash solvents.

3.2. Gas Chromatography

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 quantitation of compounds in a mixture. GC is also a frequently used technique in many environmental and forensic laboratories because it allows for the detection of very small quantities. A broad variety of samples can be analysed 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 consists of a packed column where the packing or solid support itself acts as stationary phase, or is coated with the liquid stationary phase (=high boiling polymer). Most analytical gas chromatographs use capillary columns, where the stationary phase coats the walls of a small-diameter tube directly (i.e., 0.25 µm film in a 0.32 mm tube). The separation of compounds is based on the different strengths of interaction of the compounds with the stationary phase ("like-dissolves-like"-rule). The stronger the interaction is, the longer the compound interacts 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. That is one of the main reasons why low boiling solvents (i.e., diethyl ether, dichloromethane) are used as solvents to dissolve the sample. The temperature of the column does not have to be above the boiling point because every compound has a non-zero vapour pressure at any given temperature.

The polarity of components versus the polarity of stationary phase on column:

If the polarity of the stationary phase and compound are similar, the retention time increases because the compound interacts 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 results in very short retention time but also in a very poor separation because all components mainly stay in the gas phase. If the compound does not interact with the stationary phase, the retention time

will decrease. At the same time, the quality of the separation deteriorates, because the differences in retention times are 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 a symmetric shape (Gaussian curve). If too much of the sample is injected, the peaks show 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.

3.2.1. GC-MS (Gas chromatography mass spectrometry)

Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. This makes it ideal for the analysis of the hundreds of relatively low molecular weight compounds found in environmental materials. In order for a compound to be analysed by GC/MS it must be sufficiently volatile and thermally stable. In addition, functionalised compounds may require chemical modification (derivatization), prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. Samples are usually

analysed as organic solutions consequently materials of interest (e.g. soils, sediments, tissues etc.) need to be solvent extracted and the extract subjected to a clean-up before GC/MS analysis is possible. The sample solution is injected into the GC inlet where it is vaporized and swept into a chromatographic column by the carrier gas (usually helium). The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions. The next component is a mass analyser (filter), which separates the positively charged ions according to various mass related properties depending upon the analyser used. After the ions are separated they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all of 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.

3.2.2. GC-ECD (Gas chromatography Electron capture detectors)

Electron capture detectors (ECD) are typically used in environmental testing for detecting PCB's, organochlorine pesticides, herbicides and various halogenated hydrocarbons. ECD consists of a cavity that contains two electrodes and a radiation source that emits –radiation. The collision between electrons and the carrier gas (methane plus an inert gas) produces a plasma-containing electrons and positive ions. If a compound is present that contains electronegative atoms, those electrons will be "captured" to form negative ions and the rate of electron collection will decrease. The detector is extremely selective for compounds with atoms of high electron affinity, but has a relatively small linear range.

4. Experimental Part

4.1. Reagents

The reagents used were:

n-Hexane [purity 99%], acetonitrile (ACN) [purity 99.9%] , magnesium sulphate ($MgSO_4$), sodium acetate (CH_3COONa), chloridic acid (HCl) [purity 36.5-38%], potassium hydroxide (KOH), ethyl acetate ($CH_2COOC_2H_5$) [purity 99.8%], methanol (CH_3OH), ammonium hydroxide (NH_4OH) [purity 31.2%] pH 10, methanol 5% in ethyl acetate [purity 99.5%], hexafluoro-2-propanol (HFEIP) [purity 99.8%] , N,N'-Diisopropylcarbodiimide (DIC) [purity 99%], potassium carbonate (K_2CO_3).

4.2. Sampling

7 Female rats were treated with permethrin from 6 to 21 days of life. Then 4 of them were sacrificed the day after (22 days of life) and the other 3, 14 days after the treatment (35 days of life). Hearts and urine were collected (data of the collection 29/10/2013) from these rats.

The samples were at -20°C until analysis, which were made at room temperature.

4.3. Sample preparation

In order to carry out this study, samples (rat's brain) have been homogenized with a drill by a Torax equipment and preserved in approximately 5mL of ACN at -20°C. In order to determinate the concentrations of 3-PBA, 2-PBA and permethrin, several tests were performed.

4.4. Solution preparation

12.09 mg of permethrin have been weighed in a 5 mL flacks.

With this amount of permethrin were prepared:

- Permethrin solution 1 (1209mg/L)

- Permethrin solution 2 (10mg/L)
- Permethrin solution 3 (500µg/L)
- Permethrin solution 4 (625µg/L)
- Permethrin solution 5 (125µg/L)

Preparation of microvials for calibration curve:

- Two series of standards for calibration curve were prepared:
- Concentrations: 0.3mg/L, 0.4mg/L, 0.6mg/L, 0.7mg/L, 0.8mg/L and 0.9mg/L;
- Concentrations: 0.5mg/L; 0.4mg/L; 0.3mg/L; 0.2mg/L; 0.1mg/L; 0.05mg/L; 0.02mg/L

- Buffer solution pH7:
  - 2.86mL of glacial acetic acid have been taken, put in a flask and diluted with H_2O until 500mL (solution A)
  - One the other hand 6.8g of sodium acetate have been taken, put in a flask and diluted with H_2O until 500mL (solution B)
  - The buffer solution has been prepared with 89.25 mL from solution A and 160.75mL from solution B

Moreover some solutions have been prepared with 3-PBA and with 2-PBA:

- 3-PBA solution 1: 2000mg/L,
- 3-PBA solution 2: 10mg/L
- 3-PBA solution 3: 50µg/L
- 3-PBA solution 4: 5 µg/L
- 3-PBA solution 5: 2 µg/L
- 2-PBA solution 1: 2000 mg/L
- 2-PBA solution 2: 10mg/L
- 2-PBA solution 3: 50µg/L

For 3PBA and 2PBA calibration curves were prepared:

Concentrations 3PBA: 0.01µg/L, 0,05µg/L, 0,1µg/L, 0,3µg/L, 0,5µg/L, 1µg/L, 1,5µ/L

Concentration 2PBA; 20µg/L

Ions selected for the identification of metabolites 3-PBA and 2-PBA are: 135:169; 141; 195; 197. The most abundant ion for metabolite 3-PBA is 364 the most abundant ion for metabolite 2-PBA is 195.

A NH_4OH solution with pH 10 was prepared through the dilution of pure NH_4OH.

4.5. Procedure

For preliminary studies only the buffer solution was used:

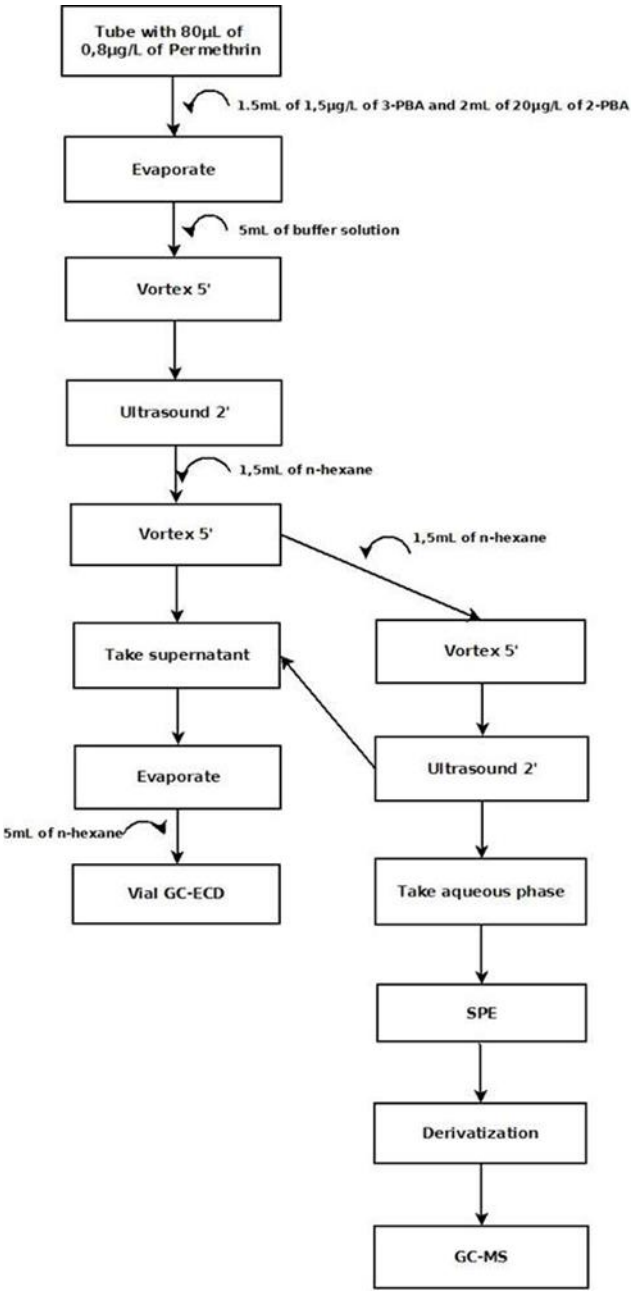


Figure 8: Scheme of procedure for preliminaries tests

The solid phase extraction (SPE) consisted in:

Conditioning:

- 5 mL of ethyl acetate
- 5 mL MeOH
- 5 mL H_20

- 5 mL of HCl

Add sample

- 5 mL of sample

Cleaning

- 5 mL of HCl
- 5 mL of NH_4OH

Drying

Elution:

- 5 mL of 5% of MeOH in AcOEt

Before injection in GC a derivatization was performed, the procedure is described in figure 9

Derivatization reactions are meant to transform an analyte for detectability in Gas Chromatography (GC) or other instrumental analytical methods. Derivatization in GC analysis can be defined as a procedural technique that primarily modifies an analyte's functionality in order to enable chromatographic separations. A modified analyte in this case will be the product, which is known as the derivative. The derivative may have similar or closely related structure, but not the same as the original non-modified chemical compound. Volatility of sample is a requirement for GC analysis. Derivatization will render highly polar materials to be sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular re-arrangement.

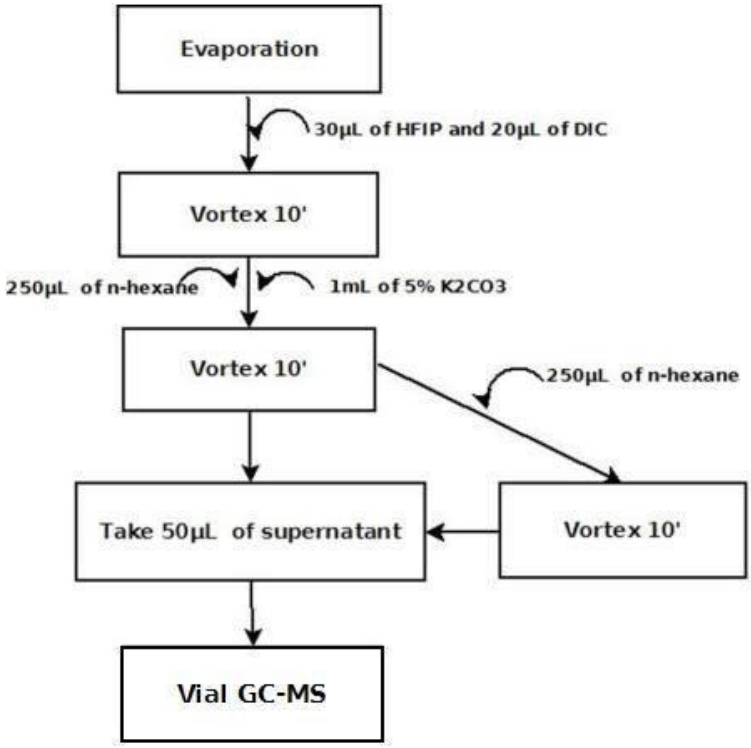


Figure 9: Scheme of derivatization procedure

Several brains obtained from rats, not treated with permethrin, were used in order to standardize the method. The general procedure with brains is described in figure 10

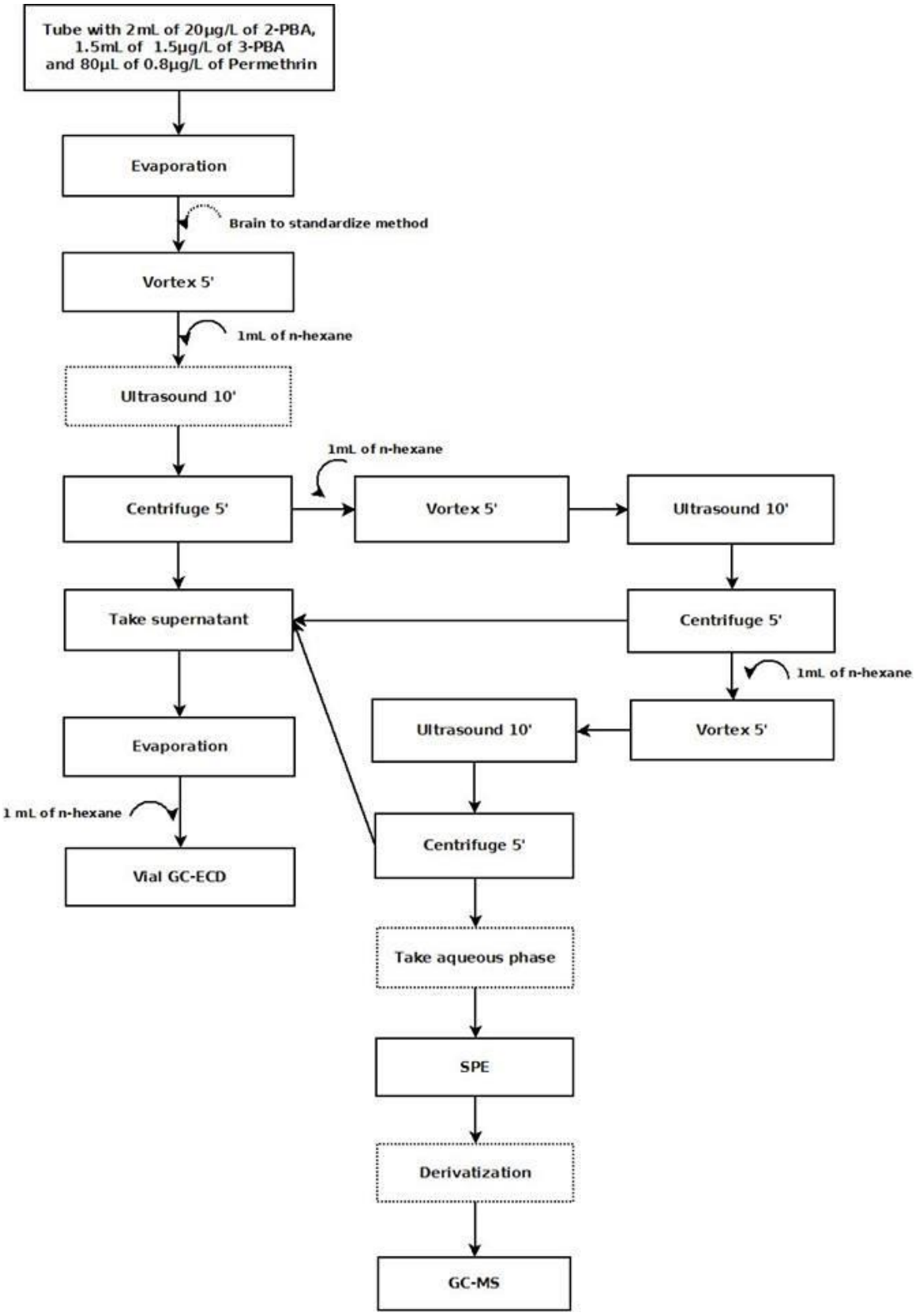


Figure 10: Scheme of procedure to standardized the method

During the tests several changes have been carried to the method, namely:

- At first samples have been filtrated with paper filter in the beginning of test. Then the aqueous phase was filtrated in order to prevent a loss of permethrin.
- The minutes of ultrasound were increased from 10’ to 20’ to have clear separation between the aqueous phase and supernatant.
- The procedure of derivatization has been modified; 150 µL of n-hexane to improve the concentration of 3-PBA and 2-PBA.

In other to verify the method, several tests were performed using the samples from rats treated with permethrin that are described in Figure 11

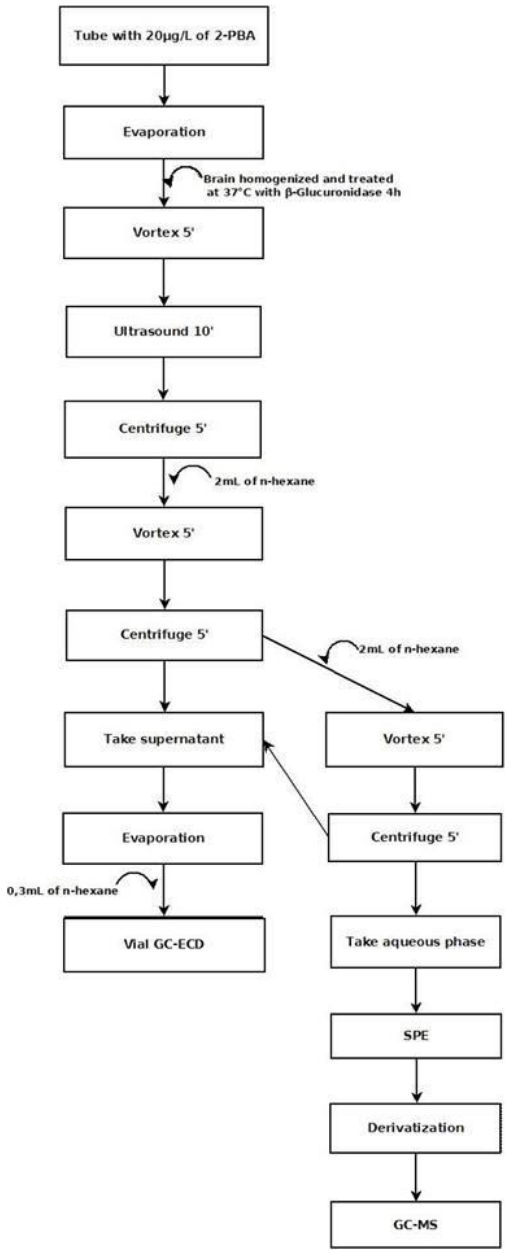


Figure 11: Scheme of procedure with sample from rats treatment with permethrin

The permethrin, 3-PBA and 2-PBA -data from brains from treated rats were obtained with the procedure described in figure 12.

A control with buffer and enzyme but without sample was performed. Only results with higher values than the achieved by the control was processed.

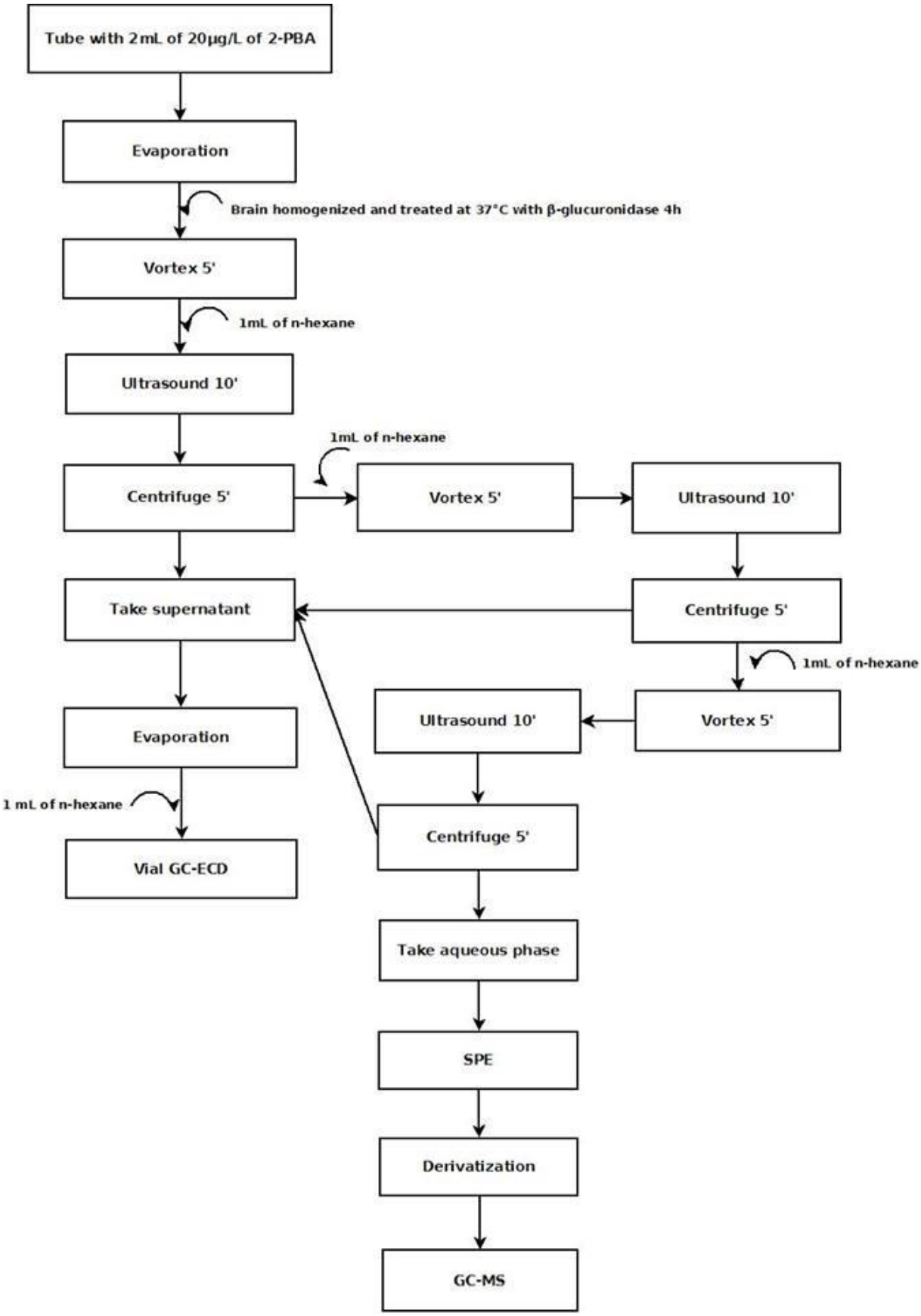


Figure 12: Scheme of procedure with rats treaded with permethrin

3-PBA values from urines were obtained with the procedure described in figure 13.

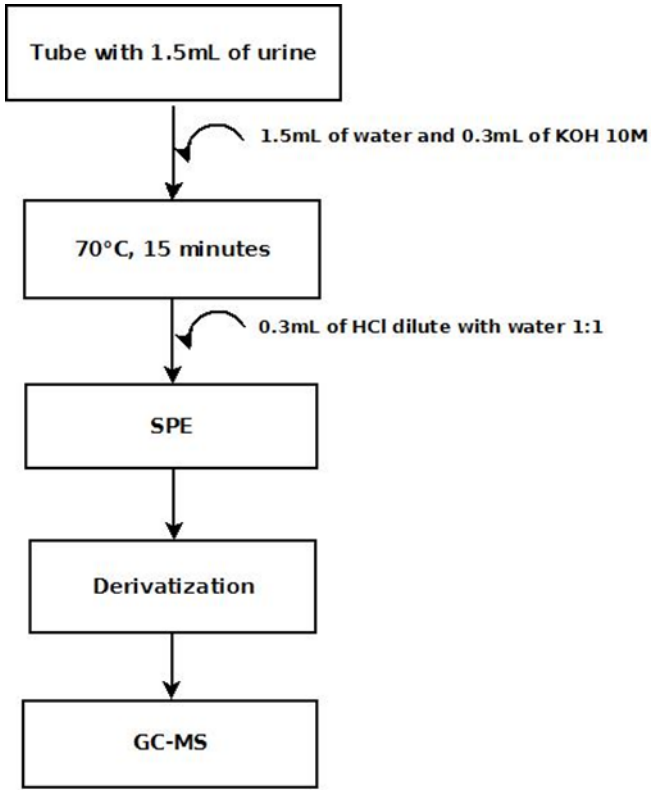


Figure 13: Scheme of procedure with urines from rats treated with permethrin

4.6. Chromatographic analysis

Permethrin was analyzed using a Shimadzu GC-2010 with ECD apparatus equipped with a capillary column ZB-XLB (30 m x 0.25 mm x 0, 25 mm) from Phenomenox. A volume of injection 1µL was used. The oven temperature was programmed starting at 60°C and held for 1'min followed by increase of 30°C/min to 250°C and then increase 10°C/min until 290°C and held for 3'. The detection was 300°C C. Helium (Linde Sogas) was used as carrier gas at a constant flow rate of 1.3 mL/min, whereas nitrogen (Linde Sogas, purityg99.999%) was employed as makeup gas at a flow of 30 mL/min. The system was operated by GCsolution Shimadzu software.

Operation of a GC/MS in SIM mode allows for detection of specific analytes with increased sensitivity relative to full scan mode. Because the instrument is set to look for only masses of interest it can be specific for a particular analyte of interest. Typically two to four ions are monitored per compound and the ratios of those ions will be unique to the analyte of interest. In order to increase sensitivity, the mass scan rate and dwell times.

The equipment that has been used for testing these samples it was GC-MS/SIM and MS/MS using a Supelco column fitted with an SLB-5MS (30 m x 0.25 mm x 0.25µm film thikness) column operating in the splitless mode. Helium was used as carrier gas at a constant flow rate of 1.3 mL/min. The oven temperature was programmed starting at 40 C and held for 1 min, followed by increases of 15 C/min to 260. The ion source and MS transfer line were at 250°C.

4.7. Recoveries evaluation

The recoveries were achieved by the relation between the area of the analyt (permethrin or 3-PBA) after the procedure and the area of a standard analyt in solution.

5. Results and discussion:

5.1. Chromatography results

5.1.1. GC-ECD, retention time, linearity, calibration curve permethrin

A solution containing permethrin was injected in GC-ECD and the retention time was recorded. Figure 14 show the chromatogram obtained with the peak of permethrin achieving 7.6 min of retention time.

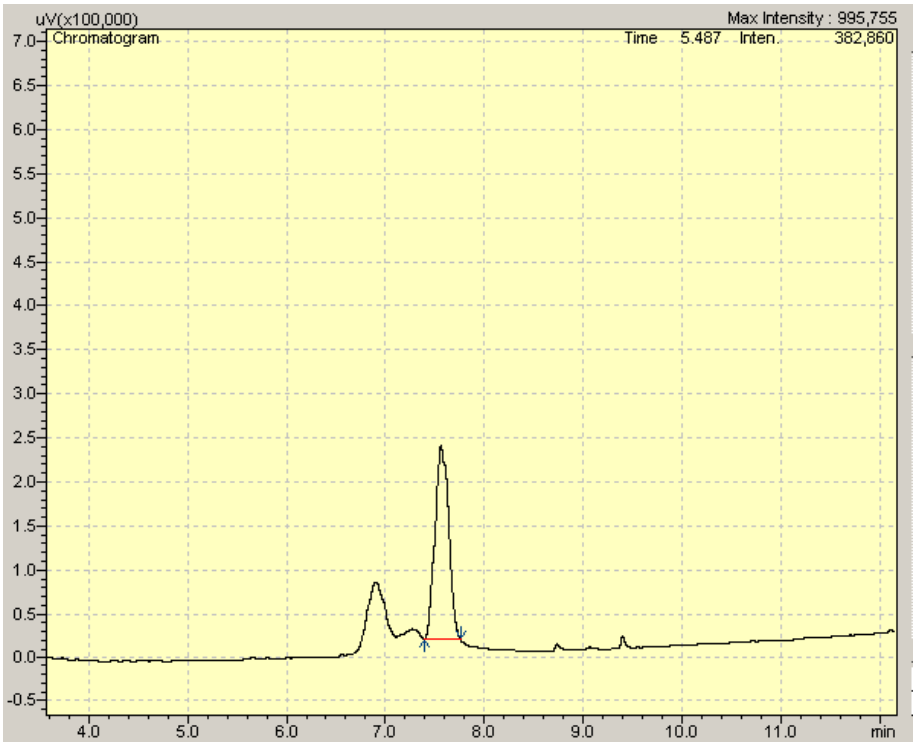


Figure 14: Chromatogram retention time permethrin

It was injected in GC-ECD five standards of permethrin (0.3 µg/L; 0.4 µg/L; 0.6 µg/L;0.8µg/L; 0.9 µg/L). Figure 15 shows the achieved calibration curve for Permethrin. It was observed linearity between 0.3 to 0.9 µg/L of permethrin in n-hexane. A R^2 higher than 0.9842 was achieved.

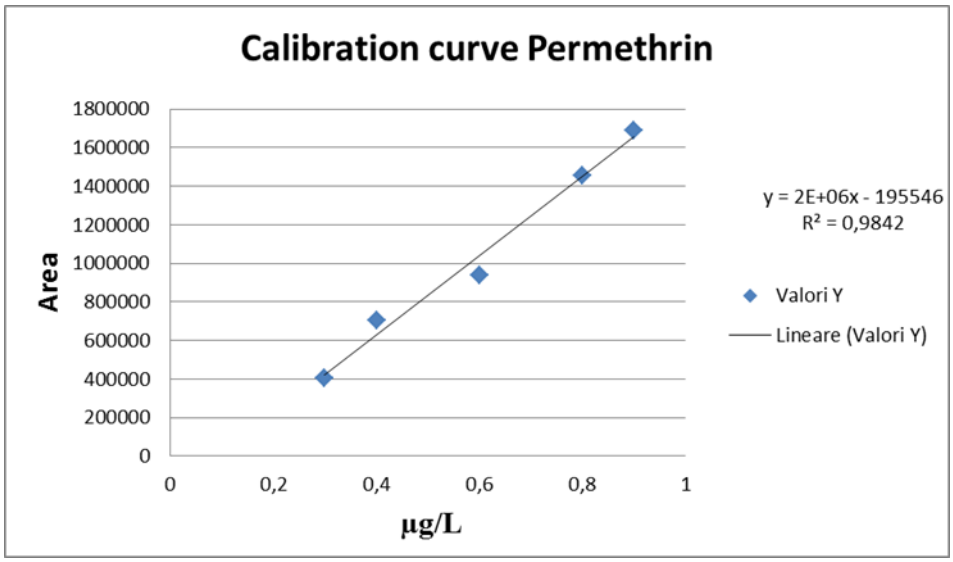
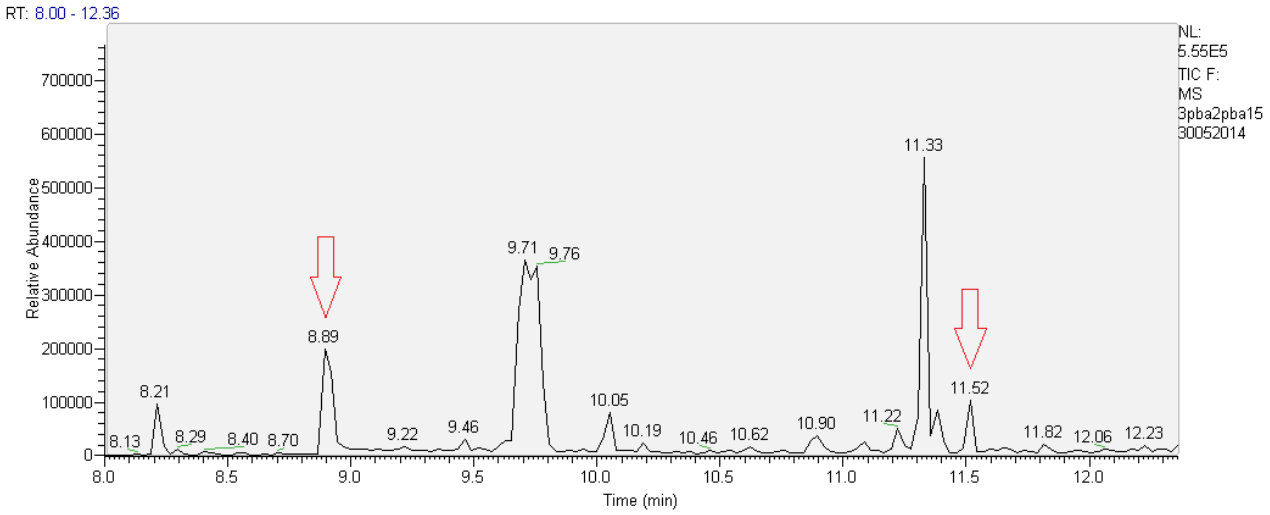


Figure 15: Calibration curve of permethrin achieved by the correlation of area versus concentration

5.1.2. GC-MS retention time, linearity, calibration curve

The retention time of 3-PBA was obtained by the injection in SIM mode of a derivatized solution.

Figure 16 shows the chromatogram obtained with the respective spectrum. The achieved retention time of 2-PBA was 8.9 min.



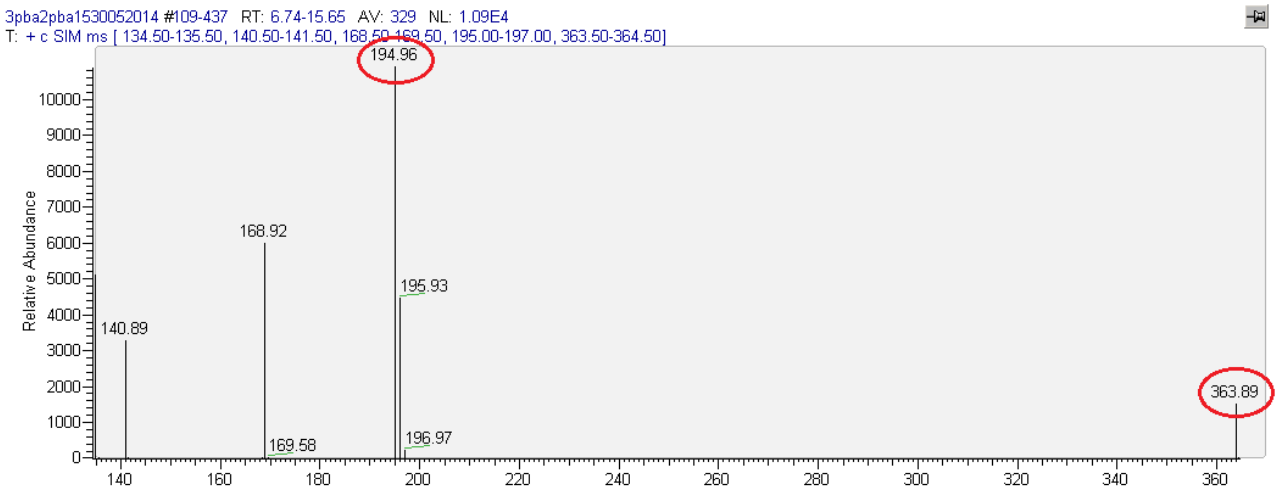


Figure 16: Chromatogram showing the peak of 3-PBA and 2-PBA (11.52 min and 8.9 min) and the spectra of 3-PBA

In figure 17 is shown the calibration curve for 3-PBA. It was observed linearity between 0.01 to 1.5 µg/L of 3-PBA. A R^2 higher then 0.9864was achieved.

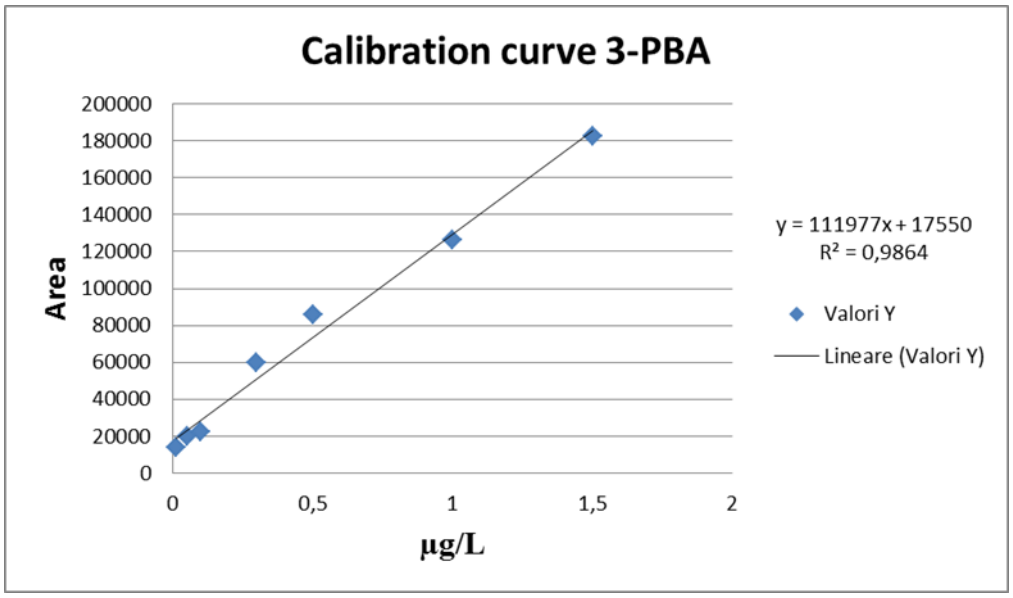


Figure 17: Calibration curve 3-PBA

The average area obtained in all chromatograms with 20 µg/L was 497601.2.

6. Recoveries:

The recoveries were obtained using the quotient between the area obtained with the tested procedure and the area obtained with a standard. The 3-PBA and 2-PBA recovery obtained with buffer solution was 78% and 58%, respectively. Identical procedure was performed with samples from rats not treated with permethrin in buffer solution. The recoveries of 3-PBA obtained with different tested procedures (ultrasounds 10' and 20') are shown in figure 18:

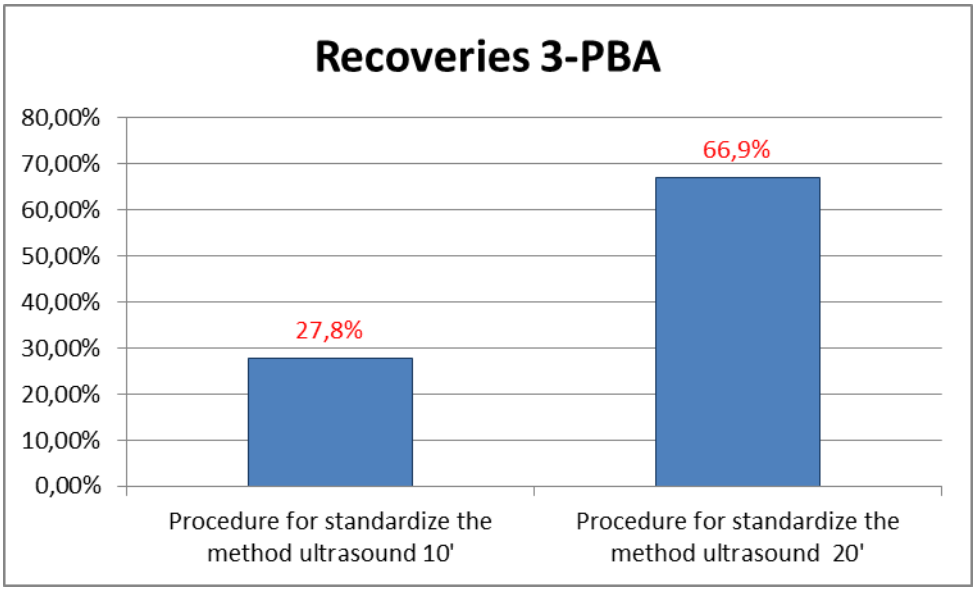


Figure 18: Recoveries of 3-PBA obtained in samples with different procedures

The highest recovery is obtained with the procedure that uses 20 min in ultrasounds, achieving 66.9 % of recovery.

The recoveries of 2-PBA obtained with different procedures are shown in figure 19.

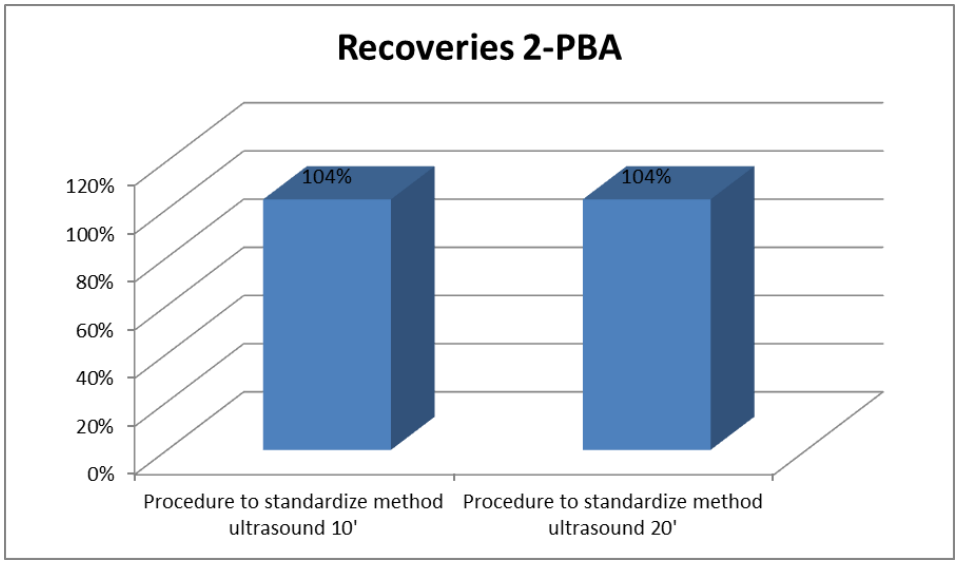


Figure 19: Recoveries of 2-PBA obtained with different times in ultrasound

For 2-PBA 10 min in ultrasounds is sufficiently to achieve 104 % of extraction. However for 3-PBA is necessary 20 min to achieve 67% of recovery, for that reason it was selected the procedure with 20 min in ultrasound.

The amounts of permethrin and 3-PBA in brains from female rats treated with permethrin were obtained using the equations from the calibration curves. Figure 20 shows the parallel between quantities of permethrin in brains of female rats treated with permethrin and sacrificed the day after the treatment (22 days of life) and brains of female rats treated with permethrin and sacrificed 14 days after the treatment (35 days of life).

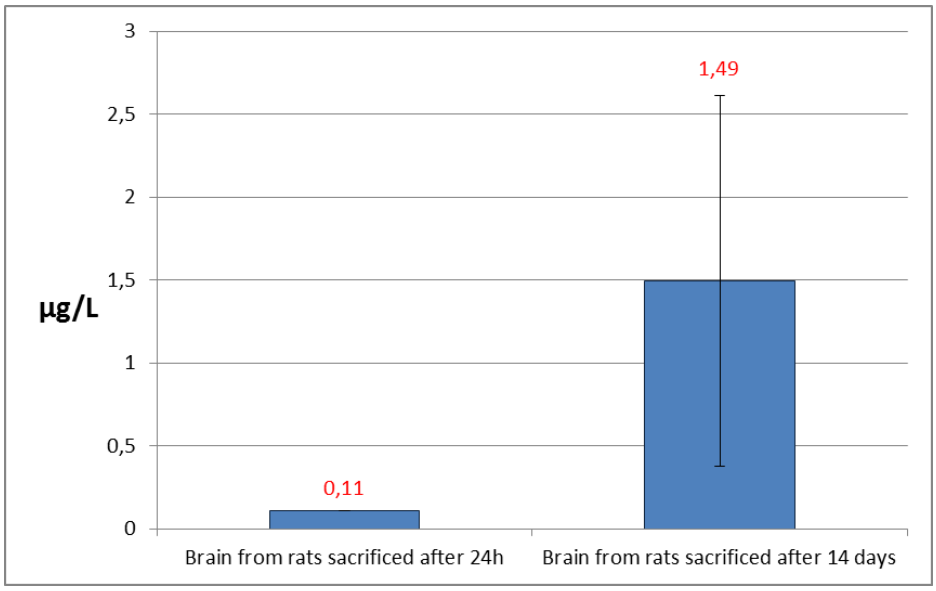


Figure 20: Permethrin concentration in rats brains after 24h and 14 days of exposure

The results demonstrate that permethrin was found in both brains of female rats sacrificed on the day after (24h after) and 14 days after the end of treatment. Such as the graph shows the concentration of permethrin in the brains from female rats sacrificed the day after the end of treatment is lower than that found in the brains of female rats sacrificed the 14th day after the end of treatment. The higher levels achieved in brains may be explained with the lipophilic character of this organ. Accordingly it can be affirm that the exposure to permethrin can represent a risk factor for the development of neuro- degenerative diseases.

The data of the metabolite of permethrin in brain is show in figure 21.

Figure 21: Concentration of 3-PBA in brains after 24 h and after 14 days of exposure.

These results show that in female rats sacrificed the day after (24h) the end of treatment, the amount of metabolite 3-PBA is high in contrast to the amount that was found in female rats sacrificed 14 days after the end of treatment. This results in agreement with other authors (58). The amount of 3-PBA 14 days after the end of the treatment is lower than the limit of detection because the area detected by the equipment was lower than that of the buffer.

The amounts of 3-PBA in urines of female rats treated with permethrin were calculated using the equations of calibration curves. Figure 22 shows the quantities of 3-PBA in urines of rats sacrificed the days after and 14 days after the treatment with permethrin.

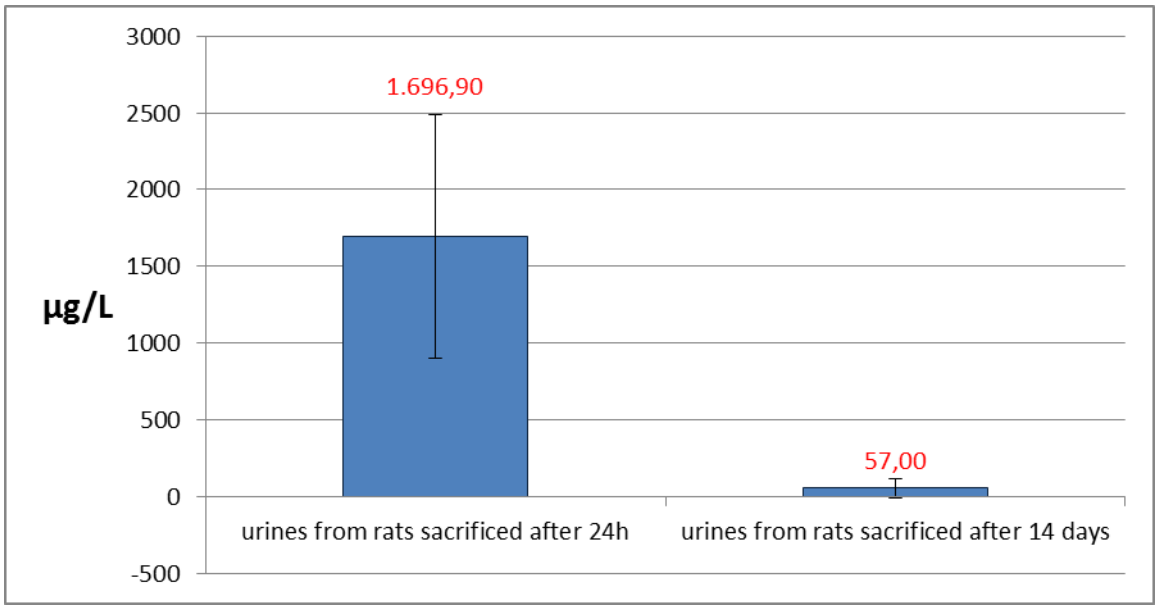


Figure 22: Concentration of 3-PBA in urines of rats

Such as is possible to see from figure 21, 3-PBA was found both in urines of rats sacrificed after 24 hours and after 14 days of the end of treatment with permethrin. The concentration of 3-PBA in urines of rats sacrificed after 24 hours is almost 30 times greater than the concentration of 3-PBA in urines of rats sacrificed after 14 days of the end of treatment. These data are in agreement with others authors (58) that described that 88% of the 3-PBA is excreted within the first 24 h following exposure.

7. Conclusion:

In this research, were studied female rats from the same treatment to investigate the effect of early life permethrin and its major metabolite on the brain. These results could confirms a neurodegenerative process associated with exposure to this pesticide, (Parkinson-like neurodegeneration). In all the brain samples treated with the same concentration the permethrin concentrations increase from 24h to 14 days 13.5 times. The metabolite achieved a maximum level at 24 h (2.6 μg/L) and none of the samples of 14 days achieved values above the limit of detection.

Therefore this results link the hypotheses that permethrin in the brain longer than expected. Accordingly it can be affirm that the exposure to permethrin can represent a risk factor for the development of neuro- degenerative diseases.

8. References

Figure 1. D.M. Soderlund et al./Toxicology 171 (2002) 3–59

Figure 2. http://www.fieldherpforum.com

Figure 3. www.inchem.org433 × 240

Figure 4. http://openi.nlm.nih.gov/detailedresult.php?img=1277854_ehp0113-000123f3&req=4

Figure 5. http://toxsci.oxfordjournals.org/content/130/1/33/F3.expansion.html

Figure 6. Current Trends in Sample Treatment Techniques for Environmental and Food Analysis

Figure7. http://separations.co.za/fileadmin/themes/default/pdf/SIDEBAR/2.%20CHROMTECHNIQUES/c.%20SPE/Strata%20-%20SPESolutionsBrochure.pdf

1. *Methods in Biotechnology, Vol. 19, Pesticide Protocols Edited by: J. L. Martínez Vidal and A. Garrido Frenich © Humana Press Inc., Totowa, NJ*

2. *2-Reigart, J., M.D. et al., Recognition and Management of Pesticide Poisonings, EPA, 1999.*

3. *Gosselin, R.E. 1984. Clinical Toxicology of Commercial Products. Williams and Wilkins. Baltimore, MD.*

4. *Reregistration 2. Eligibility 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.*

5. *K. Naumann, Synthetic Pyrethroid Insecticides: Structures and Properties, Springer-Verlag, New York, NY, USA, 1990*

6. *D. M. Soderlund and J. R. Bloomquist, Annu. Rev. En tomol. , 1989, 34, 77.*

7. *Berkowitz, Exposure to indoor pesticides during pregnancy in a multiethnic, urban cohort. Environ Health Perspect 2003; 111,1:79*

8. *R. D. Verschoyle and W. N. Aldridge, Arch. Toxicol., 1980, 45, 325.*

9. *L. J. Lawrence and J. E. Casida, Pestic. Biochem. Phvsiol., 1982, 18, 9.*

10. *Verschoyle, R.D., Aldridge, W.N., 1980. Structure–activityrelationships of some pyrethroids in rats. Arch. Toxicol.45, 325–329.*

11. *X. Wang, F. Xue, A. Hua and F. Ge, Physiol. Entomol., 2006, 31, 190.*

12. *C. Breckenridge, L. Holden, N. Sturgess, M. Weiner, L. Sheets, D.Sargent, D. M. Soderlund, J. S. Choi, S. Symington, J. M. Clark, S. Burr and D. Ray, Neurotoxicology, 2009, 30, S17.*

13. *J. R. Bloomquist, Annu. Rev. Entomol., 1996, 41, 163.*

14. *A. L. Goldin, Ann. N. Y. Acad. Sd, 1999, 868, 38.*

15. *S. J. Lozano, S. L. O'Halioran, K. W. Sargent and J. C. Brazner, E,nviron. Toxicol. Chem., 1992, 11, 35.*

16. *M.L. Falcioni, C Nasuti, C. Bergamini, R Fato, G. Lenaz, R. Gabbianelli The primary role of GSH against nuclear DNA damage of striatum induced by permethrin in rats Neuroscience, 2010, 168, 2.*

17. *V. L. Salgado, S. N. Irving and T. A. Miller, Pestie. Biochem. Physiol.,1983, 20, 169.*

18. *D. M. Soderlund, Pestic. Biochem. Physiol., 2010, 97, 78.*

19. *P. J. Forshaw, T. Lister and D. E. Ray, Toxicol. Appl. Pharmaco!., 2000, 163, 1*

20. *F. Cantalamessa Acute toxicity of two pirethyroids, permethrin and cypermethrin in neonatal and adult rats Arch. Toxicol., 1993, 67, 510.*

21. *J. G. Scott and Z. Wen, Pest Manag. Sci., 20 I, 57, 958.*

22. *D. M. Soderlund, J. M. Clark, L. P. Sheets, L. S. Mullin, V. J. Piccirillo, D. Sargent, J. T. Stevens and M. L. Weiner, Toxicology, 2002, 171, 3.*

23. *S. B. Symington, A. G. Zhang, W. Karstens, J. Van Houten and J. M. Clark, Pestie. Biochem. Physiol., 1999, 65, 181.*

24. *F. Matsumura and S. M. Ghiasuddin, J. Environ. Sci. Health B., 1983, 18, 1.*

25. *Parrón, T., Requena, M., Hernández, A.F., Alarcón, R.; Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol. Appl. Pharmacol. 2011.*

26. *Cox, C. 1998. Permethrin. Journal of Pesticide Reform 18(2): 14-20.*

27. *Blaylock, R. L, M. Abdel-Nasser, S. M. McCarty, J. A. Knesel, K. M. Tolson, P. W. Ferguson, and H. M. Mehendale. Suppression of cellular immune responses in BALB/c mice following oral exposure to Permethrin. Bulletin of Environmental Contamination and Toxicology 1995, 54 (5): 768-774.*

28. *Eil, C. and B. C. Nisula. The binding properties of pyrethroids to human skin fibroblast androgen receptors and to sex hormone binding globulin. Journal of Steroid Biochemistry 1990, 35, 409.*

29. *Kasat K., V. Go, and B. G. Pogo. Effects of Pyrethroids insecticides and estrogen on WNT10B proto-oncogene expression. Environment International, 2002, 28(5), 429.*

30. *Alavanja, M.C.R., et al. Use of agricultural pesticides and prostate cancer risk in the agricultural health study cohort. American Journal of Epidemiology. 2003, 157: 800-814.*

31. *Parrón, T., Requena, M., Hernández, A.F., Alarcón, R.,. Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol. Appl. Pharmacol. 2011.*

32. *Relton, C.L., Smith, G.D.,. Epigenetic epidemiology of common complex disease: prospects for prediction, prevention, and treatment. PLoS Med. 2010, 7, e1000356. doi:10.1371/journal.pmed.1000356.*

33. *Gabbianelli, R., Nasuti, C., Falcioni, G., Cantalamessa, F.,. Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity. Toxicology 175, 91–101; Gabbianelli, R., Lupidi, G., Villarini, M., Falcioni, G., 2003. DNA damage induced by copper on erythrocytes of gilthead sea bream Sparus aurata and mollusc Scapharca inaequivalvis. Arch. Environ. Contam. Toxicol. 2002, 45, 350–356)*

34. *N.A. Velázquez-Ulloa, N.C. Spitzer, D. Dulcis Contexts for dopamine specification by calcium spike activity in the central nervous system J Neurosci., 2011, 31, 78*

35. *T. Narahashi Neuronal ion channels as the target sites of insecticides Pharmacol. Toxicol., 1996, 79, 1.*

36. *Cox, C. Permethrin. Journal of Pesticide Reform 1998, 18(2), 14.*

37. *Vadhana, M.S.D., Carloni, M., Nasuti, C., Fedeli, D., Gabbianelli, R.,. Early life permethrin insecticide treatment leads to heart damage in adult rats. Exp. Gerontol. 2011, doi:10.1016/j.exger.2011.05.005.*

38. *T. Perlmann, A. Wallén-Mackenzie Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells Cell Tissue Res., 2004, 318, 45.*

39. *Y. Chu, W. Le, K. Kompoliti, J. Jankovic, E.J. Mufson, J.H. Kordower Nurr1 in Parkinson's disease and related disorders J. Comp. Neurol., 2006, 494 (3) 495.*

40. *Gabbianelli, R., Falcioni, M.L., Nasuti, C., Cantalamessa, F., Imada, I., Inoue, M.,. Effect of permethrin insecticide on rat polymorphonuclear neutrophils. Chem. Biol. Interact. 2009b, 182, 245.*

41. *C. Viero, G. Dayanithi Decoding calcium signals in living cells IIOAB J., 1, 2010, 11.*

42. *D.S. Woodruff-Pak, M.R. Foy, G.G. Akopian, K.H. Lee, J. Zach, K.P. Nguyen, D.M. Comalli, J.A. Kennard, A. Agelan, R.F. Thompson Differential effects and rates of normal aging in cerebellum and hippocampus Proc. Natl. Acad. Sci. U. S. A., 2010, 107, 1624.*

43. *M. Berridge Neuronal calcium signaling Neuron, 1998, 21, 13.*

44. *J.P. Kiss, E.S. Vizi Nitric oxide: a novel link between synaptic and nonsynaptic transmission. Trends Neurosci., 2001, 24, 211.*

45. *Zhang,X.,Jones,D., Gonzalez-Lima, F., Neurodegeneration produced byrotenone in the mouse retina: a potential model to investigate environmental pesticide contributions to neurodegenerative diseases. J. Toxicol. Environ. Health A 2006, 69, 1681.*

46. *W. Li, J. Xue, C. Niu, H. Fu, C.S. Lam, J. Luo, H.H. Chan, H. Xue, K.K. Kan, N.T. Lee, C. Li, Y. Pang, M. Li, K.W. Tsim, H. Jiang, K. Chen, X. Li, Y. Han Synergistic neuroprotection by bis(7)-tacrine via concurrent blockade of N-methyl-d-aspartate receptors and neuronal nitric-oxide synthase Mol. Pharmacol., 2007, 71, 1258.*

47. *VanDerRhee, 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.*

48. *Food and Drug Administration. 2003. Pesticide Program: Residue Monitoring 2001. Washington, Updated July 2004 D. C.: Food and Drug Administration, Center for Food Safety and Applied Nutrition*

49. *Anadon, A.; Martinez-Larranaga, M. R.; Diaz, M. J.; Bringas, P. Toxicokinetics of Permethrin in the Rat. Toxicol. Appl. Pharmacol. 1991, 110, 1.*

50. *Toxicological. Profile for Pyrethrins and Pyrethroids; U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry: Atlanta, 2003.*

51. *Gotoh, Y.; Kawakami, M.; Matsumoto, N.; Okada, Y. Permethrin Emulsion Ingestion: Clinical Manifestations and Clearance of Isomers. Clin. Toxicol. 1998, 36 (1), 57.*

52. *Krieger, R., Handbook of Pesticide Toxicology - Agents, 2nd ed; Academic Press: San Diego, 2001; Vol. 2, pp 1279.*

53. *Heudorf U, Butte W, Schulz C, Angerer J... Reference values for metabolites of pyrethroid and organophosphorous insecticides in urine for human biomonitoring in environmental medicine. Int J Hyg Environ Health 2006, 209, 293.*

54. *Reregistration Eligibility 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.*

55. *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 pyrethroid insecticides. Arch. Environ. Contam. Toxicol. 2004, 46, 281

56. *Casida,. Mechanism of selective action of pyrethroid insecticides. Annu. Rev. Pharmacol. Toxicol.1983 23, 413*

57. *Centers for Disease Control and Prevention (2005) Third national report on human exposure to environmental chemicals. Centers for Disease Control, Atlanta*

58. *Kühn, K.; Wieseler, B.; Leng, G.; Idel, H. Toxicokinetics of pyrethroids in humans: Consequences for biological monitoring. Bull. Environ. Contam. Toxicol. 1999, 62, 101*

59. *Bradberry, S. M.; Cage, S. A.; Proudfoot, A. T.; Vale, J. A. Poisoning due to Pyrethroids. Toxicol. Rev. 2005, 24 (2), 93.*

60. *VanDerRhee, 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.*

61. *Nakamura, Y., Sugihara, K., Sone, T., Isobe, M., Ohta, S., Kitamura, S.,. The in vitro metabolism of a pyrethroid insecticide, permethrin, and its hydrolysis products in rats. Toxicology 2007, 235, 176–184.*

62. *Leng G, Kuhn KH, Idel H. 1997. Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: applications and limitations. Sci Total Environ 199:173–181.*

63. *Catherine L. Arthur, Janusz Pawliszyn, Anal. Chem., 1990, 62 (19), 2145.*

64. *Tomlin, C. D. S. The Pesticide Manual: A World Compendium, 14th ed.; British Crop Production Council: Alton, England, 2006; pp 813-814.*

65. *Supelco (1998), Guide to Solid Phase Extraction (http:/ / www. sigmaaldrich. com/ Graphics/ Supelco/ objects/ 4600/ 4538. pdf), pp. 9,*

66. *Supelco (1998), Guide to Solid Phase Extraction (http:/ / www. sigmaaldrich. com/ Graphics/ Supelco/ objects/ 4600/ 4538. pdf), pp. 2–12*

67. *Supelco (1998), Guide to Solid Phase Extraction (http:/ / www. sigmaaldrich. com/ Graphics/ Supelco/ objects/ 4600/ 4538. pdf), pp. 3–4*

Calculations Permethrin solutions:

C1*V1=C2*V2

- Permethrin solution1= 12.090µg/10*10^-3 = 1209 mg/L
- Permethrin solution 2 = 1209mg/L *V1= 10mg *5000µL

V1= 41.36µL

Then 41.36µL from the flask and have been taken and put in another 5 ml flask,after that hexane has been added until the flask's line of demarcation (5ml).

- Permethrin solution 3= 10mg/L*V=0.5mg/L*1000µL

Vm2= 50µL

From the flask containing the mother solution 2, 50 µL have been taken and transferred then in microvial. Then hexane has been added until 1 ml.

- Permethrin solution 4= 500µg/L*V=0.625 µg/L*5000 µL

V= 6.25 µL

6.25 µL from the flask and have been taken and put in another 5 ml flask,after that hexane has been added until the flask's line of demarcation (5ml).

- Permethrin solution 5: 0.625 µg/L*V= 0.125 µg/L*5000 µL

V=1000 µL

1000 µL from the flask and have been taken and put in another 5 mL flask, after that hexane has been added until the flask's line of demarcation (5mL).

Permethrin calibration curve:

Concentration	Area
0.3µg/L	404822
0.4 µg/L	706464
0.6 µg/L	938850
0.8 µg/L	1454210
0.9 µg/L	1689112

Table 1: Permethrin area

10mg*V= 0.3µg/L*1000µL V=30µL

30µL have been taken from permethrin solution of 10mg/L and hexane was added

10mg*V= 0.4µg/L*1000µL V=40µL

40µL have been taken from permethrin solution of 10mg/L and hexane was added until 1mL.

10mg*V= 0.6µg/L*1000µL V=60µL

60µL have been taken from permethrin solution of 10mg/L and hexane was added until 1mL.

10mg*V= 0.8µg/L*1000µL V=80µL

80µL has been taken from permethrin solution of 10mg/L and hexane was added until 1mL.

10mg*V= 0.9µg/L*1000µL V=90µL

90µL has been taken from permethrin solution of 10mg/L and hexane was added until 1mL.

Calculations 3-PBA solutions:

In 5mL of ACN 10mg of 3-PBA were added.

- 3-PBA solution1 = 10000µg/ 5*10^-3L= 2000mg/L
- 3-PBA solution 2= 2000mg/L*V=10mg/L*10mL

V=0.05mL

0.05mL was taken from the 3-PBA solution of 2000mg/L. Then acetonitrile was added until 10mL.

- 3-PBA solution 3= 10mg/L*V=0.05mg/L*10mL

V=0.05mL

0.05mL was taken from the 3-PBA solution of 10mg/L. Then acetonitrile was added until 10mL.

- 3-PBA solution 4= 10mg/L*V=50µg/L*10mL

V=0.05mL

0.05mL was taken from the 3-PBA solution of 10mg/L. Then acetonitrile was added until 10mL.

- 3-PBA solution 5= 50µg/L*V=2µg/L*10mL

V=0.4mL

0.4mL was taken from the 3-PBA solution of 50µg/L. Then acetonitrile was added until 10mL.

In 5mL of ACN 10mg of 2-PBA were added.

- 2-PBA solution1 = 10000µg/ 5*10^-3L= 2000mg/L
- 2-PBA solution 2= 2000mg/L*V=10mg/L*10mL

V=0.05mL

0.05mL was taken from the 3-PBA solution of 2000mg/L. Then acetonitrile was added until 10mL.

- 2-PBA solution 3= 10000µg/L*V=50µg/L*10mL

V=0.05mL

0.05mL was taken from the 2-PBA solution of 10mg/L. Then acetonitrile was added until 10mL.

For 3PBA and 2PBA calibration curves were prepared:

Concentrations 3PBA: 0.01µg/L, 0.05µg/L, 0.1µg/L, 0.3µg/L, 0.5µg/L, 1µg/L, 1.5µ/L

Concentration 3-PBA	Area 3-PBA
0.01 µg/L	13744
0.05 µg/L	19729.67
0.1 µg/L	22358.33
0.3 µg/L	59872.5
0.5 µg/L	85899
1 µg/L	126342

1.5 µg/L	182345.7

Table 2: 3-PBA area

2µg/L*V= 0.01µg/L*5µL V=25µL

2µg/L*V= 0.05µg/L*5µL V=125µL

2µg/L*V= 0.1µg/L*5µL V=250µL

2µg/L*V= 0.3µg/L*5µL V=750µL

2µg/L*V= 0.5µg/L*5µL V=1.25mL

2µg/L*V= 1µg/L*5µL V=2.5mL

2µg/L*V= 1.5µg/L*5µL V=3.75mL

Calculation concentration of permethrin in brains of rats sacrificed after 24h:

	Area
Sample 1	25187,05
Sample 2	28025,5
Sample 3	28694,2
Sample 4	30233,4

Equation calibration curve permethrin: y=2*10^6 – 195546

Sample 1

(25187, 05+195546)/2*10^6=0, 11 µg/L

Sample 2

(28025, 5+195546)/2*10^6=0, 11 µg/L

Sample 3

(28694, 2+195546)/2*10^6=0, 11 µg/L

Sample 4

(30233, 4+195546)/2*10^6=0, 11 µg/L

Average samples: (0, 11+ 0, 11+ 0, 11+ 0, 11) = 0, 11 µg/L

Calculation concentration of permethrin in brains of rats sacrificed after 14 days:

	Area
Sample 1	5109745
Sample 2	2629519
Sample 3	658153,5

Sample 1

(5109745+195546)/2*10^6=2, 65 µg/L

Sample 2

(2629519+195546)/2*10^6=1, 41 µg/L

Sample 3

(658153, 5+195546)/2*10^6=0, 42 µg/L

Average samples: (2, 65+1, 41+0, 42)/3= 1, 49µg/L

Calculation concentration of 3-PBA in brains of rats sacrificed after 24h:

	Area

Sample 1	345170,7
Sample 2	429439,5
Sample 3	442287
Sample 4	69046

Equation calibration curve 3-PBA: y=111977x + 17550

Sample 1

(345170, 7- 17550)/ 111977 = 2, 92 µg/L

Sample 2

(429439, 5 – 17550) / 111977 = 3, 67 µg/L

Sample 3

(442287 – 17550) / 111977 = 3, 79 µg/L

Sample 4

0 µg/L

Average simples: (2, 92+ 3,67+ 3,79+ 0)/4 = 2,59 µg/L

Calculation concentration of 3-PBA in brains of rats sacrificated after 14days:

	Area
Sample 1	59368
Sample 2	115699,3
Sample 3	103765,3

Sample 1

0 µg/L

Sample 2

0 µg/L

Sample 3

0 µg/L

Average simples: 0 µg/L

Calculation of 3-PBA in urines of rats sacrificed after 24h:

	Area
Sample 1	123369516
Sample 2	181787279
Sample 3	200621820
Sample 4	358380967
Sample 5	113235427
Sample 6	162789863

Sample 1

(123369516-17550)/111977= 1101.58 µg/L

Sample 2

(181787279-17550)/111977= 1623.28 µg/L

Sample 3

(200621820-17550)/111977= 1791.48 µg/L

Sample 4

(358380967-17550)/111977= 3200.33 µg/L

Sample 5

(113235427-17550)/111977= 1011.08 µg/L

Sample 6

(162789863-17550)/111977= 1453.62 µg/L

Average samples: (1101.58+1623.28+1791.48+3200.33+1011.08+1453.62)/6= 1681.9µg

Calculation of 3-PBA in urines of rats sacrificed after 14days:

	Area
Sample 1	18628900
Sample 2	1968870
Sample 3	1656695
Sample 4	6669439
Sample 5	3077413

Sample 1

(18628900-17550)/111977= 166.21 µg/L

Sample 2

0 µg/L

Sample 3

0 µg/L

Sample 4

0 µg/L

Sample 5

0 µg/L

Average samples: (166.21+0+0+0+0)/5= 33.42 µg/L