



Determination of permethrin and its metabolites in hearts and urines of rats by GC-ECD and GC-MS

ILARIA MERCURI

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Ilaria Mercuri

(n°1131601)

Supervisor (University of Camerino) : Rosita Gabbianelli

Supervisor ISEP: Valentina Domingues

Co-supervisors : Luísa Correia-Sà

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Abstract

Pyrethroids are pesticides very used in agriculture, which tend to replace organophosphate and carbamate insecticides. These pesticides have shown to exhibit cardiotoxicity.

The aim of this study was to assess if cardiotoxicity is due to direct or indirect effects (metabolites) of permethrin on hearts. There were studies 8 rats; three of them were sacrificed after 24 of the end of treatment with permethrin and the other four were sacrificed after 14 days of the end of treatment. Afterwards hearts and urines were collected. The amounts of permethrin and its main metabolite (3-PBA) were evaluated on hearts and urines of female rats which were treated with permethrin and sacrificed the day after and 14 days after the treatment. Moreover has been highlighted the difference of amount of permethrin and its metabolite between rats sacrificed immediately at the end of treatment and those sacrificed after 14 days. The study of permethrin was accomplished by liquid-liquid extraction and GC-ECD. The evaluation of 3-PBA was performed by SPE procedure with 2-PBA as internal standard and gas-chromatography GC-MS. The concentration of permethrin in hearts is basically the same in 24h and 14 days. The 3-PBA concentration in urines decreased 50 times from 24h to 14 days. In hearts the 3-PBA level also decrease but only 2.24 times and a high variation of results were achieved in rats after 14 days.

Key-words: Pesticides, permethrin, metabolites, 3-phenoxybenzoic acid, 2-phenoxybenzoic acid, chromatography, GC-MS, GC-ECD, extraction, derivatization, heart, urine

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List of abbreviations, acronyms and symbols

PERM Permethrin

3-PBA 3-phenoxybenzoic acid

FDA Food and Drug Administration

LD50 The lethal dose

CVD Cardiovascular disease

SPE Solid-phase extraction

GC/MS Gas chromatography mass spectrometry

ECD Electron capture detectors

FAO Food and agriculture organization

1. Pesticide pyrethroid permethrin

Permethrin, also called 3-phenoxybenzyl (1*RS*, 3*RS*, 1*RS*, 3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate (IUPAC name), (1) (Fig.1) was the first synthetic pyrethroid photo-stable enough to be used in agriculture and the most used pyrethroid in USA. Synthetic pyrethroids derive from naturally occurring pyrethrins, which result from extracts of dried chrysanthemum flowers, and they are more stable and more toxic than natural pyrethroids. The most common uses of Permethrin are as an insecticide and a the treatment of ectoparasites (fleas, lice, scabie) on humans and animals, in residential sites and on clothing. Formulations of permethrin are considered pharmaceuticals and they are regulated by the United States Food and Drug Administration (FDA) (2); for example sprays, dusts, fogs, emulsifiable concentrates and creams can be found. Permethrin has a molecular weight of 391.3 g/mol , a solubility of 5.5×10^{-3} mg/L, 6×10^{-3} mg/L and four isomers: (1*S*,*trans*)(1*S*,*cis*) (1*R*,*trans*) (1*R*,*cis*).The *trans*-isomer has a lower toxicity in rats than the corresponding *cis*-isomers and a more rapid degradation.



Fig. 1 Chemical structure of permethrin

2. Toxicity and health effects

According to their chemical structure Pyrethroids can cause:

- T-syndrome (tremors, sensitivity to sensory stimuli, ataxia, convulsions and paralysis)
- CS-syndrome (3) (choreoathetosis, hypersensitivity to external stimuli, salivation and, in some cases, paralysis) (4).

Usually T-syndrome is caused by pyrethroids not containing an alpha-cyano group in their molecule and CS-syndrome derives by pyrethroids with an alpha-cyano group. Many pyrethroids, such as permethrin, exhibit both T and CS characteristics and sometimes these two syndromes combine (6). Permethrin interferes with sodium channels to disrupt the function of neurons, and causes muscles to spasm, culminating in paralysis and death (1,2). Mammals can metabolize in a good way pyrethroids, such as permethrin, before they can affect the nervous system (5). The lethal dose (LD50) for permethrin is variable, ranging from 430 mg/kg body weight to over 4000 mg/kg for rats (7).

Permethrin can induce several toxic effects:

- Neurotoxicity: At high doses tremors, loss of coordination, hyperactivity, increase of body temperature can appear (7). Moreover motor deficits, learning and memory dysfunction can be the result of neuronal cell death caused by Permethrin.
- Immune system toxicity: Even small doses of permethrin 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 Permethrin equivalent to 1/100 of the LD50 (8).

-Reproductive systems effects: Permethrin binds to receptors for androgen (9) and to the peripheral benzodiazepine receptor (which stimulates production of testosterone) (10). Moreover it inhibits the binding of estradiol to the estrogen receptor (11).

-Mutagenic effects: an increase in chromosome aberrations, chromosome fragments and DNA lesions (8) may occur after a permethrin exposure.

-Cancer: According to the World Health Organization 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 (12,13). The risk of prostate cancer in men with a family history of prostate cancer can increase because of Permethrin exposure (14).

2.1 Toxicokinetics

2.1.1 Absorption

Permethrin is absorbed from the gastrointestinal tract, minimally through intact skin and by inhalation of dust and spray mist. Human case studies involving skin applications of permethrin indicated that about 0.5% of the applied dose was absorbed dermally (15). Animal studies showed that there is a quick and substantial absorption of permethrin upon ingestion (16). In one study of rats, about 60% of the orally administered dose was absorbed with an absorption half-life of less than one hour (17). No information was found regarding absorption rates following inhalation of permethrin.

2.1.2 Distribution

Feeding studies using rats indicated that permethrin was rapidly distributed throughout the body. 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 (17,18). 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 (19).

2.1.3 Metabolism

In animal feeding studies using rats, goats, cows and hens, permethrin was metabolized quickly in the liver. Hydrolysis, hydroxylation, oxidation, and conjugation are all involved in the process of metabolism (16,20). While several metabolites of permethrin have been identified, permethrin itself is considered the only compound of toxicological significance (16,2). Permethrin is easily hydrolyzed,

and the hydrolysis product is also easily oxidised in rats in vitro. Permethrin is converted to PBAlc (3-Phenoxybenzyl alcohol) and further to PBAld and PBAcid by mixed function oxidase in rats (Fig.2). Trans-isomers are more rapidly metabolized by hydrolytic (esterase) pathways while cis-isomers are preferentially metabolized by slower oxidative (P450) pathways (21). The trans-isomer of permethrin is mainly catalyzed by the CES1 family and partly by the CES2 family. CES is a serine hydrolase and hydrolases substrates via a ping pong bi-bi mechanism, in which a tetrahedral intermediate is important (22). ES-3 and ES-10, isozymes of CES1 exhibited significant hydrolase activities. The cis-isomer of permethrin, which is metabolized predominantly by P450's in laboratory animals (21), was not detectably metabolized in human liver microsomes in the presence or absence of an NADPH regenerating system (23). 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 coworkers (2002; 2003). The alcohol generated from permethrin, PBAlc is oxidized to PBAld (3-phenoxybenzylaldehyde), and the aldehyde is further oxidized to 3PBA (3-phenoxybenzoic acid). 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 (23). The terminal phase of pyrethroid metabolism is the formation of glucuronide and glycine conjugates (24). These pathways appear similar between laboratory animals and humans. Common metabolites are found in the urine of both laboratory animals and humans (24, 25).

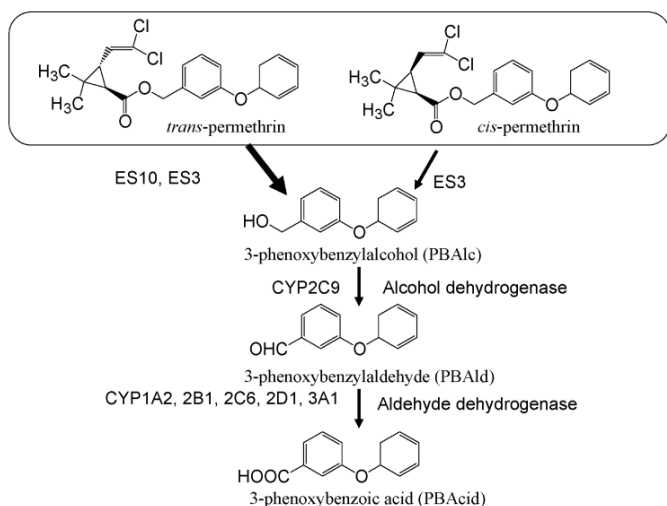


Fig. 2 metabolic pathways of *cis*- and *trans*- permethrins

The 3-phenoxybenzoic acid (3-PBA) metabolite is common to 10 of the 18 pyrethroids registered in the United States, including permethrin, cypermethrin, deltamethrin, esfenvalerate (26). 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) (27). The aromatic alcohol is further oxidized to 3-phenoxybenzoic acid (3PBA) while the cyanohydrin is able to spontaneously rearrange to the phenoxybenzaldehyde in aqueous solutions and then undergo hydroxylation to 3PBA. 3PBA 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. 3PBAcid has been detected in urine from the general population in the United States (0.32 $\mu\text{g/L}$), Germany (2.0 $\mu\text{g/L}$ reference value), Italy (0.88 $\mu\text{g/L}$ urban and 0.71 $\mu\text{g/L}$ rural) and Japan (0.29 $\mu\text{g/L}$;). Levels in occupationally exposed populations such as pest control workers tend to be higher (6.8 $\mu\text{g/g}$ creatinine) (28). Since 3-PBA is a common metabolite for most pyrethroid pesticides, it has been used as a marker of pyrethroid exposure by the Centers for Disease Control and Prevention in the National Health and Nutrition Examination Survey study (29). The elimination half-

life of about 8 h reported for 3-PBA among workers exposed to cypermethrin (30) suggests that 88% of the metabolite is excreted within the first 24 h following exposure.

2.1.4 Excretion

Permethrin and its metabolites are excreted primarily in the urine, but also in the feces (31,16). 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 certain brain and nervous tissue, including the medulla oblongata (15).

2.2 Cardiotoxicity

Cardiovascular disease (CVD) is the leading cause of death in the world. Although the largest risk factors of CVD are smoking, hypertension, diabetes, hypercholesterolemia, environmental pollutions contributing to cardiovascular defects, data from the National Institute of Environmental Health Science (NIEHS) demonstrated a relationship between the levels of airborne particles, environmental agents such as dioxins and pesticides and risk of early death for heart disease. Pyrethroid insecticides are known to modify neuronal sodium channels, where cardiac myocytes are also rich and to interact with calcium ATP-ase (32). A work on rat myocytes shows that type I and type II pyrethroids prolong action potentials and extend the duration of sodium currents measured by voltage clamp (33). In isolated hearts, they augmented the contractile variability producing a cardiac arrhythmogenic potential (33,34). Cardiovascular diseases such as coronary heart disease are associated with increased generation of reactive oxygen species (ROS) (35). Oxidative stress is dependent on the balance between oxygen free radical production and the anti-oxidant capacity of the cell; in its imbalance, oxidative stress may induce reversible or irreversible myocardial damage (36). ROS interact with intracellular signaling pathways involved in the control of vascular function, causing rapid damage to membrane lipids, proteins and DNA (37). Oxidative DNA base modifications have been demonstrated in hearts and isolated myocytes subjected to oxidant injury, including ischemia-reperfusion (38) and indicating that this cell type is susceptible to oxidant-induced DNA damage (39). A response by cells subjected to DNA damage is lesion repair rather than apoptosis or necrosis, which depends on the type and the concentration of toxin interacting with the cells. DNA repair utilizes enzymes that excise oxidized bases before DNA replication (40). Because cardiac myocytes are post-replicative, repair of DNA lesions is important to avoid deterioration of cardiac function that would eventually attend myocyte loss. The endonucleases activity increases in response to oxidative stress induced after permethrin exposure. SOD (superoxide dismutase) and Gpx (glutathione peroxidase)

enzymes play a key role in the protection against oxidative damage. In the heart of adult rats treated with permethrin (PERM) during early life, SOD activity was not modified, while a significant increase in Gpx activity and a consequent reduction in GSH levels were measured. The alteration in the enzymatic antioxidant response may be related to early life exposure to (PERM), where oxidative stress in the myocardium reflects a decrease in GSH content (41). Moreover it is well known that ROS can initiate NF- κ B-mediated transcriptional activation of inflammation genes (42). A wide range of cardiac diseases has been linked with inflammation and cytokine modulation (42). Cytokines have direct and indirect effects on myocardial function and these are time, concentration and subtype-specific (42). The relation between cytokine production and modulation of heart function is linked to the cytokine specific effect on exciting-contraction coupling, where TNF- α , IL-2 (43) and IL-6 modulate sarcoplasmic reticulum Ca²⁺ ATPase activity and IL-1 β affects the inner calcium current (44). It is worth bearing in mind that oxidative stress conditions could also result from the increased pro-inflammatory cytokines levels, because they can induce xanthine oxidoreductase, NADPH oxidase and inducible nitric oxide synthase (iNOS) that consequently increase the level of free radicals leading to deterioration of heart contractility (45). A report of FAO (46) provides that the level of permethrin residue in cardiac muscle was 0.1-0.2 % of dietary inclusion levels. Membrane fluidity of heart cells was reduced in the hydrophilic-hydrophobic region, while the hydrophobic interior results in a more fluid following PERM treatment (47). Moreover high concentration induces perturbation on heart plasma membrane.

3PBALD can cross the membrane more easily than 3PBA. So, while 3PBALD exerts its effect mainly in the hydrophobic core, 3PBA can reduce the fluidity in the inner part of the membrane only at higher concentration and 3PBA, the less lipophilic metabolite, does not exert any damage in hydrophobic region, while its effect is present in the external part of bilayer. 3PBA can induce carbonyl group formation and it 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 3PBA is 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.

3PBA is the first metabolite of permethrin deactivation process and its complete metabolization requires more steps than the other two; this more articulated deactivation process likely leads to a highest ROS formation (48). Therefore METP has the capability to modify the sodium and calcium channels located at the bilayer level, a physicochemical modification of macromolecules inside the membrane could consequently influence also channels activity. Moreover, the oxidation of proteins could contribute to modify other membrane proteins changing the signal within heart cells.

3 Extraction methods: SPE Solid-phase 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. Solid phase extraction 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 animals tissue (49). The affinity of solutes dissolved or suspended in a liquid (mobile phase) for a solid through which the sample 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 with an 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 of 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 are collected in sample tubes.. There is a variety of stationary phases which can separate 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) (49). Sodium chloride (NaCl) can be also added to the sample to improve the extraction efficiency (50). 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. It would be a good practice to transfer the upper layer (containing fewer solids) 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 sensitivity. Clean-up, trace concentration or purification, sample matrix or solvent exchange are required for SPE process. SPE is often used because it switches sample matrix to a form compatible with chromatographic analyses and concentrates analytes for increased sensitivity. Moreover SPE can remove interferences and protect the analytical column from contaminants.

3.1.1 Normal Phase SPE procedure

In a Normal Phase SPE procedure first of all the 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 sample 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 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 (49).

3.1.2 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 (49). A stationary phase of silicon with carbon chains is commonly used. Only non-polar or very weakly polar compounds will adsorb to the surface (49).

3.1.3 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.

4. Analytical methods, 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 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 consists 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 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 spend 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 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. 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.

4.1 GC-ECD

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 β -radiation (i.e., ^{63}Ni , ^3H). 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 (10^{-14} g/s), but has a relatively small linear range ($\sim 10^2$ - 10^3).

4.2 GC-MS

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 for a compound to be analysed 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 undesirable adsorption effects that would otherwise affect the quality of the data obtained. 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 molecules are retained by the column and then elute from the column at different retention times. In that way mass spectrometer capture, ionize, accelerate, deflect and detect the ionized molecules separately. 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.

5. Experimental Part

5.1 Reagents

The reagents used were: hexane, acetonitrile (ACN), magnesium sulfate (MgSO_4), PSA, sodium acetate (CH_3COONa), C18, Chloridric acid (HCl) 1M and 12M, potassium hydroxide 10M (KOH), ethyl acetate ($\text{CH}_3\text{COOCH}_2\text{CH}_3$), metanol (MeOH), ammonium hydroxide pH 10 (NH_4OH), metanol 5% in ethyl acetate, 1,1,1,3,3,3-Hexafluoro -2-propanol (HFIP), N,N-Diisopropylcarbodiimid (DIC), potassium carbonate (K_2CO_3).

5.2 Solutions preparation

12.09mg of permethrin have been weighted in a 5mL flask. With this amount of permethrin were prepared:

- Permethrin solution 1 (1209mg/L)
- Permethrin solution 2 (10mg/L)
- Permethrin solution 3 (500 $\mu\text{g/L}$)
- Permethrin solution 4 (625 $\mu\text{g/L}$)
- Permethrin solution 5 (125 $\mu\text{g/L}$)

Two series of standards for calibration curve were prepared:

1. Concentrations: 0.3mg/L, 0.4mg/L, 0.6mg/L, 0.8mg/L, 0.9mg/L;

2. 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).

On the other hand 6.8gr of CH_3COONa have been taken, put them in a flask and diluted with H_2O until 500mL (Solution B).

The buffer solution has been prepared with 89.25mL of solution A and 160.75mL of 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: 50 $\mu\text{g/L}$
- 3-PBA solution 4: 5 $\mu\text{g/L}$
- 3-PBA solution 5: 2 $\mu\text{g/L}$
- 2-PBA solution 1: 2000 mg/L
- 2-PBA solution 2: 10mg/L
- 2-PBA solution 3: 50 $\mu\text{g/L}$

For 3PBA and 2PBA calibration curves were prepared:

Concentrations 3PBA: 0.01 $\mu\text{g/L}$, 0.05 $\mu\text{g/L}$, 0.1 $\mu\text{g/L}$, 0.3 $\mu\text{g/L}$, 0.5 $\mu\text{g/L}$, 1 $\mu\text{g/L}$, 1.5 $\mu\text{g/L}$

Concentration 2PBA; 20 $\mu\text{g/L}$

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 a pH 10 was prepared through the dilution of pure NH_4OH .

5.3 Sampling

8 Female rats were treated with permethrin from 6 to 21 days of life. Then 3 of them were sacrificed the day after (22 days of life) and the other 5 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.

5.4 Sample preparation

In order to carry out this study, samples (rat's heart) 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 concentration of 3-PBA, 2-PBA and Permethrin, several tests were performed.

For preliminary studies only the buffer solution was used:

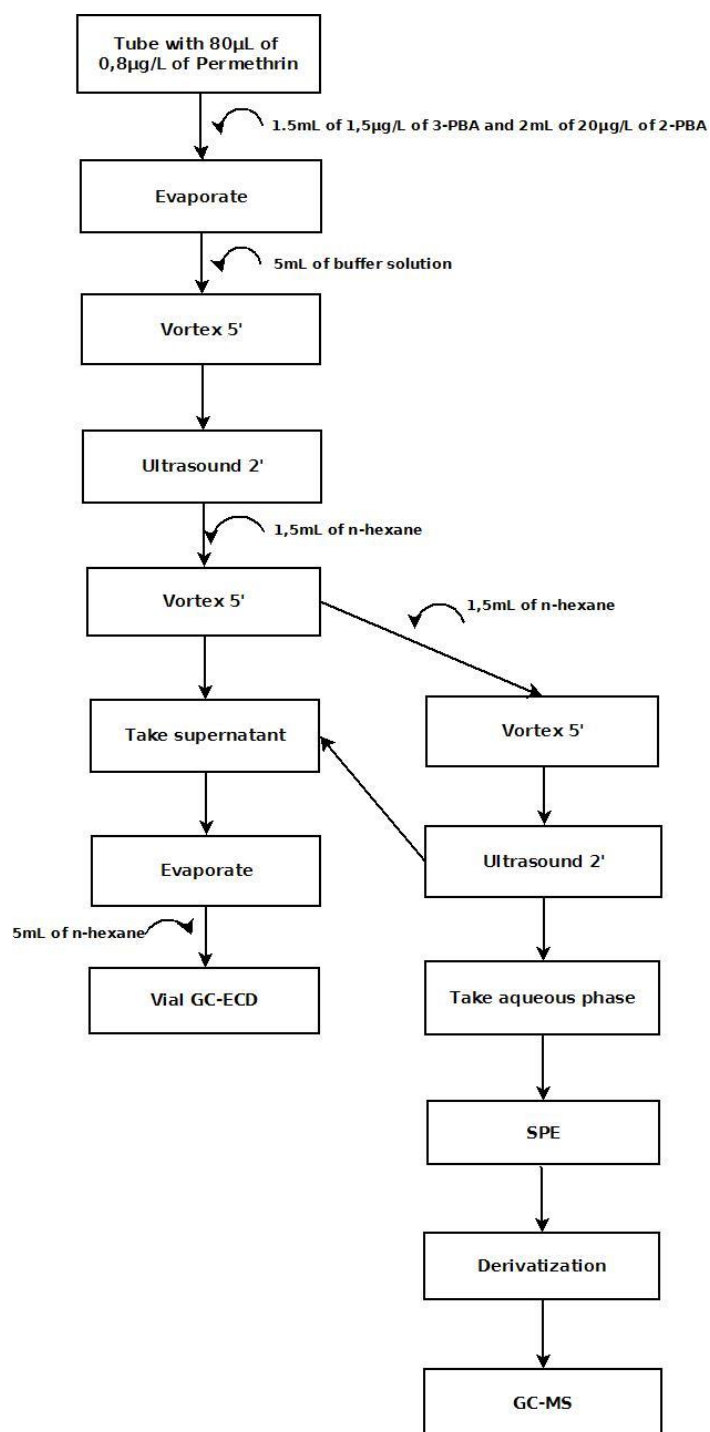


Fig. 3- Scheme of procedure for preliminaries tests

The Solid phase extraction (SPE) consisted in:

Conditioning:

- 5mL of Ethyl acetate (AcOEt)
- 5mL of MeOH
- 5mL of H₂O
- 5mL of HCl

Add sample:

- 5mL of sample

Cleaning:

- 5mL of HCl
- 5mL of NH₄OH

Drying

Elution:

- 5mL of
- 5% MeOH in AcOEt

Before injection in GC a derivatization was performed, the procedure is described in figure 4.

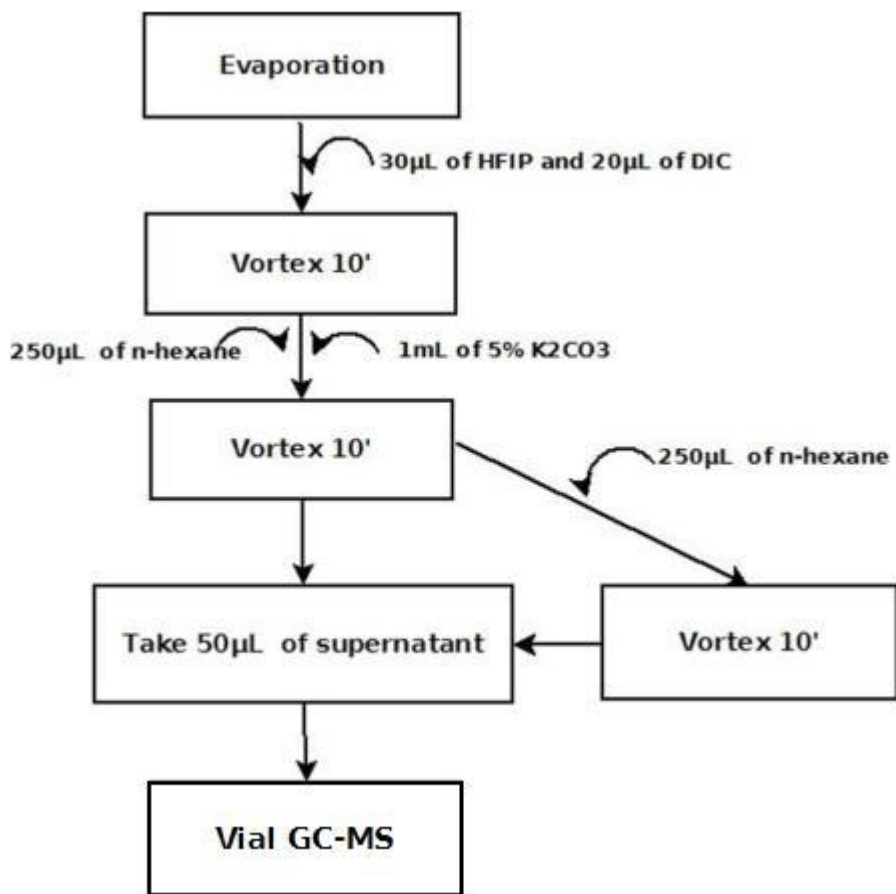


Fig.4- Scheme of derivatization procedure

Several hearts obtained from rats, not treated with permethrin, were used in order to standardize the method. The general procedure with hearts is described in figure 5.

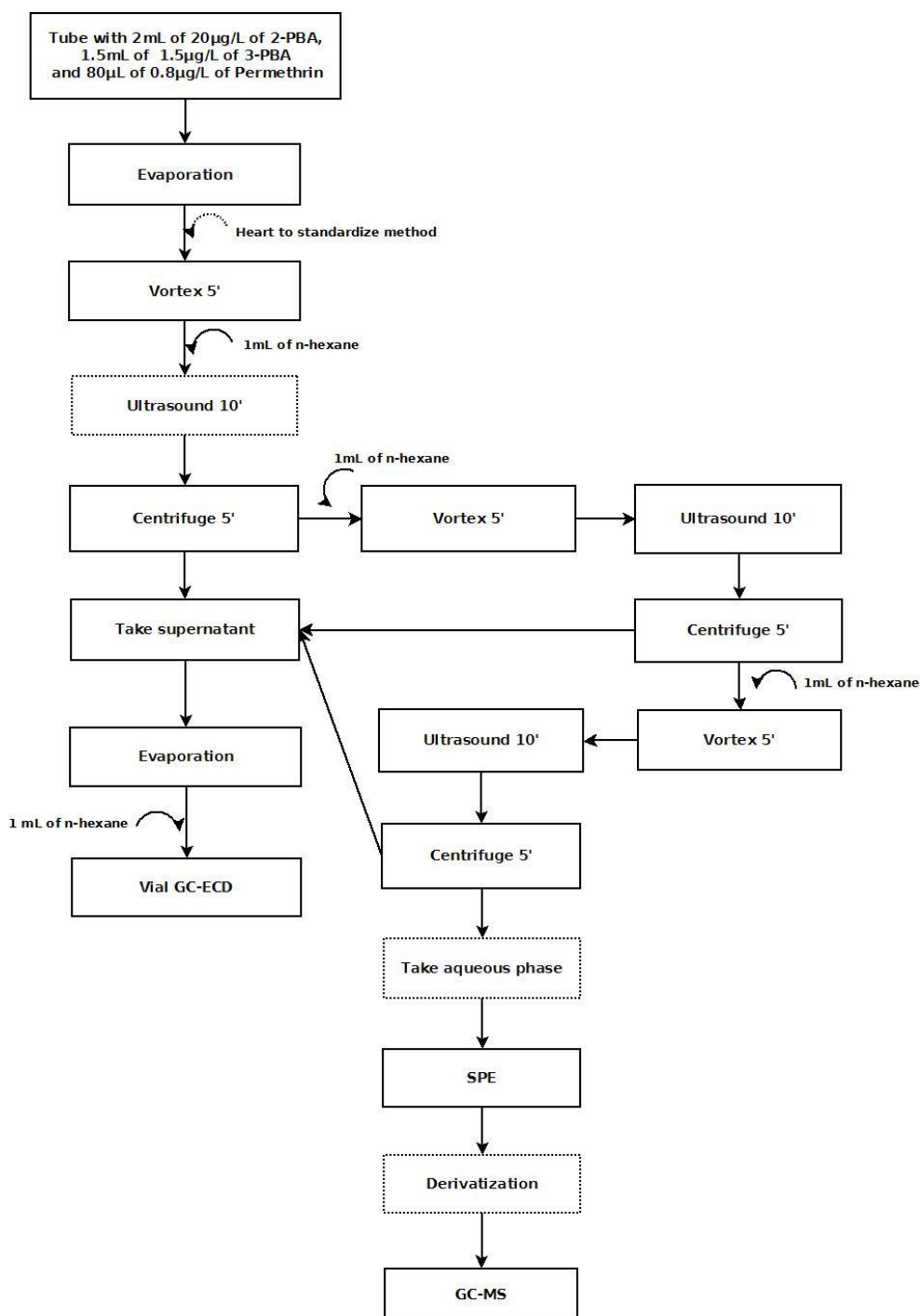


Fig. 5- Scheme of procedure to standardize the method

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

- At first samples have been filtrated with filter paper in the beginning of test.

Then only 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 a clear separation between the aqueous phase and the supernatant.
- The procedure of derivatization has been modified; 150 μ L of n-hexane were added twice during liquid-liquid extraction instead of 250 μ L of n-hexane, to improve the concentration of 3-PBA and 2-PBA.

In order to verify the method, several tests were performed using samples from rats treated with Permethrin that are described in Figure 6:

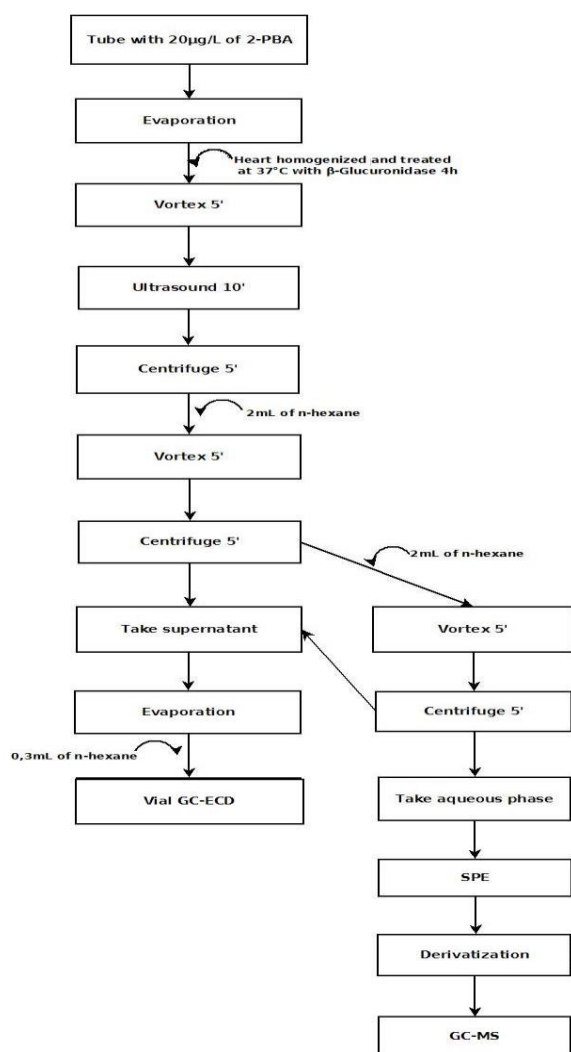


Fig.6-Scheme of procedure with samples from rats treated with permethrin

The permethrin, 3-PBA and 2-PBA -data from hearts from permethrin treated rats were obtained with the procedure described in figure 7.

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

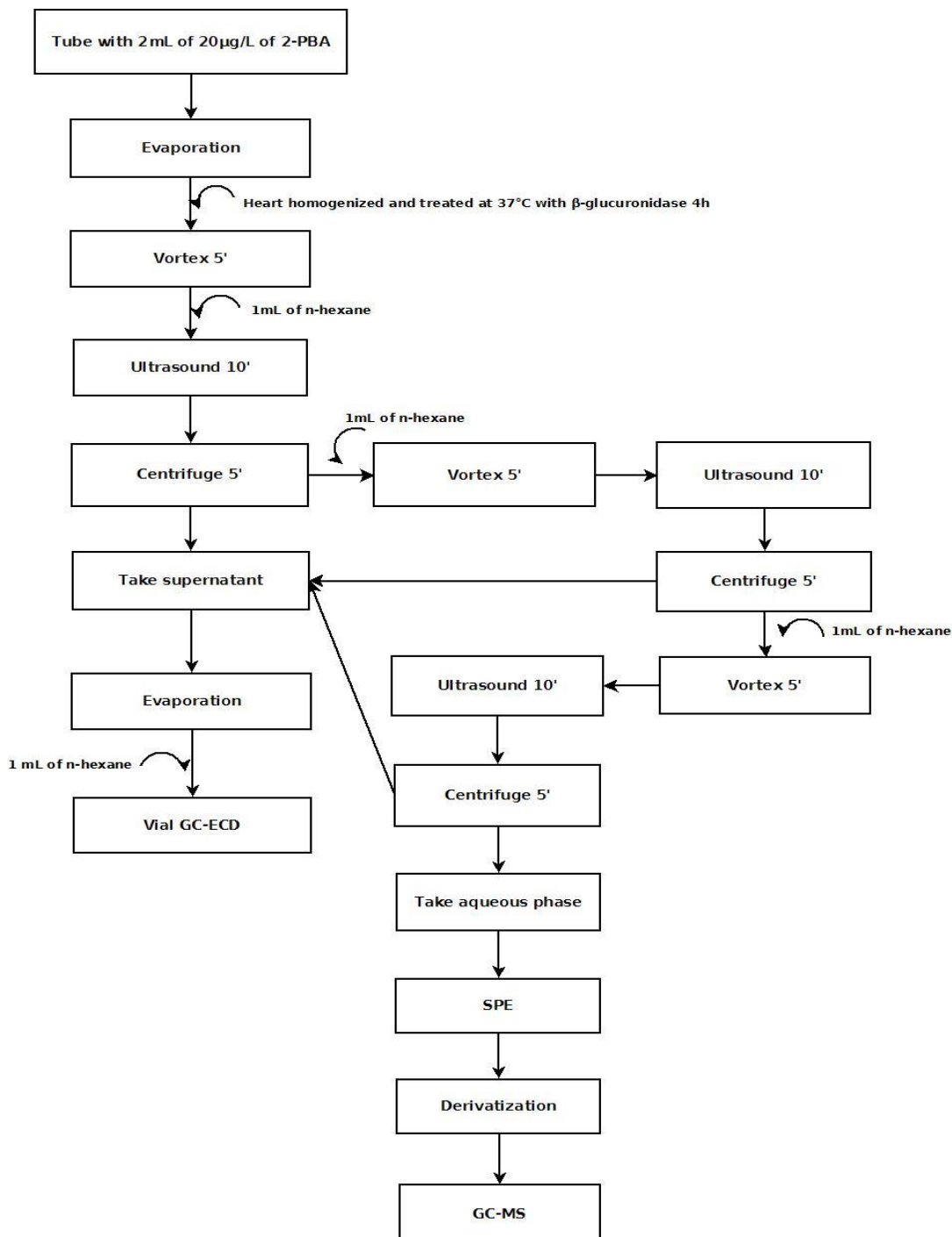


Fig.7-Scheme of procedure with samples from rats treated with permethrin

3-PBA values from urines were obtained with the procedure described in figure 8.

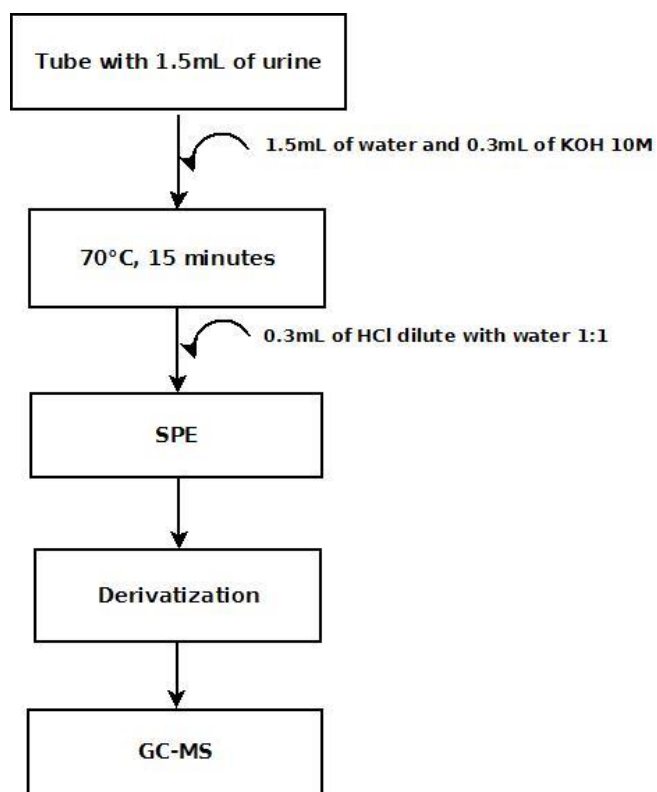


Fig.8: Scheme of procedure with urines from rats treated with permethrin

5.5 Results and discussion

5.5.1 Chromatographic results

5.5.1.1 GC-ECD retention time, linearity, calibration curve Permethrin

A solution containing permethrin was injected in GC-ECD and the retention time was recorded. Figure 9 shows the chromatogram obtained.

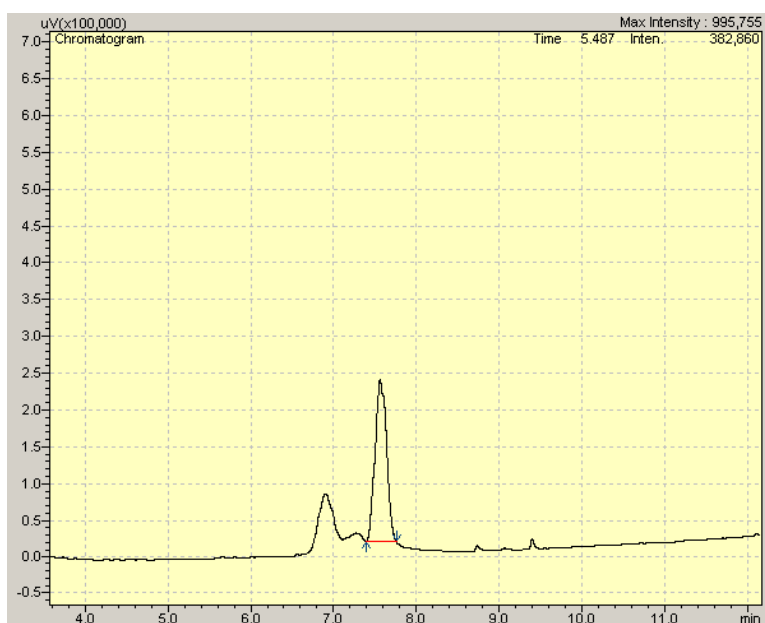


Fig.9- Chromatogram retention time permethrin

Five standards of permethrin were injected in GC-ECD. Figure 10 shows the achieved calibration curve for Permethrin. It was observed linearity between 0.3 to 0.9 $\mu\text{g/L}$ of permethrin in n-hexane. A R^2 higher than 0.9842 was achieved.

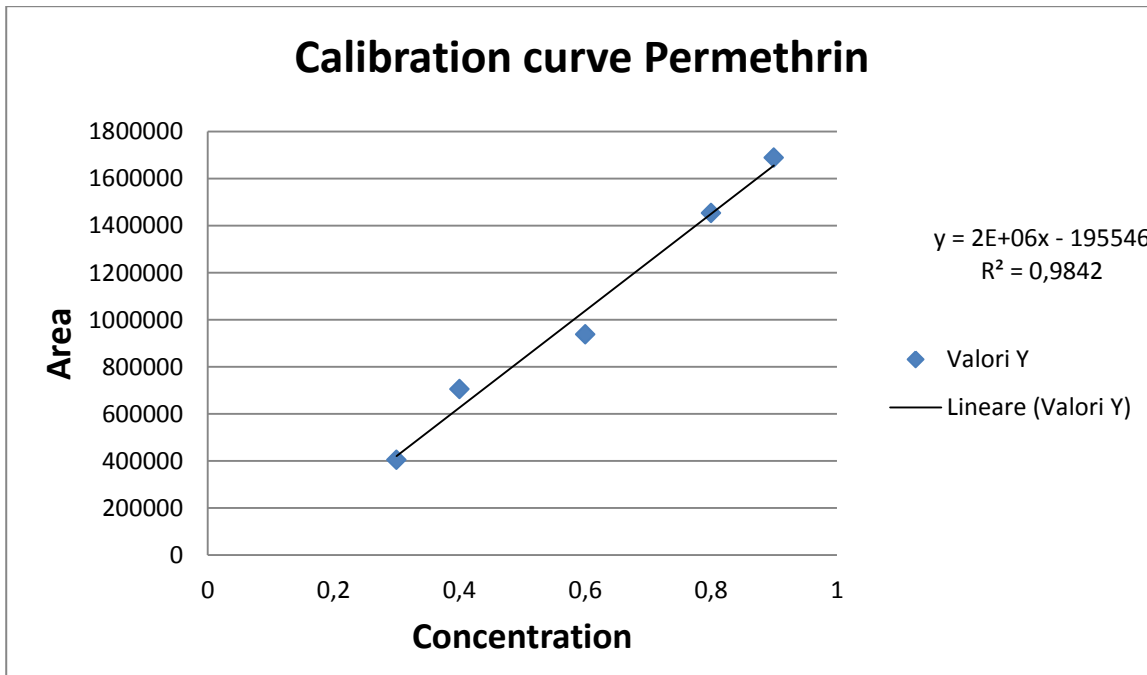


Fig. 10- Calibration curve Permethrin

5.5.1.2 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 11 shows the chromatogram obtained.

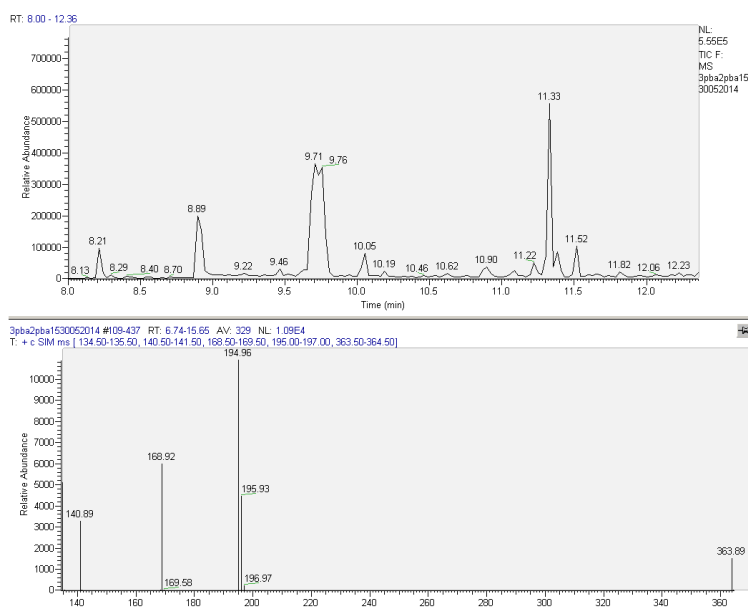


Fig.11-chromatogram retention times 3-PBA and 2-PBA

In figure 12 is shown the calibration curve for 3-PBA. It was observed linearity between 0.01 to 1.5 $\mu\text{g/L}$ of 3-PBA. A R^2 higher than 0.9864 was achieved.

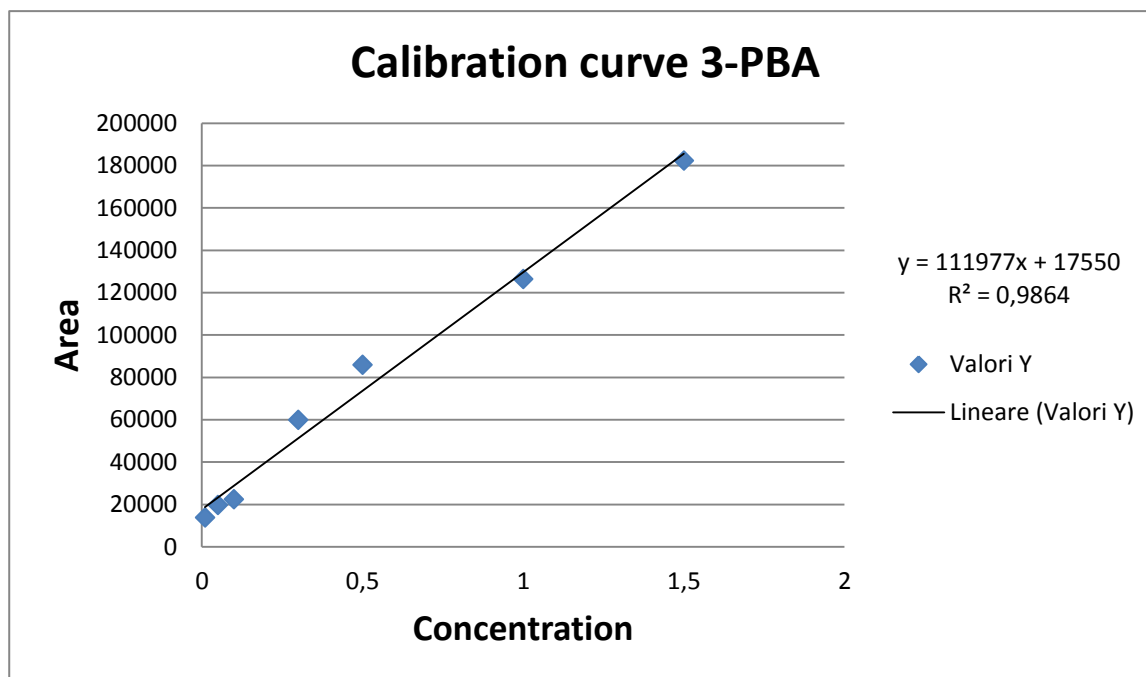


Fig. 12- Calibration curve 3-PBA

The achieved retention time of 2-PBA was 8.9 min as is shown in figure 11. The average area obtained in all chromatograms with 20 $\mu\text{g/L}$ was 497601.2.

5.5.2 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 recoveries 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 13:

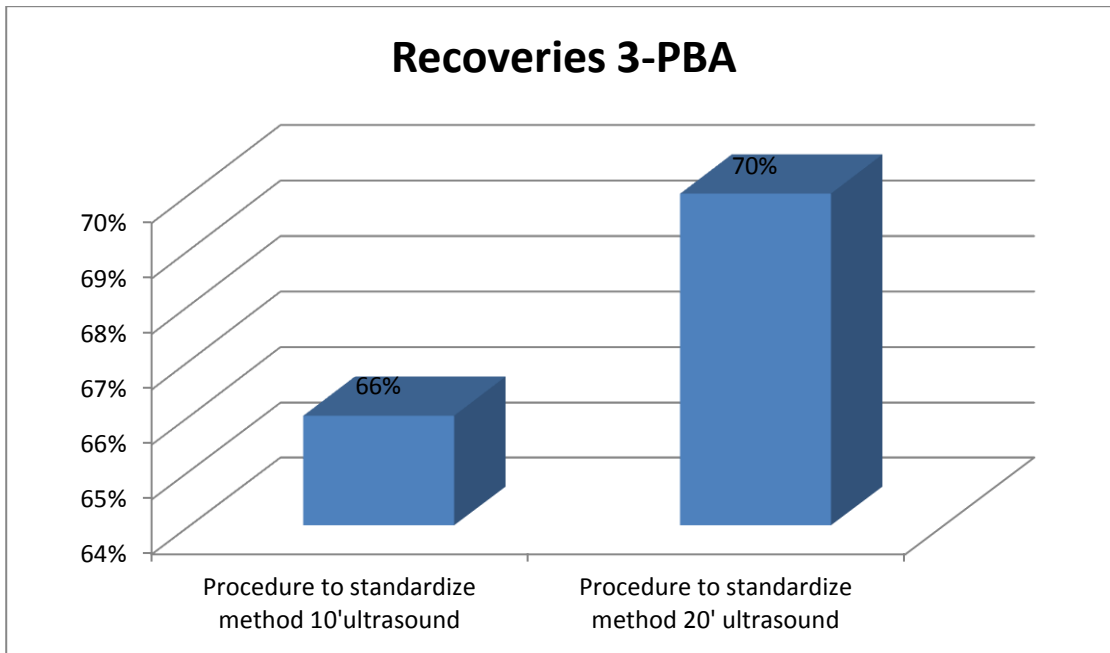


Fig. 13- Recoveries of 3-PBA obtained in samples with different procedures

The highest recovery is obtained with the procedure that uses 20 min in ultrasounds, achieving 70% of recovery.

The recoveries of 2-PBA obtained with different procedures are shown in figure 14:

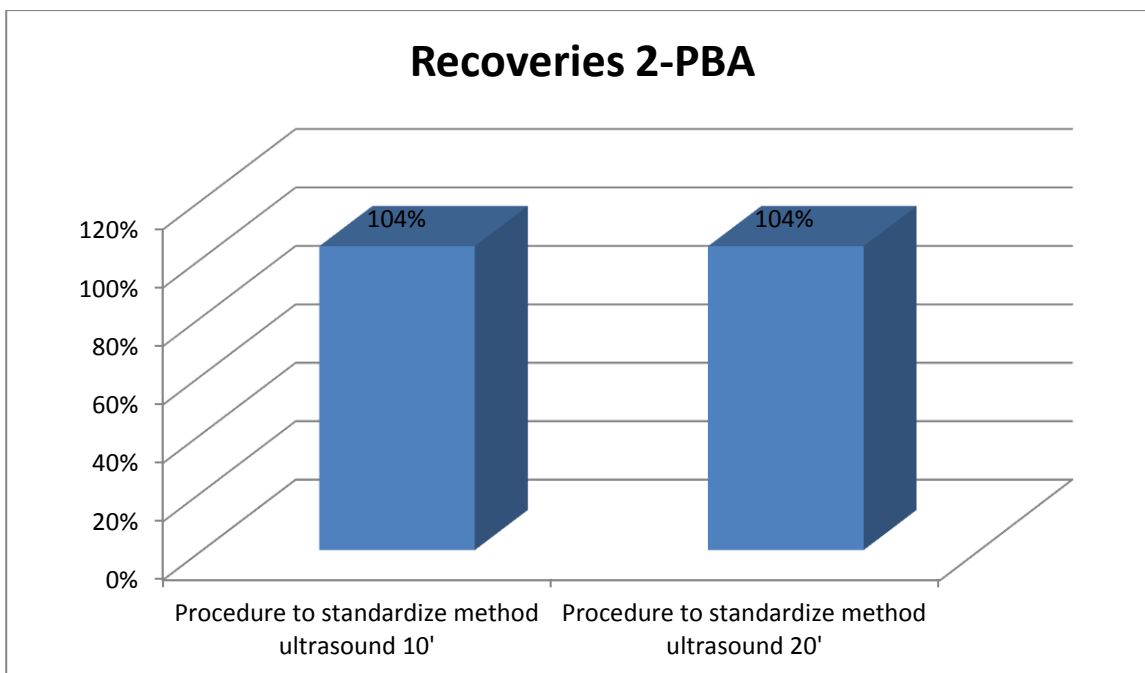


Fig. 14-Recoveries of 2-PBA obtained with different procedures

Both procedures, 10 minutes and 20 minutes in ultrasounds achieved recoveries of 2-PBA near 100%. It was selected 20 minutes because 3-PBA reached better results.

The amounts of Permethrin and 3-PBA in hearts from female rats treated with permethrin were obtained using the equations from the calibration curves. Figure 15 shows the parallel between quantities of Permethrin in hearts of female rats treated with permethrin and sacrificed the day after the treatment (22 days of life) and hearts of female rats treated with permethrin and sacrificed 14 days after the treatment (35 days of life).



Fig. 15: Concentrations of Permethrin in hearts

Permethrin was found in hearts of rats sacrificed after 24h of the end of treatment with permethrin and even in hearts of rats sacrificed after 14 days. The concentration of Permethrin in rats sacrificed after 24 hours is a little bit lower than the concentration of permethrin in hearts of rats sacrificed after 14 days.

Figure 16 shows the parallel between quantities of 3-PBA in hearts of female rats treated with permethrin and sacrificed the day after the treatment (22 days of life) and

hearts of female rats treated with permethrin and sacrificed 14 days after the treatment (35 days of life).

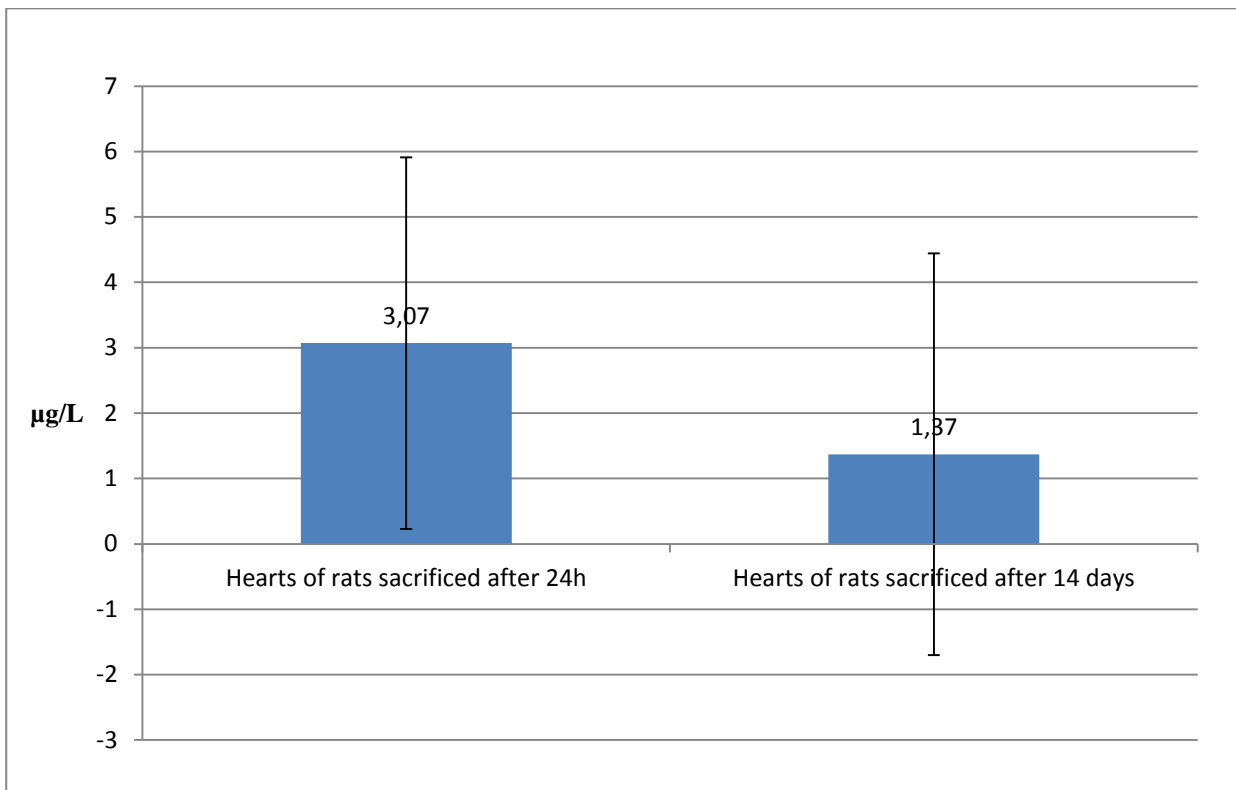


Fig.16- Concentrations of 3-PBA in hearts

3-PBA was found both in hearts of rats sacrificed after 24 hours and after 14 days of the end of treatment with permethrin. The concentration of 3-PBA in hearts of rats sacrificed after 24 hours is higher than that one in hearts of rats sacrificed after 14 days (2.24 times greater). This results are in agreement with literature (30) where the excretion half time of permethrin's metabolite is measured at 24 hours following exposure.

This work demonstrated as other studies, that both Permethrin and its metabolite 3-PBA get to the heart. Consequently it can be deduced that cardio-toxicity is due both to direct and indirect effects of Permethrin.

The amounts of 3-PBA in urines of female rats treated with permethrin were calculated using the equations of calibration curves.

Figure 17 shows the quantities of 3-PBA in urines of rats sacrificed the days after and 14 days after the treatment with permethrin.

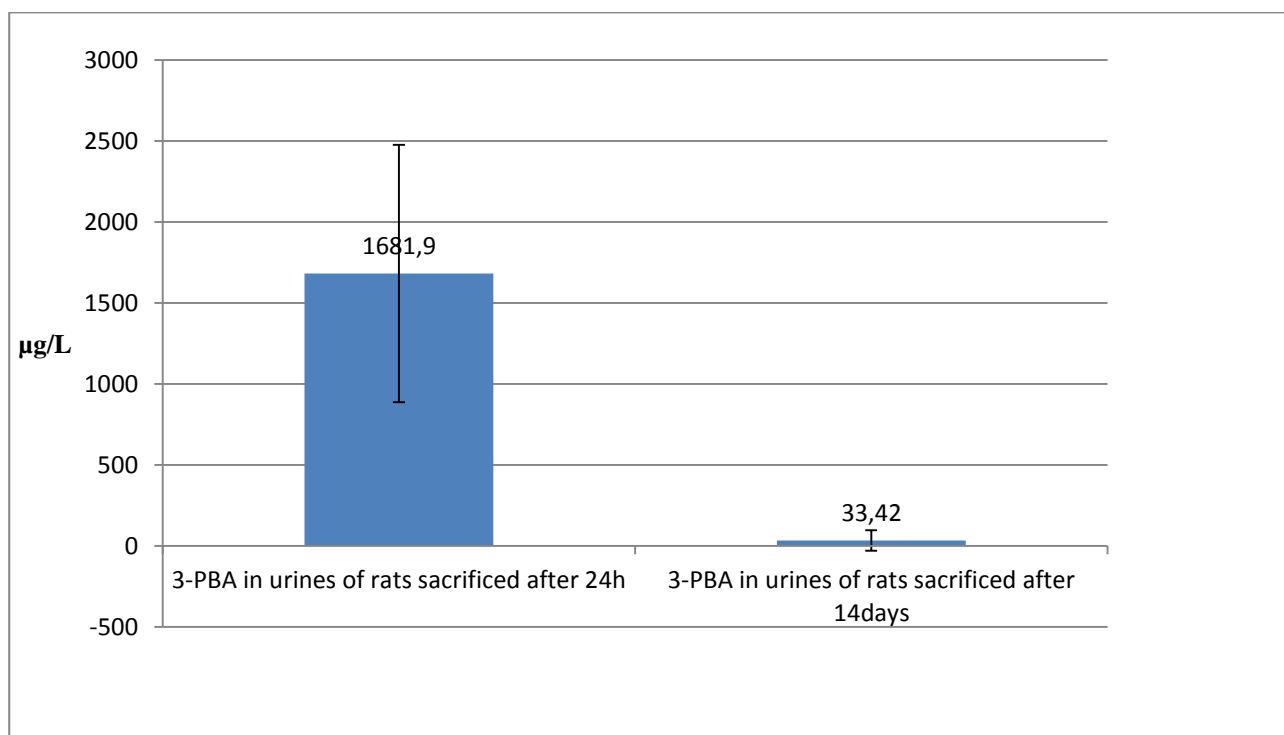


Fig. 17: Concentrations of 3-PBA in urines

As it's possible to see from figure 17, 3-PBA was found both in urines of rats sacrificed after 24 hours and after 14days of the end of treatment with permethrin. The concentration of 3-PBA in urines of rats sacrificed after 24hours is almost 50 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 results published by other authors (30).

6. Conclusions

A concentration of 0.11 µg/L of Permethrin was found in hearts of rats sacrificed after 24 hours. A similar concentration (0,12 µg/L) has been found in hearts of rats sacrificed after 14 days of the end of treatment.

The pyrethroid metabolite 3-PBA has been also found both in hearts of rats sacrificed after 22 days of life and after 35 days of life. The concentration of 3-PBA found in hearts of rats sacrificed after 24 hours resulted higher than the concentration of 3-PBA in hearts of rats sacrificed after 14 days. In urines of rats sacrificed 24 hours after the end of treatment was found a concentration of 3-PBA almost 50 times greater than the concentration of 3-PBA in urines of rats sacrificed after 14 days. While the concentration of permethrin in hearts grows slightly day by day, the concentration of 3-PBA decreases. Moreover even the concentration of 3-PBA in urines decreases between rats sacrificed after 24 hours and rats sacrificed after 14 days after the treatment with permethrin. That results are in agreement with the time of excretion of 3-PBA, which is within the first 24h following permethrin exposure.

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Calculations Permethrin solutions:

$$C1*V1=C2*V2$$

- Permethrin solution 1 = $12.090\mu\text{g}/10*10^{-3} = 1209 \text{ mg/L}$

- Permethrin solution 2 = $1209\text{mg/L} * V1 = 10\text{mg} * 5000\mu\text{L}$

$$V1 = 41.36\mu\text{L}$$

Then 41.36 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 3 = $10\text{mg/L} * V = 0.5\text{mg/L} * 1000\mu\text{L}$

$$V_{m2} = 50\mu\text{L}$$

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 = $500\mu\text{g/L} * V = 0.625 \mu\text{g/L} * 5000 \mu\text{L}$

$$V = 6.25 \mu\text{L}$$

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: $0.625 \mu\text{g/L} * V = 0.125 \mu\text{g/L} * 5000 \mu\text{L}$

$$V = 1000 \mu\text{L}$$

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

$$10\text{mg} \cdot V = 0.3\mu\text{g/L} \cdot 1000\mu\text{L} \quad V=30\mu\text{L}$$

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

$$10\text{mg} \cdot V = 0.4\mu\text{g/L} \cdot 1000\mu\text{L} \quad V=40\mu\text{L}$$

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

$$10\text{mg} \cdot V = 0.6\mu\text{g/L} \cdot 1000\mu\text{L} \quad V=60\mu\text{L}$$

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

$$10\text{mg} \cdot V = 0.8\mu\text{g/L} \cdot 1000\mu\text{L} \quad V=80\mu\text{L}$$

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

$$10\text{mg} \cdot V = 0.9\mu\text{g/L} \cdot 1000\mu\text{L} \quad V=90\mu\text{L}$$

90 μ L 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 solution 1 = $10000\mu\text{g} / 5 \times 10^{-3}\text{L} = 2000\text{mg/L}$
- 3-PBA solution 2 = $2000\text{mg/L} * V = 10\text{mg/L} * 10\text{mL}$

$V = 0.05\text{mL}$

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

- 3-PBA solution 3 = $10\text{mg/L} * V = 0.05\text{mg/L} * 10\text{mL}$

$V = 0.05\text{mL}$

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

- 3-PBA solution 4 = $10\text{mg/L} * V = 50\mu\text{g/L} * 10\text{mL}$

$V = 0.05\text{mL}$

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

- 3-PBA solution 5 = $50\mu\text{g/L} * V = 2\mu\text{g/L} * 10\text{mL}$

$V = 0.4\text{mL}$

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

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

- 2-PBA solution 1 = $10000\mu\text{g} / 5 \cdot 10^{-3}\text{L} = 2000\text{mg/L}$
- 2-PBA solution 2 = $2000\text{mg/L} \cdot V = 10\text{mg/L} \cdot 10\text{mL}$

$V = 0.05\text{mL}$

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

- 2-PBA solution 3 = $10000\mu\text{g/L} \cdot V = 50\mu\text{g/L} \cdot 10\text{mL}$

$V = 0.05\text{mL}$

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: 0.01 μ g/L, 0.05 μ g/L, 0.1 μ g/L, 0.3 μ g/L, 0.5 μ g/L, 1 μ g/L, 1.5 μ g/L

3-PBA calibration curve

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

$$2\mu\text{g/L} * V = 0.01\mu\text{g/L} * 5\mu\text{L} \quad V = 25\mu\text{L}$$

$$2\mu\text{g/L} * V = 0.05\mu\text{g/L} * 5\mu\text{L} \quad V = 125\mu\text{L}$$

$$2\mu\text{g/L} * V = 0.1\mu\text{g/L} * 5\mu\text{L} \quad V = 250\mu\text{L}$$

$$2\mu\text{g/L} * V = 0.3\mu\text{g/L} * 5\mu\text{L} \quad V = 750\mu\text{L}$$

$$2\mu\text{g/L} * V = 0.5\mu\text{g/L} * 5\mu\text{L} \quad V = 1.25\text{mL}$$

$$2\mu\text{g/L} * V = 1\mu\text{g/L} * 5\mu\text{L} \quad V = 2.5\text{mL}$$

$$2\mu\text{g/L} * V = 1.5\mu\text{g/L} * 5\mu\text{L} \quad V = 3.75\text{mL}$$

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

	Area
Sample 1	17525.13
Sample 2	27390
Sample 3	27245.45

Table 3: Permethrin area in hearts of rats sacrificed after 24hours

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

Sample 1

$$(17525.13+195546)/ 2*10^6= 0.107\mu\text{g/L}$$

Sample 2

$$(27390+195546)/ 2*10^6= 0.112\mu\text{g/L}$$

Sample 3

$$(27245.45+195546)/ 2*10^6= 0.111\mu\text{g/L}$$

Average samples: $(0.107+0.112+0.111)/3= 0.110 \mu\text{g/L}$

Calculation concentration of permethrin in hearts of rats sacrificed after 14days

	Area
Sample 1	32633.4
Sample 2	33668.8
Sample 3	11623.1
Sample 4	35224.6

Sample 5	56397.4
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Table 4: permethrin area in hearts of rats sacrificed after 14 days

Sample 1

$$(32633.4+195546)/ 2*10^6= 0.114\mu\text{g/L}$$

Sample 2

$$(33668.8+195546)/ 2*10^6= 0.115\mu\text{g/L}$$

Sample 3

$$(11623.1+195546)/ 2*10^6= 0.104\mu\text{g/L}$$

Sample 4

$$(35224.6+195546)/ 2*10^6= 0.115\mu\text{g/L}$$

Sample 5

$$(56397.4+195546)/ 2*10^6= 0.126\mu\text{g/L}$$

$$\text{Average samples: } (0.114+0.115+0.104+0.115+0.126)/5= 0.12\mu\text{g/L}$$

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

	Area
Sample 1	436054.7
Sample 2	236594
Sample 3	645277.7

Table 5: 3-PBA area in hearts of rats sacrificed after 24 hours

$$\text{Equation calibration curve 3-PBA : } y=111977x + 17550$$

Sample 1

$$(436054.7-17550)/111977= 3.59 \mu\text{g/L}$$

Sample 2

$$0 \mu\text{g/L}$$

Sample 3

$$(645277.7-17550)/111977= 5.61 \mu\text{g/L}$$

$$\text{Average samples : } (3.59+5.61+0)/3= 3.07\mu\text{g/L}$$

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

	Area
Sample 1	86913
Sample 2	787280
Sample 3	197933.3
Sample 4	269918.5
Sample 5	121842.3

Table 6: 3-PBA area in hearts of rats sacrificed after 14 days

Sample 1

$$0\mu\text{g/L}$$

Sample 2

$$(787280-17550)/111977= 6.87 \mu\text{g/L}$$

Sample 3

$$0\mu\text{g/L}$$

Sample 4

0µg/L

Sample 5

0µg/L

Average samples : $(0+6.87+0+0+0)/5= 1.37 \mu\text{g/L}$

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

	Area
Sample1	123369516
Sample 2	181787279
Sample 3	200621820
Sample 4	358380967
Sample 5	113235427
Sample 6	162789863

Table 7: 3-PBA area in urines of rats sacrificed after 24 hours

Sample 1

$(123369516-17550)/111977= 1101.58 \mu\text{g/L}$

Sample 2

$(181787279-17550)/111977= 1623.28 \mu\text{g/L}$

Sample 3

$(200621820-17550)/111977= 1791.48 \mu\text{g/L}$

Sample 4

$(358380967-17550)/111977= 3200.33 \mu\text{g/L}$

Sample 5

$$(113235427-17550)/111977= 1011.08 \mu\text{g/L}$$

Sample 6

$$(162789863-17550)/111977= 1453.62 \mu\text{g/L}$$

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

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

Table 8: 3-PBA area in urines of rats sacrificed after 14 days

Sample 1

$$(18628900-17550)/111977= 166.21 \mu\text{g/L}$$

Sample 2

0 $\mu\text{g/L}$

Sample 3

0 $\mu\text{g/L}$

Sample 4

0 $\mu\text{g/L}$

Sample 5

0 µg/L

Average samples: $(166.21+0+0+0+0)/5= 33.42 \text{ µg/L}$