INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO

MESTRADO EM ENGENHARIA QUÍMICA

isep

Otimização metodológica para a avaliação do metabolito de permetrina em pêlo de rato

CLAUDIA PELLEGRINI

março de 2017

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Methodology optimization for the evaluation of permethrin metabolite in rat's hair

By Claudia Pellegrini

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Finally, I want express my deep thankfulness for my parents, that gave me the opportunity to find my way always holding my hand. I could not be more proud to be your daughter.

ABSTRACT

Pyrethroids are insecticides with worldwide commercial and retail usage and they provide benefits such as increased supply of crops, fruits and vegetables, as well as control of diseases transmitted through insects that affect humans and livestock. Despite their benefits, many pesticides are neurotoxic compound that induce acutely toxic effect at high doses and can potentially exert more subtle effects at lower doses though different exposure routes. The permethrin is one of the most widely used pyrethroids that in mammals, once absorbed, is rapidly distributed to various organs/tissues and is transformed into metabolites, 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid (DCCA) and the 3-phenoxybenzoic acid (3-PBA). The 3-PBA has been used in numerous studies for the biomonitoring of exposure in occupationally and environmentally exposed individual. The aim of our work was to develop an analytical method for the quantification of 3-PBA in hair samples. Hair is a non-invasive biological material useful in the biomonitoring of trace elements because it is a vehicle for substance excretion from the body, and it permits evaluating long-term exposure. Our animal's model are rat exposed to permethrin in early days of them life and we took hair rat's samples after 22 and 60 days. The hair has been analyzed with the purpose to assess if 3-PBA content could be used as biomarkers on this sample. The analytical method comprises five main steps: preparation of hair samples, decontamination step, extraction of analytes, derivatization and subsequent instrumental analysis by gas chromatography (GC) and detection using ion trap mass spectrometry (MS). The analytes were isolated from hair with recoveries of 78%. The limit of detection and quantification ensured by the method are 0.92 µg/mg and 3.06 µg/mg, respectively. The results presented in this work show the potential of GC-MS based method for the extraction of 3-PBA in hair of rat aged 22 days, but I could not be determined the 3-PBA concentration in hair of rat aged 60 days.

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CHAPTER 1: BACKGROUND MOTIVATION AND PROJECT GUIDELINE

1.1. Background motivation

In modern agriculture, chemical pesticides are widely used and play an essential role in agriculture production. Most chemical pesticides are designed to be toxic to a lot of organisms, so by their very nature pose risks to environment and the human health.

Exposure to pyrethroids, a class of insecticides, has been widely documented in humans, including exposure of pregnant women, infants, and children (Yang et al., 2016).

Pyrethroids induce an interference with sodium channels, receptor-ionophore complexes, and neurotransmitters. They slow the activation, or opening, of VSSCs (voltage-sensitive sodium channels). In addition, they retard the rate of VSSC inactivation. The result is that sodium channels open at more hyperpolarized potentials and are held open longer, allowing more sodium ions to cross and depolarize the neuronal membrane (Imamura et al., 2000).

There is increasing evidence showing an association between pesticides exposures and human health problems, such as neurotoxicity, immunotoxicity, cardiotoxicity, hepatotoxicity, cytotoxicity, digestive system toxicity and reproductive, genotoxic, and haematotoxic effects. Further are not less important the cancer and nervous system disorders that they come after the resumed contact with pyrethroids (Wang et al., 2016).

In this regard, it was proved that the exposure to various pyrethroids are associated with increased risk for Parkinson disease, a progressive movement disorder that is attributed to the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (Ascherio et al., 2006).

One of the most widely used pyrethroids is permethrin (**PER**). Products containing permethrin are very common (there are currently more than 1400 registered products

containing it). They may be used in public health mosquito control programs and they can be present in a lot of ambience, as food and feed crops, ornamental lawns, livestock, pets and clothing. In mammals, once absorbed, permethrin is rapidly distributed to various organs/tissues and is transformed into metabolite, **DCCA** [3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid] and 3-phenoxybenzoic alcohol, oxidized to form **3-PBA** (3-phenoxybenzoic acid) (Nakamura et al., 2007).

Several analytical strategies have been proposed to measure the different permethrin isomers and their metabolites in biological matrices. As permethrin is a non-polar and semivolatile compound, it is more commonly analysed by gas chromatography (GC) coupled with flame ionization detection (FID), electron capture detection or mass spectrometry (MS) (Du, Yan, She, Liu, & Yang, 2010).

The metabolites cis-DCCA, trans-DCCA and 3-PBA can also be analysed by GC using a derivatization step (Corrion, Ostrea, Bielawski, Posecion, & Seagraves, 2005).

Permethrin has been previously monitored in blood or plasma and organs such as the brain, whereas metabolites have been primary measured in urine or plasma.

In this thesis we want prove if it is possible to evaluate the permethrin exposition by analyse the metabolite in rats hair.

Hair is a biological material that can be easily acquired by non-invasive sampling for biomonitoring of many microelements (as drugs of abuse, medicines, metals, various xenobiotics, environmental pollutants) (Kavvalakis et al., 2014) because it is a vehicle of substance excretion from the human body and it permits evaluating long-term exposure of metals and microelement accumulation.

Moreover, hair is a biological material better accepted by the population, than blood samples, and its transport are very simple. Besides it is stable matrix and it is available for repeated determinations over time (Nasuti, Ferraro, Giovannetti, Piangerelli, & Gabbianelli, 2016a).

For this reason, it represents an attractive choice for occupational and environmental surveys.

1.2. Thesis objectives

The objective of this project was to develop an analytical method for observe the metabolite of permethrin accumulation in rat’s hair. The method was applied on various type of samples, with different age (22 and 60 days), exposed to several substances. In the specific the rats were exposed to permethrin, permethrin-vitamin E and permethrin-alkaline water. Vitamin E and alkaline water were and compound with antioxidant effect, for that reason we chose them to verify if they can influence the metabolism of the permethrin. The hair was submitted to distinct phases: decontamination step, for remove eventually irrelevant materials; extraction step, using a ultrasonic bath for 2 h; derivatization process followed by analysis for separated and identified the several molecules. Specifically these analysis were performed by gas chromatography and detection using ion trap mass spectrometry.

1.3 Organization and structure

The thesis is compound of 6 chapters. The first concern the background motivation and project guideline, specifically it explains the important of 3-PBA assay in rat’s hair and the target of the current project.

The chapters 2 introduce a lots of signifying subjects, like pyrethroids type I and II, permethrin, Vitamin E, alkaline water and gas chromatography mass spectrometry, frequently mentioning in the thesis and the analytical methods used for the treatment of hair sample.

The third chapter show the previous studies regarding the determination of 3-PBA in biological samples (as urine, blood, plasma).

The chapter number 4 is divided in several paragraphs to uncover the materials and methods used in our project. This chapter is composed by materials (reagents, samples and equipment) and methods (optimization of extraction procedure, recoveries, preparation of hair samples, decontamination step, extraction step, derivatization process, calibration curve, samples analysis and conversion of the concentration)

The chapter number 5 is results and discussion and in this are shown the results of our work and it is explained how we have proceeded for the elaboration of the method.

In particular all the results are reported in graphs and there are different type of chromatography for highlight how it was possible to get the results by the gas chromatograph mass spectrometry.

In the chapter number 6 are exposed our conclusions about the project and the results obtained and the potentials of the gas chromatography mass spectrometry method for the extraction of 3-PBA in rat's hair.

Bibliography used during the development of this work may be consulted at the References section and additional information are in the annex section.

CHAPTER 2: INTRODUCTION

2.1 Pyrethroids

Pyrethroids are a class of neurotoxics insecticides with worldwide commercial and retail usage. They were derived in the 1920s by Hermann Straudinger and Leopold Ružička from natural compounds, the pyrethrins, isolated from plants of the Chrysanthemum genus.

The first-generation of pyrethroids were developed in the 1960s by a team of Rothamsted research scientists and included bioallethrin, tetramethrin, resmethrin and bioresmethrin. They are more active that the natural pyrethrin but are unstable in sunlight. By 1974, the Rothamsted team had discovered a second generation of more persistent compounds notably: permethrin, cypermethrin and deltamethrin. They are substantially more resistant to degradation by light and air, thus making them suitable for use in agriculture (Matsuda, 2011).

The main commercially available pyrethroids include allethrin, bifenthrin, cyfluthrin, λ cyhalothrin, cypermethrin, deltamethrin, permethrin, d-phenothrin, resmethrin, and tetramethrin.

All pyrethroids contain several common features: an acid moiety, a central ester bond, and an alcohol moiety (Figure 1). The acid moiety contains two chiral carbons; thus, pyrethroids typically exist as stereoisomeric compounds. Furthermore, some compounds also contain a chiral carbon on the alcohol moiety, which allows for three chiral carbons and a total of eight different stereoenantiomers. The mechanism of pyrethroid action is through interference with VSSCs (voltage sensitive sodium channels) and it is stereospecific, indicating the presence of specific binding sites (Hossain, Liu, & Richardson, 2016).

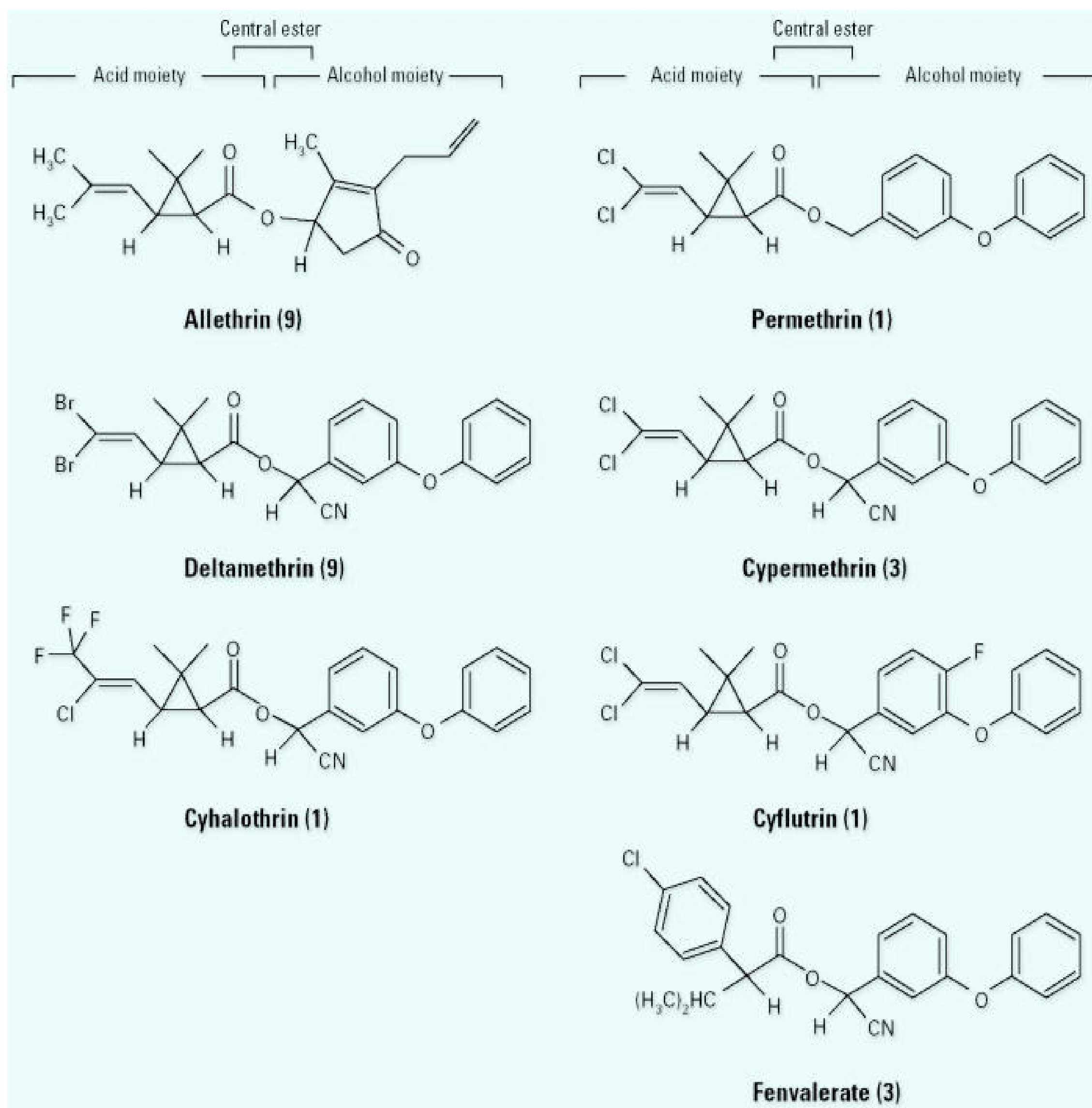


Figure 1. *Structures of pyrethroids which have been examined developmental neurotoxicity. Developmental neurotoxicity studies have been conducted using either technical compound or formulations of the seven pyrethroids illustrated; the numbers in parentheses after each compound name indicate the number of studies that have been conducted using that compound or a formulation containing that compound* (Shafer, Meyer, & Crofton, 2005)

Based on toxic signs in the rat, pyrethroids have been divided into two types:

a) compounds that produce a syndrome consisting of aggressive sparring, increased sensitivity to external stimuli, and fine tremor progressing to whole-body tremor and prostration (type I or T syndrome).

b) compounds that produce a syndrome consisting of pawing and burrowing, profuse salivation, and coarse tremor progressing to choreoathetosis and clonic seizures (type II or CS syndrome) (Shafer et al., 2005).

Pyrethroids slow the activation, or opening, of VSSCs. In addition, they slow the rate of VSSC inactivation (or closing) and shift to more hyperpolarized potentials the membrane potential at which VSSCs activate (or open).

The result is that sodium channels open at more hyperpolarized potentials (after smaller depolarizing changes in membrane potential) and are held open longer, allowing more sodium ions to cross and depolarize the neuronal membrane. In general, type II compounds delay the inactivation of VSSCs substantially longer than do type I compounds. Type I compounds prolong channel opening only long enough to cause repetitive firing of action potentials (repetitive discharge), whereas type II compounds hold open the channels for such long periods of time that the membrane potential ultimately becomes depolarized to the point at which generation of action potentials is not possible (Figure 2).

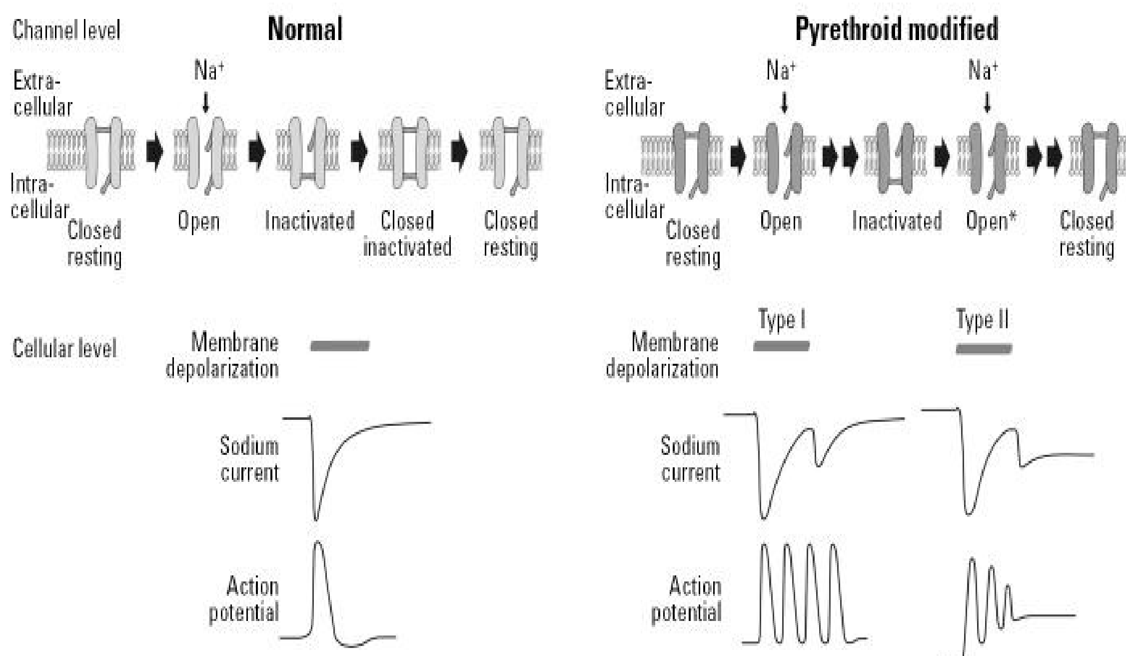


Figure 2. *Pyrethroid effects on neuronal excitability. This schematic depicts pyrethroid effects on individual channels, whole-cell sodium currents, and action potentials. Depolarization opens VSSCs (top left) allowing sodium to enter the cell. To limit sodium entry and depolarization length, VSSCs inactivate and must return to a "resting" state before reopening. Pyrethroids inhibit the function of two different "gates" that control sodium flux through VSSCs (top right), delaying inactivation (indicated by double arrows between states) of the channel and allowing continued sodium flux (Open*). If sodium current through an entire cell is measured, depolarization leads to a rapidly inactivating current under normal circumstances (bottom left, Sodium current). Pyrethroid-modified VSSCs remain open when depolarization ends (bottom right, Sodium current), resulting in a "tail" current (the notch at the end of example currents). If membrane voltage is examined, depolarization under normal circumstances generates a single action potential (bottom left). VSSCs modified by type I compounds (bottom right, Action potential) depolarize the cell membrane above the threshold for action potential generation, resulting in a series of action potentials (repetitive firing). Type II compounds cause greater membrane*

depolarization, diminishing the sodium electrochemical gradient and subsequent action potential amplitude. Eventually, membrane potential becomes depolarized above the threshold for action potential generation (depolarization-dependent block) (Shafer et al., 2005)

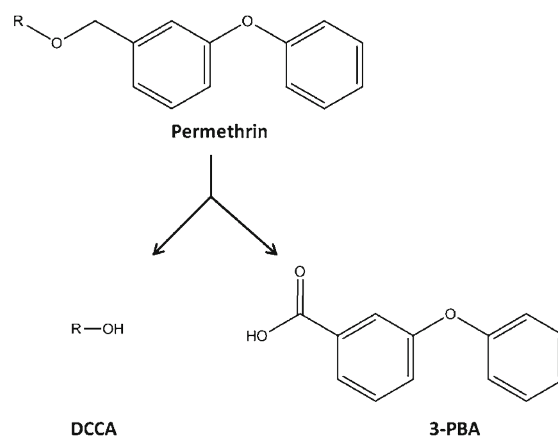
These differences in prolongation of channel open times are hypothesized to contribute to the differences in the CS and T syndromes after exposure to type II and I pyrethroids, respectively.

2.2 Permethrin and 3-PBA

Permethrin (PER) [-3-phenoxybenzyl (1RS, 3RS; 1RS, 3SR) -cis, trans-3-(2,2dichlorovinyl) 2,2dimethylcyclopropanecarboxylate-] is a type I pyrethroid that has been widely used in agricultural and residential setting.

Permethrin is a chiral compound with two stereocentres in the cyclopropane ring (Figure 3) (Ross, Borazjani, Edwards, & Potter, 2006) and is usually produced as a mixture of the two stereoisomers (cis/ trans ratios of 40:60, 80:20 or 25:75).

In mammals, once absorbed, permethrin is rapidly distributed to various organs/tissues (liver, brain, adipose tissues, etc.) and is transformed into metabolites that are subsequently excreted in urine and faeces (Tornero-Velez et al., 2012).



Where **R** may be: R1 : [1R,3R] cis R2 : [1S,3S] cis R3 : [1R,3S] trans R4 : [1S,3R] trans

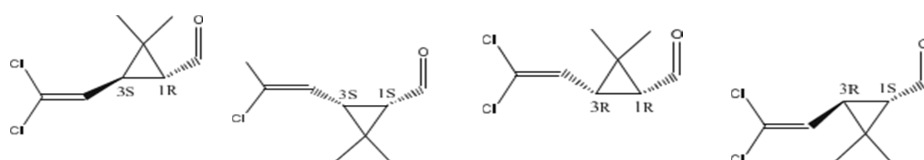


Figure 3. Metabolic pathway of permethrin with the formation of 3-phenoxybenzoic acid (3-PBA) and 3-(2,2-dichlorovinyl)-2,2dimethyl-(1-cyclopropane) carboxylic acid (DCCA)

In rats, cis-permethrin and trans-permethrin are hydrolysed in the blood, small intestine and liver by an ester cleavage, due to the presence of carboxylesterases, to cis3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid (**cis-DCCA**), trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid (**trans-DCCA**) and 3-phenoxybenzoic alcohol (Crow, Borazjani, Potter, & Ross, 2007). 3-Phenoxybenzoic alcohol is then oxidized to form 3-phenoxybenzoic acid (**3-PBA**) in the liver by cytochromes P450 or dehydrogenases (Figure 3) (Nakamura et al., 2007).

Even though they share the same metabolic pathways, the cis isomer is metabolized three times more slowly than the trans isomer (Scollon, Starr, Godin, DeVito, & Hughes, 2009). These major metabolites have been used as biomarkers to monitor acute or short-term exposure in adults and children from the general population and in workers after use of the pesticide.

2.3. Vitamin E

Vitamins are organic compounds and a vital nutrients that organisms obtain for the most part with food. Once growth and development are completed, vitamins remain essential nutrients for the healthy maintenance of the cells, tissues, and organs that make up a multicellular organism; they also enable a multicellular life form to efficiently use chemical energy provided by food it eats, and to help process the proteins, carbohydrates, and fats required for respiration.

Vitamins are classified by their biological and chemical activity and usually are distinct as water-soluble (Thiamine B1, Riboflavin B2, Niacin B3, Pantothenic acid B5, Biotin B7, Folic acid B9, vitamin B12) and fat-soluble (Vitamin A, C, D, E, K). In our study we selected the Vitamin E, a naturally occurring antioxidant nutrient, refers to a group of compounds that include tocopherols and tocotrienols (Figure 4), that plays important roles in animal health by inactivating harmful free radicals produced through normal cellular activity and from various stressors.

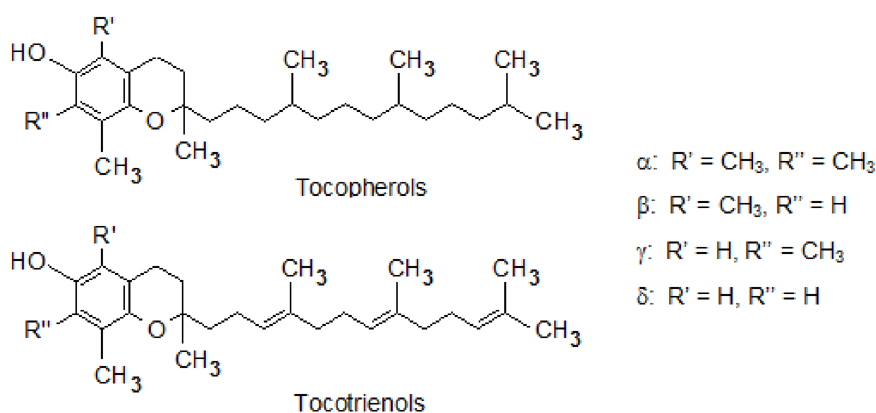


Figure 4. Chemical structure of the components of the Vitamin E, Tocopherols and Tocotrienols.

The antioxidant function of vitamin E could, at least in part, enhance immunity by maintaining the functional and structural integrity of important immune cells (Yousef, 2010).

The action mechanism of Vitamin E is to inhibit reactive oxygen species (ROS)-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of

Polyunsaturated Fatty Acids (PUFA) in membrane phospholipids, from oxidative damage of plasma very low-density lipoprotein, cellular proteins, DNA and from membrane degeneration (El-Demerdash, 2007). Moreover, when taken at therapeutic doses over a sustained period of time, vitamin E has been implicated in a decreased risk of cardiovascular diseases and some cancers, improved immune function, and slowing of the progression of a number of degenerative conditions associated with aging (Singh, Kumar, 2013).

2.4 Alkaline water and Electrolyzed reduced water

The alkaline reduced water (ARW) is a mineral combination of Electrolyzed reduced water (ERW) made by putting mineral combinations into water bottle. The pH of water is increased up to 10.5 and oxidation–reduction potential (ORP) is decreased until -200mv. The mineral combinations is easy to carry and less expensive than the system to produce ERW. Studies concerning ARW showed significant anticancer effect like tumor growth delay, survival span lengthened and inhibition of metastasis always for the reduced of ROS and for the stimulation of systemic cytokines, such Th1 (IFN-γ, IL-12) and Th2 (IL-4, IL-5), suggesting strong immune-modulation effect (Lee, Park, Kim, Kim, & Ryang, 2004).

ERW was generated in a cell containing inert positively charged and negatively charged electrodes separated by a septum which was first used about 1900 in the soda industry (Figure 5).

According to the principles and characteristics of electrolysed water, two types of water are produced simultaneously, that is the electrolysed oxidising water (EOW) and the electrolysed reducing water (ERW) (RATANA-ARPORN & JOMMARK, 2014).

The EOW is generated at the anode side with low pH value (2.3–2.7), high ORP (>1000 mV) and hypochlorous acid which has been determined to have a strong bactericidal effect on most known pathogenic bacteria and used as an alternative to conventional fungicides for controlling plant diseases. The ERW is generated at the cathode side

with high pH value and low ORP, which has limited application in the food industry due to the lack of strong antibacterial activity (Han, Song, An, & Pan, 2016).

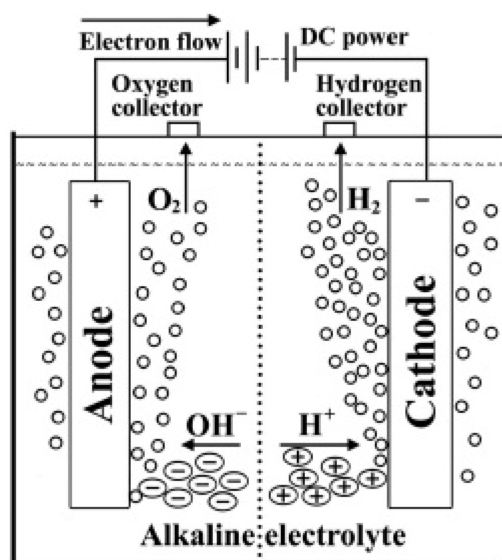


Figure 5. Basic scheme of a water electrolysis system

ERW was demonstrated to scavenge reactive oxygen species (ROS) in several cell types and is a promising candidate for antioxidant therapies because it has the potential to cross the BBB (blood-brain barrier), which protects the brain from toxins and reduces the bioavailability of exogenous antioxidants in the brain. ERW scavenged intracellular ROS and exhibited a protective effect against neuronal network damage caused by 200μM H_2O_2 in N1E-115 cells. ERW significantly suppressed NO-induced cytotoxicity in PC12 cells despite the fact that it haven't the ability to scavenge intracellular NO and it significantly suppressed both glutamate induced Ca^{2+} influx and the resulting cytotoxicity in primary cells (Kashiwagi et al., 2014).

Besides, hydrogen-rich water was demonstrated to have salutary effects in prevention of lifestyle diseases such as type 2 diabetes, insulin resistance and liver inflammation (Shirahata et al., 2011).

Furthermore, (Hao et al., 2011) reported that the cleaning property of ERW made it a potential means to eliminate pesticides from fresh fruits and vegetables, and they

found the ERW (pH 11.6, ORP: −860 mV) could effectively reduce the pesticide residues in spinach, cabbage and leek.

2.5. Analytical method described

2.5.1. Hair sample treatment

This type of procedure was previously applied for the monitoring the pyrethroid and his metabolites in hair of laboratory animals (rabbits) exposed by diet to CPMN at 40 (low dose) and 80 (high dose) mg/kg weight/day for 16 weeks. The hair sample treatment comprises four main steps: isolation of samples from hair, decontamination steps, extraction steps and samples derivatization (Kavvalakis et al., 2014).

In this study, hair samples collections were performed, before the first dose administration and at the end of second and fourth month of treatment, from two different anatomical sites (neck and lower back) of each animal. Hair of these two anatomical sites were mixed and analysed as one for each one sampling. At the end of experiment (fourth month) an additional sampling was taken place, collecting total length hair sample representative to all the four months of exposure, from the same anatomical sites. Hair specimens were stored in paper envelopes in a dry place, at room temperature until analysis. Another important step is the removal of the external contaminants with water and methanol. After that the samples are dried in the oven and cut in small pieces (some mm) (Kavvalakis et al., 2014).

To allow the extraction of the pesticides, the hair sample is incubated with methanol in an ultrasonic bath for two hours.

Once filtered the procedure is repeated put other methanol and after the it is evaporated with dryness under a nitrogen stream. At last there is the derivatization, explained accurately in the chapter 4.1.4 (Kavvalakis et al., 2014).

2.5.2 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. The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas.

The sample flows through the column and the compounds comprising the mixture of interest are separated by 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 (Figure 6) where compounds eluting from the column are converted to ions. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

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. Several types of analyser exist, quadrupoles, ion traps, magnetic sector, etc. We use the ion traps, a device that uses an oscillating electric field to store ions. It works by using a radio-frequency quadrupolar field that traps ions in two or three dimensions.

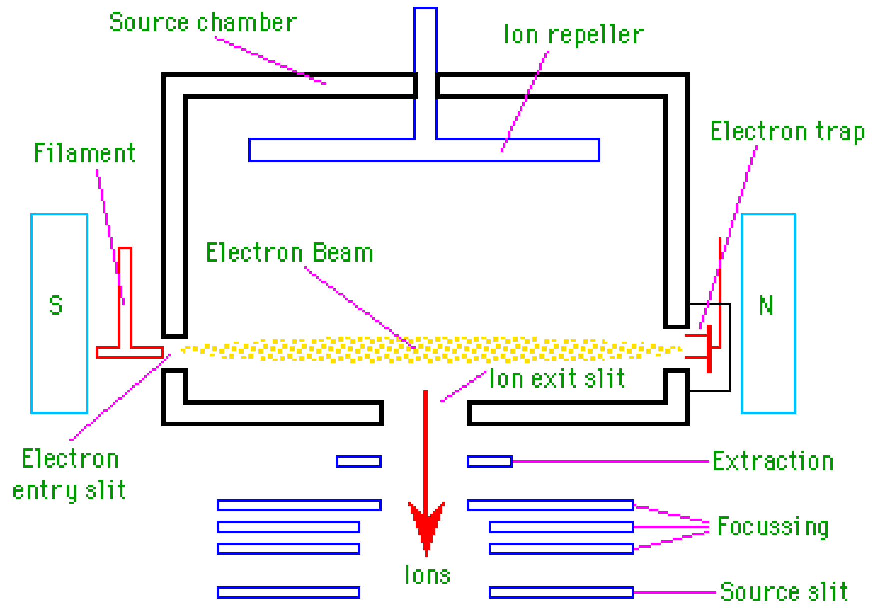


Figure 6. A schematic of an ion source

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 data produced (Edmond de Hoffman, Vincent Stroobant, 2007).

CHAPTER 3: STATE OF THE ART

Hitherto there were a whole lot of studies concerning the determination of 3-PBA in biological samples, like urine, blood and plasma, but in that thesis there were taken into considerations only the studies published in the last decade. Commonly the 3-PBA was analysed in the urine because it has been shown that more than 60% of an oral dose of permethrin in rats were excreted in urine as metabolites (Ratelle, Côté, & Bouchard, 2015).

In 2016 a German group of research has developed a procedure for the simultaneous determination of eight urinary metabolites of synthetic pyrethroids in human urine. In that study, they had investigated a group of 38 persons (15 females, 23 males) without known exposure to synthetic pyrethroids. The use of tandem mass spectrometry allowed a substantial improvement in sensitivity, while the use of two isotopically labelled internal standards considerably improved precision and accuracy of the method. However, it must be emphasised that LC/MS/MS methods are highly susceptible to ion suppression effects in complex matrices like urine that can only be efficiently accounted for by a labelled internal standard. Thought, the application of this method it was very signifying because confirmed previous reports in a background exposure of the general population to synthetic pyrethroids (Schettgen, Dewes, & Kraus, 2016).

Another study was focused about the pyrethroid pesticide exposure of pregnant women, that may be a particularly vulnerable population, as *in utero*-fetal exposure, that is a critical periods of development and affect long-term neurobehavioral function. The research scientists measured maternal urinary 3-PBA concentrations during the third trimester of pregnancy as a measure of *in utero*-pyrethroid exposure to the fetus among participants in an established Mexico City birth cohort (n=187). They proved that there was an association between third trimester 3-PBA concentrations and children's scores on the Mental Development Index (MDI) and Psychomotor Development Index (PDI). The result of the search showed that 3-PBA was detected in 46% of all urine samples and that participants in the medium and high 3-PBA categories (≥LOD) had lower MDI scores at 24 months (Watkins et al., 2016).

Our research team pursued a very interesting project in which has tested 3-PBA in urine and Mg in hair and they were changed in ASD children (Autism Spectrum Disorders) relative to control ones. Gas chromatography coupled with trap mass detector was used to measure the level of 3-PBA in a group of ASD patients, while optical emission spectrometry analysis was employed to estimate the level of metals and microelements in hair in a different group of ASD children. The presence of 3-PBA in urine seems to be independent of age in ASD children, while a positive correlation between 3-PBA and age was observed in the control group of the same age range. Urine concentration of 3-BPA in ASD children had higher values than in the control group, which were marginally significant ($p = 0.054$). Mg results were significantly decreased in ASD with respect to controls, while V, S, Zn, and Ca/Mg were marginally increased, without reaching statistical significance. Results of Principal Component (PC) analysis of metals and microelements in hair were not associated with either age or health status (Domingues et al., 2016).

The aim of other study was to identify a plasma biomarker of exposure to pyrethroids insecticides. A major metabolite, 3-PBA, can be detected in urine but urinary 3-PBA cannot be used to assess the active dose. The 3-PBA-adduct represents a much more persistent class of biomarkers than metabolites excreted into urine, having half lives up to several weeks or months. This group of research developed an enzyme-linked immunosorbent assay (ELISA) for total 3-PBA including adduct formed after alkaline hydrolysis, liquid-liquid extraction (LLE) and solid phase extraction (SPE) of the sample. The method was successfully applied to the detection of the target in real samples obtained from consumers (n=50) and farmers (n=50). The results were 30- to 47-fold more sensitive than previous studies and the developed method could separate more than 80% of 3-PBA from adduct form (Thiphom et al., 2012).

Another group of research developed a sensitive method to detect several classes of pesticides and their metabolites in maternal and cord whole blood using electron-impact gas chromatography–mass spectrometry (GC–MS). The method can detect parent and metabolite compounds at levels of <0.10 and 0.20 µg/mL, respectively, with high accuracy and recovery. Analysis of blood from mother–infant dyads from an

area of high pesticide use in the Philippines showed detectable levels of propoxur, 3-phenoxybenzoic acid (3-PBA), and 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p*'-DDE) in maternal and umbilical cord blood. GC–MS analysis of several classes of parent pesticides and their metabolites in maternal and cord blood provides a sensitive and specific method to detect pesticide exposure during pregnancy (Corrion et al., 2005).

The University of Camerino researchers has been analyzed that hair from an animal model of neurodegeneration, induced by early life permethrin treatment from the sixth to 21th day of life, with the aim to assess if metal and microelement content could be used as biomarkers. A hair trace element assay was performed by the ICP-MS technique in 6- and 12-month-old rats. A significant increase of As, Mg, S and Zn was measured in the permethrin-treated group at 12 months compared to 6 months, while Si and Cu/Zn were decreased. K, Cu/Zn and S were increased in the treated group compared to age-matched controls at 6 and 12 months, respectively. Cr significantly decreased in the treated group at 12 months. PCA analysis showed both a best difference between treated and age-matched control groups at 6 months. The present findings support the use of hair analysis to identify biomarkers of neurodegeneration induced by early life permethrin pesticide exposure (Nasuti, Ferraro, Giovannetti, Piangerelli, & Gabbianelli, 2016)

Another study on rat with 60 days exposed to two pyrethroids, cypermethrin and permethrin, showed that oral treatment with 150 mg/kg body weight/day of permethrin, induced a significant increase in all comet parameters. No lymphocyte DNA damage was measured after treatment with 25 mg/kg body weight/day of cypermethrin for the same period. A higher dose of permethrin (300 mg/kg body weight/day), for a shorter period (22 days), did not induce lymphocyte DNA damage, while supplementation with 200 mg/kg of Vitamins E and C protected erythrocytes against plasma membrane lipids peroxidation. But the work led an important discovery, namely that the treatment with Vitamins E and C maintained the activity of glutathione peroxidase, which was reduced in the presence of permethrin, and reduced the osmotic fragility, which had increased following permethrin treatment (Gabbianelli, Nasuti, Falcioni, & Cantalamessa, 2004).

CHAPTER 4: MATERIALS AND METHODS

4.1. Materials

4.1.1 Reagents

The reagents used were:

n-Hexane, Unisolv [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, Romil [purity 99.5%]; hexafluoro-2-propanol, Fluka (HFEIP) [purity 99.8%]: N,N'-Diisopropylcarbodiimide, Aldrich (DIC) [purity 99%]; potassium carbonate (K_2CO_3); 3-phenoxybenzoic alcohol (3-PBA); 2-phenoxybenzoic alcohol (2-PBA); vitamin E; tocopherol; alkaline water.

4.1.2. Samples

Hair sample collection was performed before the first dose administration and on rats aged 22 and 60 days exposed to permethrin, permethrin and vitamin E, permethrin and tocopherol, permethrin and alkaline water and only tocopherol. In order to assess the bioaccumulation of target metabolites, hair sample were collected from the neck of our experimental animals, the rats.

-In February 2015 we collected the hairs of rats aged 60 days; we took five samples from control group, five samples treated with 100 mg/kg of tocopherol, four treated with permethrin and 31 mg/kg of tocopherol and five treated with permethrin and 100 mg/kg of tocopherol.

-In December 2015 we collected the hairs to eight female adult, mother of rats aged 22 days; we took ten samples from control group, ten treated with permethrin and alkaline water and ten treated with permethrin and vitamin E.

-Still in December 2015 we collected the hairs to eight female adult rats aged 60 days; we took two samples from rats treated with permethrin, three treated with permethrin and alkaline water and eleven treated with permethrin and vitamin E.

-in August 2016 we collected the hairs to rats aged 60 days; we took eleven samples to control group, ten samples treated with permethrin and twelve treated with permethrin and alkaline water.

Hair specimens were labelled and stored in paper envelopes in a dry place, at room temperature until analysis.

Rat aged 60 days:

- Control
- Permethrin
- Permethrin + Vitamin E
- Permethrin + Alkaline water
- Permethrin + 31 mg/kg Tocopherol
- Permethrin + 100 mg/kg Tocopherol
-

Rat aged 22 days:

- Control
- Permethrin
- Permethrin + Vitamin E
- Permethrin + Alkaline water

4.1.3 Equipment

3PBA and 2PBA were analysed using a Thermo Trace-Ultra gas chromatograph coupled to an ion trap mass detector Thermo Polaris (Dreieich, Germany), operated in the electron impact ionization at 70 eV. The ion source temperature and the MS transfer temperature were at 250°C.

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 for compound and the ratios of those ions will be unique to the analyte of interest.

The system was operated by Xcalibur v 1.3 software. Confirmation of residues was carried out by GC-MS/SIM and MS/MS using a Supelco column (Laborche mikalien, Germany) 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 160 and held for 0.50 min. Then followed by increases of 15 °C/min to 180 and held for 1 min. Finally, followed by increasing of 20 °C/min to 250. The ion source and MS transfer line were at 250°C.

Column previously cut around 2 m in the injector side. The injector was maintained at 240C.

For the identification of 2-phenoxybenzoic alcohol (2-PBA), our internal standard, and 2-phenoxybenzoic alcohol (3-PBA), the retention time for the 2-PBA was 9:50 and for the 3-PBA 11:70 min. The MS/MS conditions were fixed for each compound, for the 2-PBA was 196 and for 3-PBA is 141 and 364.

The LOD was calculated as three times higher than the level of noise (equation 1), and the LOQ was defined to ten times of the noise level (equation 2).

All tests were conducted at least in triplicate with the standard of the lowest concentration.

$$LOD = \frac{3 \times conc}{S/N} \qquad (1)$$

$$LOQ = \frac{10 \times conc}{S/N} \qquad (2)$$

4.2 Methods

4.2.1. Optimization of extraction procedure

Before beginning the procedure on the rat's hair, the first tests were made using human hair samples, implemented to optimize the extraction procedure. We tested a human hair samples added 200 µL of 2-PBA and 90 µL of 3-PBA and a control sample spiked with 200 µL of 2-PBA and 90 µL of 3-PBA.

After that we had to verify if plastic or glass compounds could alter the extraction procedure of 3-PBA and 2-PBA. To do that we used different ways:

- in the first we use a plastic test-tube and we put in only 200 µL of 2-PBA and 90 µL of 3-PBA without human hair samples;
- in the second we use a glass test-tube and we inserted only 200 µL 2-PBA and 90 µL 3-PBA without human hair samples.

It was checked if the washing with water and methanol bring loss of 3-PBA. So we tested our method on human hair with the addiction to 90 µL of 3-PBA and 200 µL of 2-PBA, the internal standard. We prepared three various samples:

- the first test 200 mg of human hair samples were washed with water and methanol;
- the second test 200 mg of samples were washed with water, without methanol;
- the last test 200 mg of hair were washed with methanol, without water.

4.2.2 Recoveries

The recoveries were achieved using two hair control samples.

In one of them it was added 90 µL of 3-PBA the day before the washing procedure, as it normally done for the other analysis of hair control samples. In the second control samples it was added 90 µL of 3-PBA after the washing procedure.

It was possible to obtained the recoveries by the relation between the area of the analyte 3-PBA in the first samples and the area of the analyte 3-PBA in the second samples.

4.2.3. Preparation of hair samples

For the derivatization step, the hair samples were prepared the day before: 200 mg of hair samples were weighed, cute in small pieces (some mm) and inserted in a plastic test-tube. After that the hair samples were put in contact with 200 µL of 2-PBA solution 3 (50 µg/L). The hair samples needed to remain in contact with the 2-PBA for one day to adhere each other.

4.2.4 Decontamination step

The day after, before starting with the extraction step, it was necessary remove the external contaminants on the rat hair.

The hair samples were washed in 5 mL of distilled water at room temperature (20-25 °C), centrifuged for 2 minutes and left with the water for 10 minutes. Subsequently, the supernatant was take out and the hair samples were washed in 5 mL of methanol, centrifuged for 2 minutes and left with the methanol for 2 minutes. Taken out the supernatant, the hair samples were washed with other 5 mL of methanol, centrifuged for 2 minutes and separated from supernatant. The hair samples were put in the oven at 40-50 °C (temperature did not exceed 50 °C).

After the hair samples were dry, 100 mg of hair were weighed and transferred in a plastic test-tube with screw-top.

4.2.5. Extraction step

Hair samples were incubated with 2 mL of methanol at room temperature in an ultrasonic bath (H-D P-Selecta with 40 KHz and 120W) at 15-20 °C for 2 h. The temperature of the bath during the ultrasonic extraction did not exceed 40 ^{o}C. The methanol extract was filtered through 0.2 µm membrane filter to a glass test-tube. After that other 2 mL of methanol were add in the plastic test-tube with hair samples. The test-tube was put in the ultrasonic bath for 2h always at 15-20°C. The other 2 mL of methanol were filtered in the glass test-tube used before. Finally the 4 mL of methanol in the glass test-tube were evaporated to dryness under a gentle nitrogen stream at room temperature.

4.2.6. Derivatization process

Once the samples were dry, in the same glass test-tube used before were added 30 µL of Hexafluoro-2-propanol (HFEIP) [purity 99.8%] and 20 µL of N,N'-Diisopropylcarbodiimide (DIC). Whereupon the glass test tube was put on the vortex for 10 minutes. The previous solution was added 1 mL of 5% Potassium carbonate (K_2CO_3) and 250 µL of n-hexane [purity 99.9%]. The K_2CO_3 had to be with pH=12 and was made with 5 g of K_2CO_3 in 100 mL of deionized water. The solution was put on vortex for 5 minutes. After that it was form a supernatant, with a pipette, 100 µL of the supernatant was removed and inserted into a small glass vial. Afterwards at the first solution, in the big glass tube, was added another 250 µL of n-Hexane and put on vortex for 5 minutes. Other 100 µL of new supernatant, which had formed in the big glass tube, was taken, inserted in the small glass vial and saved for the next day (Figure 7).

The following day, 50 µL of supernatant were added with 50 µL of n-Hexane in a vial with an insert ready to be analysed by GC-MS.

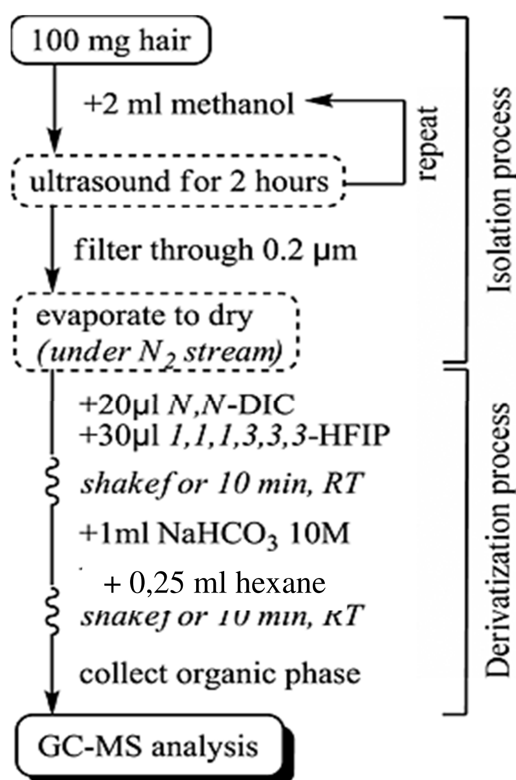


Figure 7.Flow chart of the experimental procedure followed for Permethrin metabolites extraction from hair samples.

* **2-PBA solution 3** was made with 50 µL of 2-PBA solution 2 (10 mg/L) in 10 mL of acetonitrile (ACN) [purity 99,9].

** **3-PBA solution 5** was made with 400 µL of 3-PBA solution 3 in 10 mL of ACN.

*** **3-PBA solution 3** was made with 50 µL of 3-PBA solution 2 in 10 mL of ACN.

4.2.7. Calibration curves

Two calibrations curves were performed. The first curve was performed with different concentration of 3-PBA (between 0.25 µg/L and 5 µg/L) and the same amount of 2-PBA (as internal standard) (200 µL of a solution of 20 µg/L), evaporation the n-hexane and followed by the derivatization.

For the calibration curve in the matrix, the extraction was performed as described in Figure 7, and the 500 µL of the supernant was added at each vial before evaporation.

4.2.8. Samples analysis

To evaluate 3-PBA in rat's hair it was used the "control" samples from rat not exposed to permethrin, to control any cross contamination.

All samples were analysed as previously described.

4.2.9. Conversion of the concentration

It was necessary to convert the concentration of 3-PBA presents inside of the samples from µg/L in µg/mg of hair. To do what we used this formula:

$x = [y*(1*10^6)/500\mu L]/100mg$

x= concentration of 3-PBA in µg/mg of hair

y=concentration of 3-PBA in µg/L of solution

500 µL was the volume of solution and 100 mg the quantity of a hair sample used for analysis.

CHAPTER 5: RESULTS AND DISCUSSION

5.1. Optimization with human hair samples

5.1.1 Tube materials

The analysis to verify if plastic or glass compounds could alter the extraction procedure of 3-PBA and 2-PBA showed that the materials did not affect the procedure as it is reported in the figure 8. For this reason we proceeded using the plastic tube for the first phases of procedures and we used the glass tube for the nitrogen drying, the derivatization and the GC analysis.

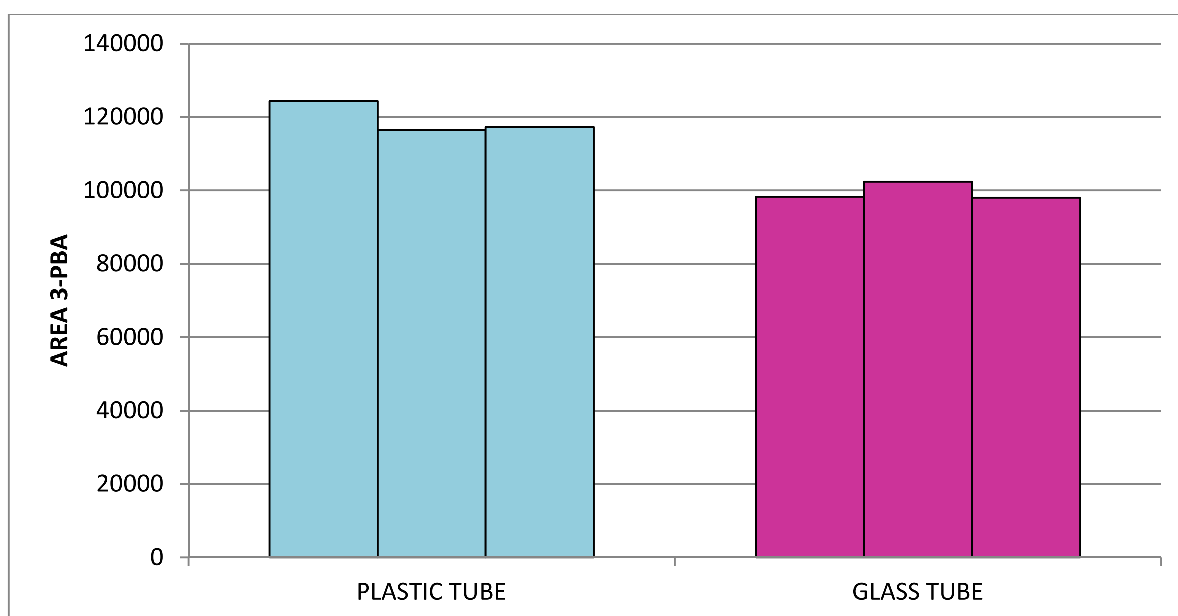


Figure 8. Area of 3-PBA extract with plastic test-tube and glass test-tube.

5.1.2 Washing solvents selection

The test of the three human hair samples washed with water and methanol, only water and only methanol showed only two results. It was not possible detect the area

of 3-PBA on the sample washed only with methanol. Furthermore, the sample washed with water and methanol had the higher area of 3-PBA, as shown in the figure 9. After that analysis we considered necessary wash the samples with water and methanol to remove all the contaminants and prevent loss of 3-PBA.

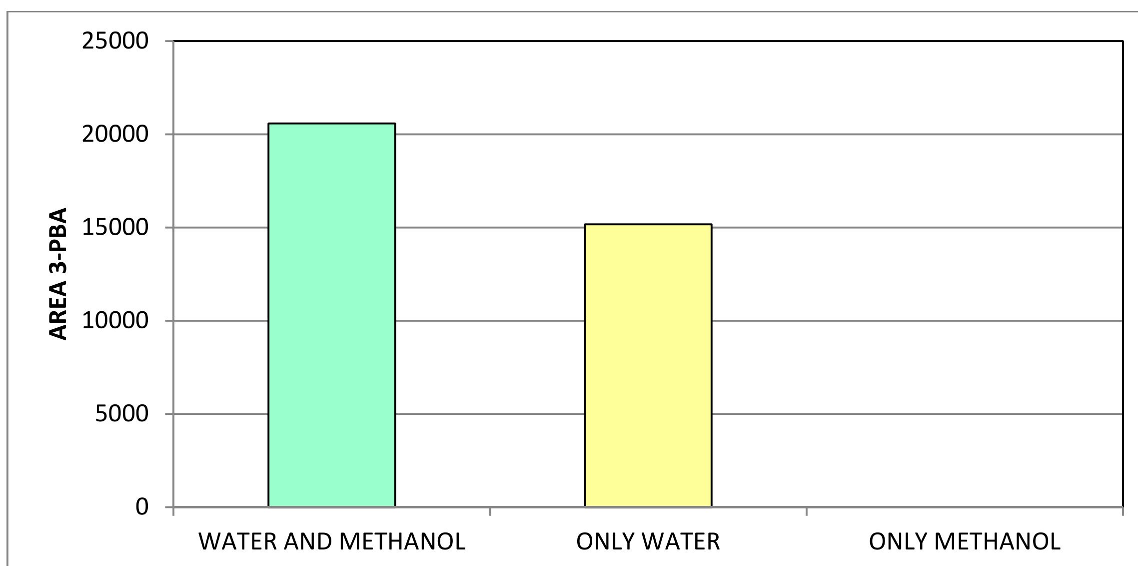


Figure 9. Area of 3-PBA of samples washed with water and methanol (50/50) and samples washed only with water.

5.1.3 Recovery

The first test done on human hair sample obtained positive results. We compared the area of 3-PBA in the human hair sample and in a control sample. How is explained in the figure 10, the 3-PBA concentration was slightly lower in the control compared to the hair sample.

These results gave the confirmation that it was possible to apply this method of 3-PBA extraction on human hair.

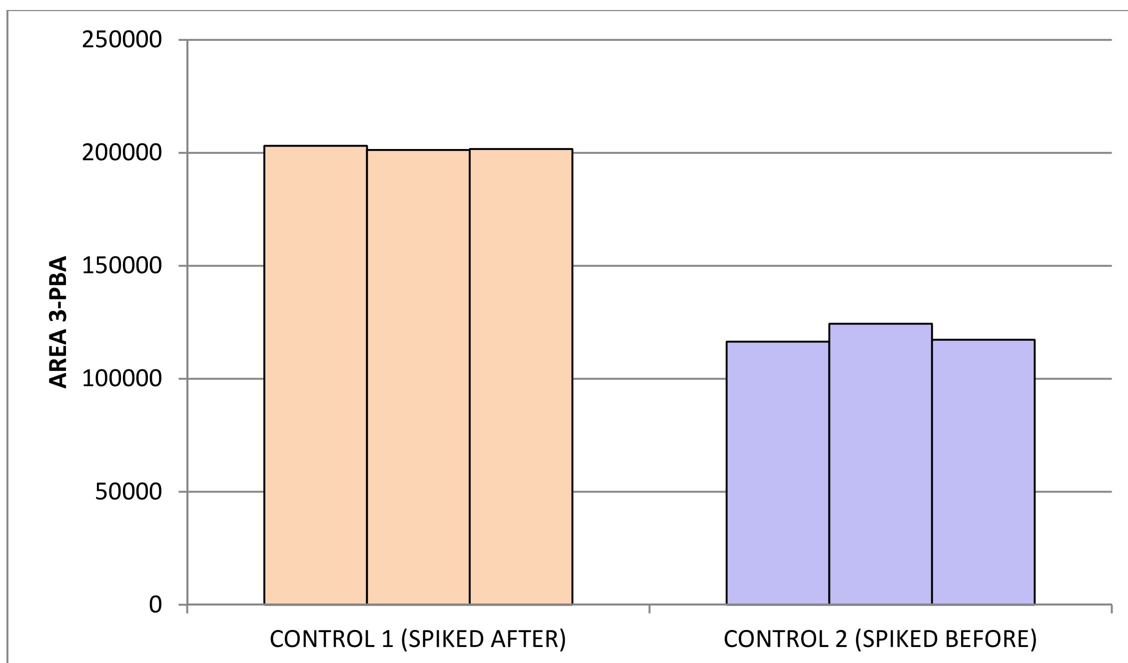


Figure 10. Area of 3-PBA in hair sampleS spiked with 90 µL of 3-PBA: Control 1 (spiked after washing procedure) and control 2 (spiked before washing procedure).

According this data the recovery in this method is 78%, obtained with the average of area of 3-PBA in control sample number 1 and control sample number 2 .

5.2. Limits of detection and quantification

The determination of the limit of detection and quantification were obtained using the equation 1 and 2.

The ratios S/N were obtained by the Xcalibur software.

The LOD of the equipment is 0.036 µg/L and LOQ is 0.119 µg/L.

Considering the recovery of 78%, the LOD of the method is 0.046 µg/L and the LOQ of the method is 0.153 µg/L.

According the amount of sample it was used, the LOD with this sample is 0.92 µg/mg and the LOQ is 3.06 µg/mg.

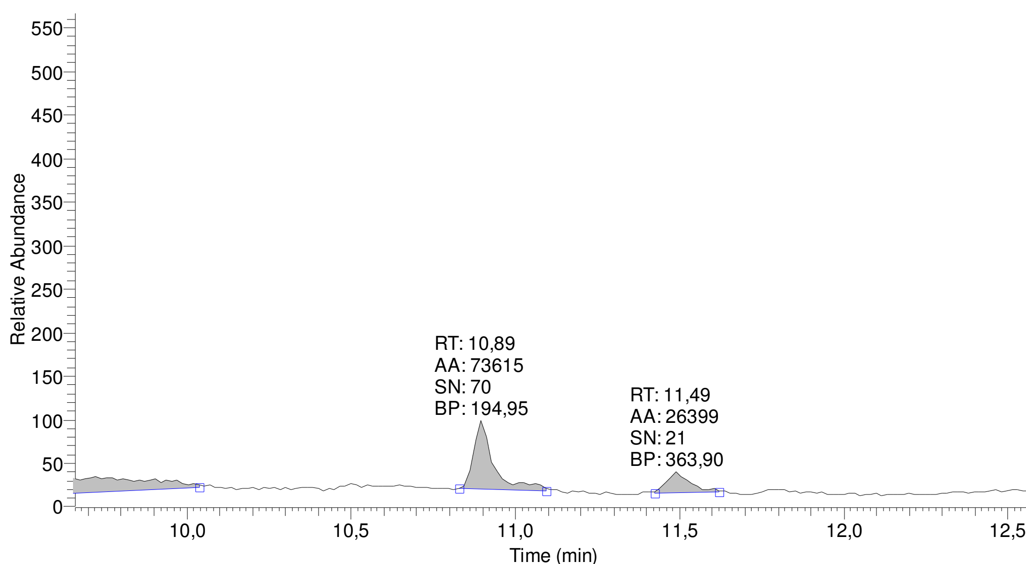


Figure 11. Chromatogram of the lowest concentration of 3-PBA (0.25 µg/L) obtaining a S/N ration of 21.

5.3. Calibration curves

A calibration curve was obtained for 3-PBA, by injections in triplicate of at five calibration levels.

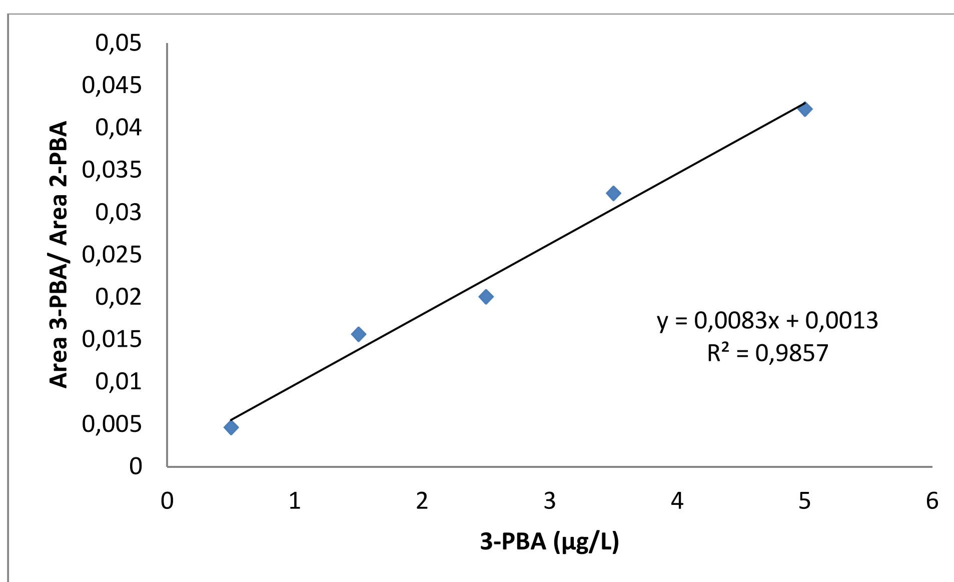


Figure 12 Calibration curve obtained of 3-PBA in solvent.

For the calibration curve in the matrix, a poor R^2 was obtained (0.882) and a equation y=0.0246x – 0.0428.

5.4. Results of rat's hair samples

5.4.1 Rat's hair samples with 60 days

All samples of rats exposed 60 days before the hairs collection were analysed and the signal were lower than limit of detection of the method.

5.4.2 Rat's hair samples with 22 days

These samples were analysed with the same procedure. 3-PBA was detected by the retention time and by produced ions. In Figure 12 is shown a chromatogram of a sample detected with 3-PBA.

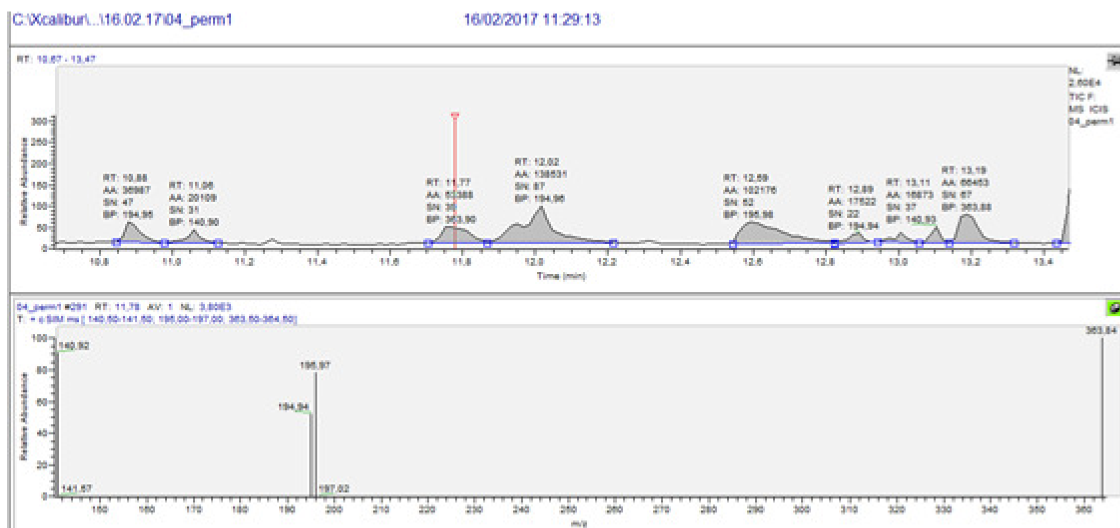


Figure 13. Chromatogram and spectrum obtained from sample collected from rats with 22 days exposed to permethrin.

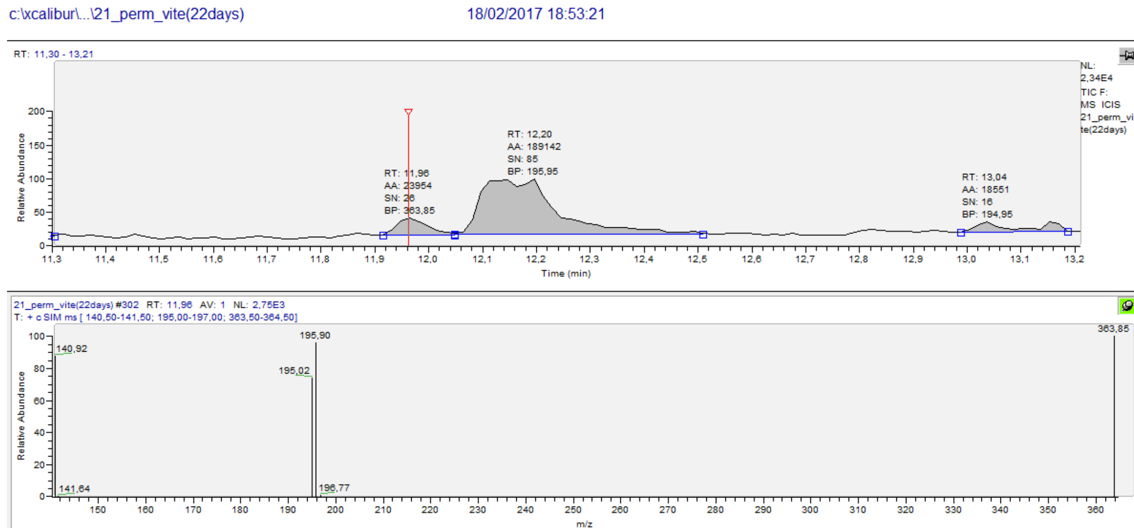


***Figure 14**. Chromatogram and spectrum obtained from sample collected from rats with 22 days exposed to permethrin and vitamin E.*

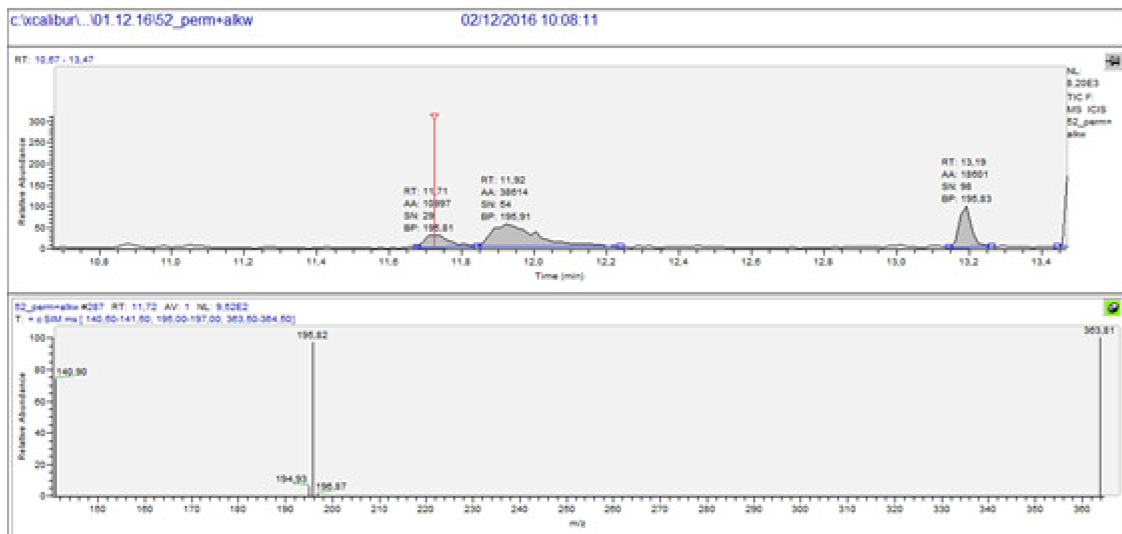


Figure 15. Chromatogram and spectrum obtained from sample collected from rats with 22 days exposed to permethrin and alkaline water.

The analysis of hair samples of rats treated with permethrin, permethrin + vitamin E and permethrin + alkaline water with 22 days of age has led to the positive results described in Figure 13.

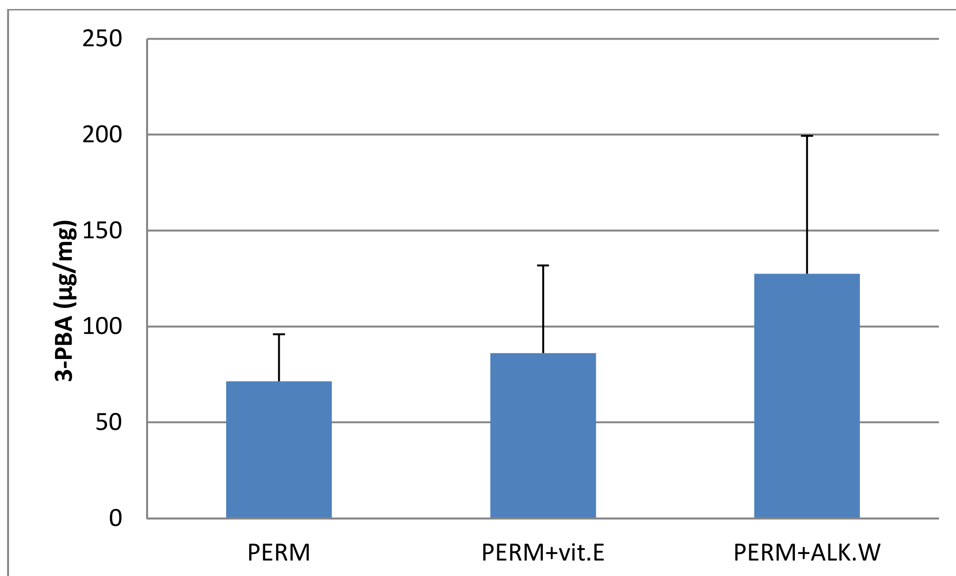


Figure 16. Concentrations in µg/mg of rat hair aged 22 days of age treated with permethrin, permethrin + Vitamin E and permethrin + alkaline water.

From these data we can conclude that rats simultaneously exposed to permethrin and Vitamin E or alkaline water shows higher amount of permethrin metabolite.

Taking account the hair grow, it can be hypothesised, that these compounds (Vitamin E and alkaline water) may increase the detoxification process of this pesticide.

CHAPTER 6: CONCLUSION

The current project had the purpose to obtain a method for the analysis of 3-PBA in hair samples using a gas chromatography mass spectrometry.

Based on the results reported in the section optimization with human hair samples, we noticed that the realization of the procedure with a plastic test tube it resulted in a better extraction of 3-PBA from hair. For that reason we considered more convenient for the proceedings use a plastic tube and we discarded the glass tube for the extraction process because the glass tubes, probably, may affect the recovery. Concerning the solvent used for wash the sample, the solvent water and methanol (50/50) was the more appropriate for washing samples. Using two control hair samples, one of them spiked with 2-PBA and 3-PBA after the washing procedure and the other spiked before the washing procedure, we obtained different area of 3-PBA that permitted us to calculate the recovery. In particular this method allowing a recovery of 78%. The limit of detection and quantification was achieved with the formula reported in the experimental section and was 0.046 μg/L the LOD of the method and 0.153 μg/L the LOQ of the method. According the amount of sample it was used, the LOD with this sample is 0.92 μg/mg and the LOQ is 3.06 μg/mg. The analytical method optimized was used for the extraction of 3-PBA in rat's samples. How is reported in the figure 16, the concentration expressed in μg/mg of 3-PBA in the rat's hair with 22 days is lower in the samples exposed only to permethrin, slightly higher in the samples exposed to permethrin and vitamin E and greater in the samples exposed to permethrin and alkaline water. We believe that antioxidant power of vitamin E and alkaline water can influence the metabolism of the permethrin and, for that reason, the concentration of her metabolite, the 3-PBA, change considerably in that samples. On the contrary we could not detected area and consequently concentrations of 3-PBA in the samples exposed to permethrin, permethrin and vitamin E and permethrin and alkaline water, but aged 60 days. Maybe the change of rat's hair happens before the sixtieth day.

According with these considerations we believe that this process have the potential to be used for the extraction of 3-PBA in hair, however it should be kept in mind that the

present results are only preliminary studies and further experiments should be implemented to verify the accuracy of the method.

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ANNEX 1

Preparation of solution for the calibration curve

Add 200 µl of 2-PBA solution 3 (for 20µg/L in 500 µl) and add:

1. for 3-PBA standard 0.25 µg/L: 125 µl of 3-PBA solution 5;
2. for 3-PBA standard 0,5 µg/L: 10 µl of 3-PBA solution 3;
3. for 3-PBA standard 1,5 µg/L: 30 µl of 3-PBA solution 3;
4. for 3-PBA standard 2,5 µg/L: 50 µl of 3-PBA solution 3;
5. for 3-PBA standard 3,5 µg/L: 70 µl of 3-PBA solution 3;
6. for 3-PBA standard 4,5 µg/L: 90 µ of 3-PBA solution 3;
7. for 3-PBA standard 5 µg/L: 100 µl of 3-PBA solution 3.

3-PBA solution 5 (2µg/L): 400 µL of 3-PBA solution 3 in 10 mL of ACN.

3-PBA solution 3 (50µg/L): 50 µL of 3-PBA solution 2 in 10 mL of ACN.

ANNEX 2

Table A. Area of 2-PBA and 3-PBA, 3-PBA/2-PBA, concentration in µg/L and real concentration of samples tested with a plastic test-tube**.**

	area 2-PBA	area 3-PBA	3-PBA/2-PBA	conc µg/L	real conc
Plastic tube	1409286	116456	0,082634753	9,14830585	11,7286
	1466350	124359	0,084808538	9,38983758	12,03825
	1448462	117302	0,08098383	8,96486997	11,49342
Glass tube	1964464	102344	0,052097671	5,75529683	7,378586
	1821615	98267	0,053944988	5,96055426	7,641736
	1821615	98057	0,053829706	5,94774512	7,625314

Table B. Area of 2-PBA and 3-PBA, 3-PBA/2-PBA, concentration in µg/L and real concentration

	area 2-PBA	area 3-PBA	3-PBA/2-PBA	conc µg/L	real conc
methanol and water	1152383	6332	0,005494701	0,577189	0,739986
	1236426	20573	0,016639087	1,81545413	2,327505
	1229446	0	0	0	0
only water	1724480	15172	0,008798014	0,94422383	1,210543
	1767859	0	0	0	0
	1574667	0	0	0	0
only methanol	1686022	0	0	0	0
	1706425	0	0	0	0
	1715672	0	0	0	0

Table C. Rat aged 60 days

Table C 1. Area of 2-PBA and 3-PBA in hair samples of rats exposed to permethrin aged 60 days.

Permethrin		
	area 3-PBA	**area 2-PBA**
sample 1	n.d	753472
	n.d	770136
	n.d	790569
sample 2	n.d	907399
	n.d	928010
	n.d	896114
sample 3	n.d	949385
	n.d	1047587
	n.d	1092110

sample 4	n.d	27883
	n.d	752072
	n.d	912591
sample 5	n.d	1189974
	n.d	1747402
	n.d	1944115
sample 6	n.d	193483
	n.d	1571737
	n.d	1697075

Table C 2. Area of 2-PBA and 3-PBA in hair samples of rats exposed to permethrin and vitamin E aged 60 days.

Permethrin + vitamin E

	area 3-PBA	area 2-PBA
sample 1	n.d	573122
	n.d	656003
	n.d	688155
sample2	n.d	761668
	n.d	1041069
	n.d	1052971
sample3	n.d	n.d
	n.d	558742
	n.d	574298
sample4	n.d	273203
	n.d	379867
	n.d	354854
sample5	n.d	183290
	n.d	40523
	n.d	n.d
sample6	n.d	1916085
	n.d	1948215
	n.d	1866231

Table C 3. Area of 2-PBA and 3-PBA in hair samples of rats exposed to permethrin and alkaline water aged 60 days.

Permethrin + alkaline water

	area 3-PBA	area2-PBA
sample 1	n.d	35256
	n.d	n.d
	n.d	88272
sample 2	n.d	1432597
	n.d	1468324
	n.d	1555453
sample3	n.d	1138936

	n.d	1202107
	n.d	1162423
sample 4	n.d	n.d
	n.d	n.d
	n.d	n.d
sample5	n.d	88483
	n.d	n.d
	n.d	n.d
sample 6	n.d	30050
	n.d	48837
	n.d	n.d

Table C 4. Area of hair samples of rats exposed to permethrin and 100 mg/kg tocopherol aged 60 days.

Permethrin+tocopherol 100mg/kg	**area 3-PBA**	**area 2-PBA**
sample 1	n.d	525633
	n.d	498353
	n.d	952294
sample 2	n.d	n.d
	n.d	n.d
	n.d	n.d
sample 3	n.d	n.d
	n.d	n.d
	n.d	n.d

Table C 5. Area of hair samples of rats exposed to permethrin and 31 mg/kg of tocopherol aged 60 days.

Permethrin + tocopherol 31 mg/kg	**area 3-PBA**	**area 2-PBA**
sample 1	n.d	33593
	n.d	121324
	n.d	448861
sample 2	n.d	n.d
	n.d	n.d
	n.d	n.d
sample 3	n.d	919875
	n.d	930285
	n.d	894140
sample 4	n.d	919875
	n.d	930285
	n.d	894140

Table D. Rat aged 22 days

Table D 1. Area of 3-PBA and 2-PBA, ratio, concentration in µg/L, concentration in µg/mg of hair and average of the concentration in µg/mg of hair of hair samples of rats exposed to permethrin aged 22 days.

Permethrin	**3-PBA**	**2-PBA**	**ratio**	**conc µg/L**	**conc µg/g**	**avarage conc µg/mg**
sample 1	n.d	151368	0	0	0	0
	n.d	134685	0	0	0	
	n.d	147760	0	0	0	
sample 2	n.d	91755	0	0	0	0
	n.d	310166	0	0	0	
	n.d	255378	0	0	0	
sample 3	n.d	190899	0	0	0	79,21749
	10479	208118	0,050351	3,881301794	77,62603587	
	10960	202298	0,054178	4,040729188	80,81458377	
sample4	53388	719475	0,074204	4,875171132	97,50342263	102,526453
	48105	672580	0,071523	4,763462091	95,26924183	
	60428	636298	0,094968	5,740336708	114,8067342	
sample5	3837	150287	0,025531	2,847131267	56,94262533	54,8469
	3830	171649	0,022313	2,713040662	54,26081325	
	4205	198305	0,021205	2,666862913	53,33725826	
sample6	4436	287854	0,015411	2,425441254	48,50882508	48,8604933
	5129	295388	0,017364	2,506816797	50,13633594	
	4248	288517	0,014724	2,396815381	47,93630762	

Table D 2. Area of 3-PBA and 2-PBA, ratio, concentration in µg/L, concentration in µg/mg of hair and average of the concentration in µg/mg of hair of hair samples of rats exposed to permethrin and vitamin E aged 22 days.

Permethrin + vitamin E	**3-PBA**	**2-PBA**	**ratio**	**conc µg/L**	**conc µg/mg hair**	**avarege conc µg/mg**
sample 1	269886	2173345	0,12418	6,9575003	139,15	144,68
	271043	2139998	0,126656	7,0606552	141,21	
	283081	1999138	0,141602	7,6833971	153,67	
sample 2	214019	2484741	0,086133	5,3722218	107,44	99,99
	169327	2325044	0,072827	4,81781	96,36	
	179811	2476267	0,072614	4,8089057	96,18	
sample 3	n.d	2467781	0	0	0,00	0
	n.d	2208622	0	0	0,00	
	n.d	2364243	0	0	0,00	
sample 4	n.d	2506582	0	0	0,00	0
	n.d	2466142	0	0	0,00	
sample 5	n.d	1735968	0	0	0,00	0

	n.d	1671164	0	0	0,00	
sample 6	n.d	625486	0	0	0,00	
	25537	1545804	0,01652	2,4716752	49,43	49,43
sample 7	24482	1379917	0,017742	2,5225686	50,45	50,21
	23954	1396001	0,017159	2,4982922	49,97	

Table D 3. Area of 3-PBA and 2-PBA, ratio, concentration in µg/L, concentration in µg/mg of hair and average of the concentration in µg/mg of hair of hair samples of rats exposed to permethrin and alkaline water aged 22 days.

Permethrin+alkaline water	**3-PBA**	**2-PBA**	**ratio**	**conc µg/L**	**conc µg/mg hair**	**avarege conc µg/mg**
sample 1	n.d	1189668	0	0	0	0
	n.d	1172936	0	0	0	
	n.d	1218064	0	0	0	
sample 2	n.d	35238	0	0	0	0
	n.d	31857	0	0	0	
	n.d	39193	0	0	0	
sample 3	n.d	455857	0	0	0	0
	n.d	493108	0	0	0	
	n.d	464920	0	0	0	
sample 4	4752	149385	0,03181	3,1087676	62,18	80,713333
	10641	153123	0,069493	4,6788814	93,58	
	8974	147477	0,06085	4,3187569	86,38	
sample 5	10997	51798	0,212305	10,629396	212,59	234,726667
	12524	53531	0,233958	11,531579	230,63	
	14014	51836	0,270353	13,048027	260,96	
sample 6	n.d	51798				
	4008	53531	0,074873	4,903021	98,06	98.06
	n.d	51836				
sample 7	n.d	458495				
	34388	452747	0,075954	4,9480883	98,96	96,325
	33206	476923	0,069625	4,6843956	93,69	