UNIVERSIDADE DA BEIRA INTERIOR

ANALYSIS OF ORGANOPHOSPHOROUS PESTICIDES IN POSTMORTEM BIOLOGICAL FLUIDS

RAQUEL HELENA CARVALHO SILVA RAPOSO

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Trabalho elaborado sob a supervisão e orientação científica do Mestre Mário João Dias, Director do Serviço de Toxicolgia Forense da Delegação Sul do Instituto Nacional de Medicina Legal e da Prof. Doutora María Eugenia Gallardo Alba, Faculdade de Ciências da Saúde da Universidade da Beira Interior

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ABBREVIATIONS

1-NAP - 1-(dimethylamino) ethyl phenol

3-Me-PNP - 3-methyl-4-nitrophenol

3-PBA - 3-phenoxybenzoic acid

ACh - Acetylcholine

AChE – Acetylcholinesterase

ADHP - 2-amino-5,6-dimethyl-4-hydroxypyrimidine

AM – Atrazine mercapturate

AP – Acephate

Br2CA - cis-3-(2,2-dibromo-vinyl)-2,2-dimethyl-cyclopropane carboxylic acid

BRP – Naled

BTA - 1,2,3-benzotriazine-4-one

BuChE – Butyrylcholinesterase

C.V. - Coefficient of variation

CGC – Capillary Gas Chromatography

CIT - 5-chloro-1,2-dihydro-1-isopropyl-(3H)-1,2,4-triazol-3-one

CMHC - 3-chloro-4-methyl-7-hydroxycoumarin

CNS – Central Nervous System

CVMP – tetrachlorvinphos

CW – CarbowaxTM

DAT – Dialkylphosphate

DCA - Malathion dicarboxylic acids

DCM – Dichloromethane

DDE – Dichlorodiphenyldichloroethylene

DDT – Dichlorodiphenyltrichloroethane

DDVP – 2,2-dichlorovinyl dimethyl (Dichlorvos)

DEAMPY - 2-diethylamino-6-methyl pyrimidin-4-ol

DEDTP - O,O-Diethyldithiophosfate

DEP – O,O-Diethyl phosphate

DEP - O,O-Diethylphosfate

DETP - O,O-Diethylthiophosphate

DMDTP – O,O-Dimethyldithiophosfate

DMP – O,O-Dimethyl Phosphate

DMTP – O,O-Dimethyl thiophosphate

DVB – Divinylbenzene

EBDC - Ethylene-bis-dithiocarbamate

EC - European Communities

EDDP – Edifenphos

EI-MS - Electron Ionization – Mass Spectrometry

ENP - 1, 1-bis-p-ethoxyphenyl)-2-nitropropane

EPN – Ethyl p-nitrophenyl thionobenzenephosphonate

ETU – Ethylenethiourea

FAO - Food and Agriculture Organization

FDA – U.S. Food and Drug Administration

F-PBA - 4-fluoro-3-phenoxy-benzoic acid

GA – Ethyl-dimethylamidocyanophosphate (Tabun)

GABA - Gamma-aminobutyric acid

GB - Isopropyl methylphosphonofluoridate (Sarin)

GC – Gas Chromatography

GD - Pinacolyl methylphosphonofluoridate (Soman)

HCB - Hexachlorobenzene

HCH– Hexachlorocyclohexane

HCOOH – Methanoic acid

HPLC – High Performance Liquid Chromatography

IMPY - 2,3-dihydro-1H-imidazo (1,2-b)pyrazole

IS – Internal Standard

IUPAC - International Union of Pure and Applied Chemistry

LD50 – Median lethal dose

LLE – Liquid liquid extraction

LLOQ – Lower Limit of Quantification

LOD - Limit of Detection

LOQ – Limit of Quantification

MCA - Malathion monocarboxylic acids

MCPA – 2-methyl-4-chlorophenoxyacetic acid

MDHP - 2-methylamino-5,6-dimethyl-4-hydroxypyrimidine

MeOH – Methanol

MEP – Fenitrothion

METH – Methamidophos

MIP – Molecular Imprinted Polymer

MMP – Methamidophos

MPP – Fenthion

MS – Mass Spectrometry

MS/MS – Tandem Mass Spectrometry

OCP – Organochlorine pesticides

OMS - WHO

OP – Organophosphorous pesticides

PAP – phenthoate

PBB – Polybrominated biphenyl

PBDE – Polybrominated diphenyl ethers

PCB – Polychlorinated biphenyls

PCDD – Polychlorinated dibenzo-p-dioxin

PCDFs – Polychlorinated dibenzofurans

PDMS – Polydimethylsiloxane

PNP - 4-nitrophenol

SIM – Selected Ion Monitoring

SPE – Solid-Phase Extraction

SPME – Solid-phase microextraction

TCP orTCPy - 3,5,6-trichloro-2-pyridinol

TEPP - Tetraethyl pyrophosphate

ULOQ – Upper Limit of Quantification

UV – Ultra Violet

VX - O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothiolate

WHO – World Health Organization

ABSTRACT

Following the intensification of agriculture and the promotion of agro-chemicals in low and middle income countries, acute pesticide poisoning has become a major public health problem with more than 300,000 deaths each year around the world.

The easy availability of highly toxic pesticides in the homes of farming communities has made pesticides a preferred choice for suicide with an extremely high case fatality. In fact, the World Health Organization (WHO) indicates that there may be 1 million serious unintentional poisonings each year and in addition 2 million people hospitalized for suicide attempts with pesticides.

The goal of this work was the detection and quantification of eight organophosphorous pesticides in blood samples using solid phase extraction and gas chromatography-mass spectrometry.

The studied analytes were omethoate, dimethoate, diazinon, chlorpyrifos, parathion, clorfenvinphos, quinalphos and azinphos. Ethion was used as internal standard (IS).

The analytes and IS were extracted by solid-phase extraction using Oasis® HLB extraction cartridges, and the extracts were analyzed by gas chromatography-electron ionisation-mass spectrometry (GC/EI-MS). Calibration curves were established using a weighed linear calibration model (except for omethoate, for which a power regression was used) between 0.05 and 25.00 µg/mL. The correlation coefficients were higher than 0.991. Precision (intraday and intermediate) and accuracy were in conformity with the criteria normally accepted in bioanalytical method validation. Limits of quantification were 50 ng/mL for all compounds, except for ometoathe, for which 100 ng/mL were obtained.

Because of its simplicity and speed, the proposed method can be applied in the determination of these compounds in post-mortem blood samples, and is suitable for application in toxicology routine analysis.

RESUMO

Seguindo a intensificação da agricultura e da promoção de agro-químicos em países de baixo e médio rendimento, o envenenamento agudo por pesticidas tem vindo a tornar-se um grande problema de saúde pública com mais de 300 000 mortes por ano a nível global.

O fácil acesso a pesticidas altamente tóxicos tornou-os numa escolha de eleição para o suicídio, com uma casuística de intoxicação extremamente elevada.

De facto, a Organização Mundial de Saúde (OMS) indica que é possível que haja um milhão de envenenamentos acidentais graves todos os anos e ainda dois milhões de pessoas hospitalizadas por tentativas de suicído com pesticidas.

O objectivo deste trabalho foi a detecção e quantificação de oito pesticidas organofosforados (OP) em amostras de sangue usando extracção em fase sólida e a cromatografia gasosa-espectrometria de massa.

Os analitos estudados foram o ometoato, o dimetoato, o diazinão, o clorpirifos, o paratião, o clorfenvinfos, o quinalfos e o azinfos. O Etião foi usado como padrão interno.

Os analitos e PI foram extraídos por extracção em fase sólida usando as colunas de extracção Oasis® HLB, e os extractos foram analisados através de cromatografia gasosa com ionização de electrões em espectrometria de massa (GC/EI-MS). As curvas de calibração foram estabelecidas usando um modelo de calibração linear ponderado (excepto para o ometoato, para o qual foi usada uma regressão em potência) entre 0,05 e 25.00 µg/mL. Os coeficientes de correlação foram superiores a 0,991. Os valores de precisão (intradia e intermédia) e exactidão estão de acordo com os critérios normalmente aceites em validação de métodos bioanalíticos. Os limites de quantificação foram de 50 ng/mL, excepto para o ometoato em que os limites foram de 100 ng/mL.

Devido à sua simplicidade e rapidez, o método proposto pode ser aplicado na determinação destes compostos em amostras de sangue post-mortem, e é apropriado para aplicação em análises de rotina de toxicologia.

JUSTIFICATION AND OBJECTIVES

Although pesticides have always been the preferred method for suicidal purposes in agricultural areas, they do not represent a high social impact group which requires immediate by any laboratory of toxicological service. However, it is important not to ignore the fact that such group exists, and therefore it may be necessary to determine these compounds precise and accurately. Advantages in determining these compounds reside on the fact that they are not produced endogenously. For such reason, any detected quantity derives, undoubtedly, from an exogenous toxic source, unlike it happens with other chemical groups. The objective of this was the detailed study of a representative group of the most common pesticides in intoxication cases in Portugal, and also in the development and validation of an analytical methodology according to the international and laboratory guidelines of the hosting institution, the Laboratory of Forensic Toxicology, South Branch, National Institute of Legal Medicine.

Organophosphorous pesticide intoxication occurs either by accidental exposure, usually related to the workplace environment, deliberated ingestion in suicidal cases, or even homicides (which are rare events, due to the intense flavor and scent of these compounds).

In emergency rooms, high mortality of the intoxication cases appears as a result of a delayed or even wrong diagnosis. The scarce knowledge of these compounds' metabolism increases the probability of not only delayed or wrong diagnosis, but also misinterpretation of cause of death. For these reasons, fast precise and accurate analytical methods are needed for the detection of organophosphorous insecticides in biological matrices, enabling the correct diagnosis of intoxication and application of maintenance measures.

I - LITERATURE REVIEW

1. INTRODUCTION

The word pesticide has fallen into the quotidian life with great ease brought, though, by the press in a rather infamously way. In general knowledge of population most define pesticide as a substance or mixture that can kill a pest, being the later defined as any threat posed by animals, bacteria, fungi, insects, etc, that endangers the growth or production of a certain agricultural product. The infamous reputation of pesticides comes from its widespread use in the past, when unintended targets, such as human life, were also affected.

A pesticide is by definition "any substance or mixture of substances intended for preventing, destroying or controlling pests, including vectors of human or animal disease, unwanted species of plants or animals causing harm during (or otherwise interfering) the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs; or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport." (FAO, 2002). Therefore, it includes a wide variety of substances from simple minerals to more complex synthetic substances or mixtures.

The earliest evidence found was in Homer's literature, implying the use of sulfur in China 1000 B.C. as a fumigant. Subsequently, in the 19th century in Europe similar compounds were used as fungicides. But it wasn't until the 1930s that the so-called "pesticide revolution" began, giving birth to first synthetic pesticides. It is not a coincidence that the previous and, more importantly, the following years were of warfare (World Wars I and II). Whereas the unraveling of more

effective pesticide compounds brought on a shadier side pesticide's history, for some of their offspring were the infamous Tabun (GA - ethyl dimethylamidocyanophosphate), Sarin (GB - isopropyl methylphosphonofluoridate), Soman (GD - pinacolyl methylphosphonofluoridate), VX (o-ethyl S-(2-diisopropylaminoethyl) methylphosphonothiolate) and TEPP (tetraethyl pyrophosphate). Further researches lead to an increased specificity in intended targets, and with so the birth of pioneer pesticides of modern ages. (Marrs and Ballantyne, 2004; Casarett, et al, 2001).

2. CLASSIFICATION AND CATEGORIZATION

The pesticides can be classified and categorized according to the following: chemical structure, target organism in which their action is most effective, mode of action, toxicity, or even a combination of these. This diversity in the classification is due to the different needs in pesticide classification e.g. for the scientific community the chemical structure gives a better insight of the compound's chemical behavior, and therefore is preferred; however, with commercial purposes, the categorization according to toxicity and targeted organisms is preferable, due to the need to adjust the pesticide to the crops. Table 1 illustrates different categorization types as well as pesticide toxicity (WHO, 2004).

Table 1 - Different types of pesticide classification (chemical structure, toxicity, organism of action, mode of action). (Marrs and Ballantyne, 2004).

CHEMICAL STRUCTURE	TOXICITY (WHO)	ORGANISM OF ACTION	MODE OF ACTION
Organophosphorous	Ia - Extremely hazardous	Herbicide	Anticholinesterase
Organochlorine	Ib - Highly hazardous	Fungicide	GABA blocker
Carbamates	II - Moderately hazardous	Insecticide	Chitin synthesis inhibitor
Pyrethoids	III - Slightly hazardous	Roedenticide	Anticoagulant
Bipyridyls		Acaricide	Glutamine synthetase inhibitor
Organometallics		Nematicide	RNA-polymerase inhibitor
Phenols		Molluscicide	Ecdysone agonist
Morpholines			Juvenile hormone analogues
Phenoxy			Steroid demethylation inhibitor
Azoles			Protoporphyrinogen oxidase inhibitor
Ureas/thioureas			Thiol reactant
Anilines			Protein synthesis inhibitor
Chloronitrile			Photosynthetic electron transport inhibitor
Chloroalkylthiols			Mitochondrial respiration inhibitor

3. ORGANOPHOSPHOROUS PESTICIDES

3.1. STRUCTURE

Organophosphorous pesticides are, as the name implies, compounds containing an organic carbon bonded with phosphorus. This bond can be direct or indirect, and depends on the location of the phosphorus element within the molecule, which can vary greatly. The vast numbers of different organophosphorous pesticides within the family is brought by the diversity of possible groups of the radicals X/Y and Z (Figure 1), and these originate different physical-chemical properties. (INCHEM, 1999; Casarett, et al, 2001).

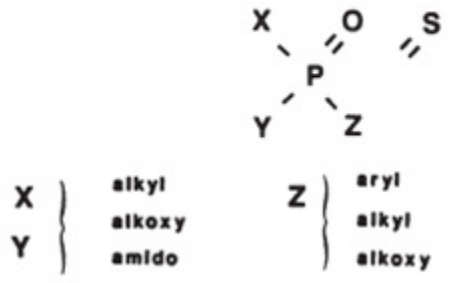
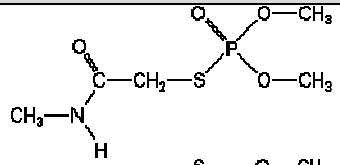
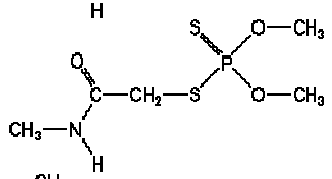
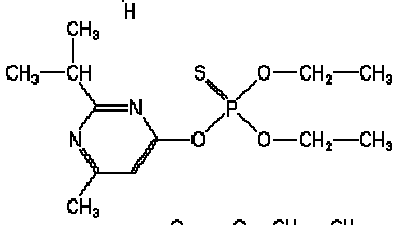
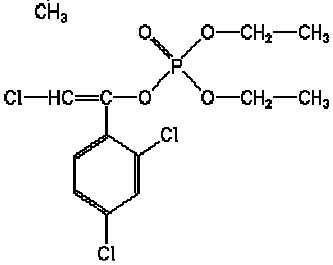
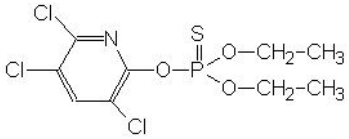
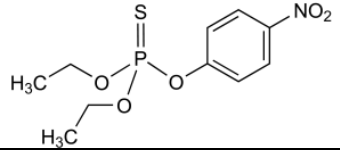


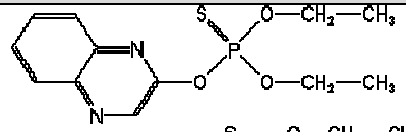
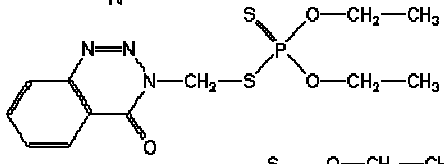
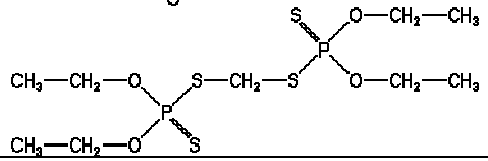
Figure 1 – Structural formula of organophosphorous pesticides. (Casarett, et al, 2001)

3.2. PHYSICAL-CHEMICAL PROPERTIES

Most organophosphorous, have very low solubility in water thus conferring them hydrophobic characteristics granting them higher affinity for organic solvents (e.g. parathion-ethyl nearly insoluble in water, though readily soluble in a vast variety of organic solvents such as alcohols, ethers, esters, ketones and aromatic hydrocarbons (INCHEM, 1999)). The majority of these compounds also possess low vapor pressure, which enhances highly their evaporation at room temperature. Most of organophosphorous can also be hydrolyzed, originating hydrophilic compounds (Gallardo, 2005; INCHEM, 1999; Casarett, et al, 2001). Table 2 resumes the physico-chemical properties of the compounds studied.

Table 2 - Illustrating some of the more useful physico-chemical properties of the OP. (IUPAC FOOTPRINT Pesticide Properties Database – 2009)

Organophophorous Pesticide	Chemical Structure	Molecular Weight (g/mol)	Solubility in water at 20°C (mg/L)	Vapour pressure at 25°C (mPa)	Melting Point (°C)	Boiling Point (°C)	pKa
Omethoate		213.2	10000	3.3	-28	135	-
Dimethoate		229.26	39800	0.247	50.5	Decomposes before boiling	-
Diazinon		304.35	60	11.97	Not applicable	Decomposes before boiling	2.6
Chlorfenvinphos		359.6	145	0.53	-20	167	-
Chlorpyrifos		350.89	1.05	1.43	41.5	Decomposes before boiling	-
Parathion-ethyl		291.26	12.4	0.89	6.1	-	-

Organophophorous Pesticide	Chemical Structure	Molecular Weight (g/mol)	Solubility in water at 20°C (mg/L)	Vapour pressure at 25°C (mPa)	Melting Point (°C)	Boiling Point (°C)	pKa
Quinalphos		298.3	17.8	0.346	31.5	-	-
Azinphos-ethyl		345.38	4.5	0.32	50	111	-
Ethion		384.48	2	0.2	-12	165	-

3.3. MECHANISM OF TOXIC ACTION

Organophosphorous pesticides are also known as acetylcholinesterase (AChE) agents. Their main mechanism of toxic action is through the inhibition of the enzyme acetylcholinesterase, which is the enzyme responsible for terminating the biological activity of the neurotransmitter acetylcholine (ACh). This enzyme is localized in the pre and postsynaptic membranes of cholinergic neurons, but it's also found in erythrocytes. Acetylcholine is released from the presynaptic membrane to the synaptic cleft after stimulation, activating the receptors at the postsynaptic membrane; ACh is then degraded by stopping the activation of the receptors. Organophosphorous insecticides act by inhibition of the AChE, leading to a saturation of ACh in the synaptic cleft and an overstimulation of the receptors. At first, involuntary muscle contraction occurs, and this is followed by desensitization (and thus paralysis) if the inhibition persists. The effects of this inhibition through the body are related to the location of ACh receptors (Gallardo, 2005; Widmaier et al, 2006; Siegel, et al, 1998; Purves et al, 2004), which is summarized in Figure 2.

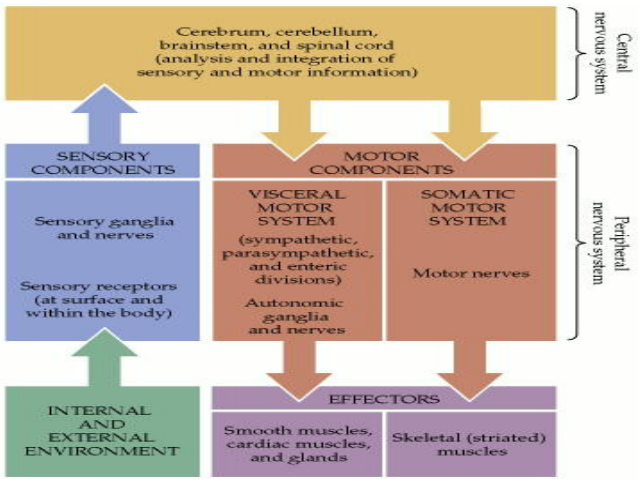


Figure 2 - Location and Function of Cholinergic Receptors in the Nervous System. (Purves et al, 2004)

Organophosphorous insecticides bind so strongly to AChE that the inhibition is considered irreversible, impairing the enzymes for long periods of time, around 20 or 30 days until new AChE is synthetized tocompensate for the excess of ACh accumulated throughout the body. Though AChE is not the only enzyme capable of degrading acetylcholine, it is truly the one with higher affinity for this neurotransmitter, and more widespread throughout the body. For instance, butyrylcholinesterase (BuChE) can partially compensate the absence of AChE though it has lower affinity for acetylcholine, and thus limited in its capacity for its degradation. Therefore, these enzymes are called pseudocholinesterases. These are also affected by the organophosphorous pesticides, however to a lesser extent; Due to its location in the body (liver and plasma) it is highly unlikely that this enzyme can replace effectively the activity of the AChE (Casarett, et al, 2001, Gallard, 2005).

There are two subtypes of ACh receptors: muscarinic (from the toxin extracted of the *Amanita muscaria*) and nicotinic (from nicotine). On one hand, nicotinic receptors are ionotropic, which produce sudden changes in membrane potentials causing a fast depolarization, resulting in a rapid response but usually of low endurance. Muscarinic receptors are, on the other hand, metabotropic, usually associated with G proteins; this requires intermediate activation, which slightly slows the process of depolarization but increases its persistence. The differences ~~in~~ between these receptors will influence the wide variety of symptoms observed and the treatment of the intoxication, which will be addressed later (Gallardo, 2005, Widmaier, et al, 2006, Siegel, et al, 1998, Purves, et al, 2004).

Muscarinic receptors are distributed in different regions of brain (hippocampus, cerebral cortex, cerebellum, brainstem, striatum, central nervous system), sympathetic and parasympathetic systems (effector tissues, innervating many glands and organs), visceral smooth muscle, cardiac

muscle, secretory glands and endothelium cells (Widmaier et al, 2006; Siegel, et al, 1998; Purves et al, 2004).

Nicotinic receptors are distributed in peripheral ganlia, skeletal muscle, reward pathways of the brain, sympathetic and parasympathetic ganglia, adrenal glands, central nervous system and renshaw cells (Widmaier et al, 2006; Siegel, et al, 1998; Purves et al, 2004).

3.4. TOXICOKINETICS

3.4.1. ABSORPTION

As already stated, most of the organophosphorous pesticides are lipophillic, and therefore can be easily absorbed through the majority the entrance routes in the organism (dermal, respiratory and digestive). Since the typical form of application of pesticides is spraying, the main route of entrance will be the respiratory tract, due to the high blood irrigation of the lungs and associated regions. Dermal absorption is, perhaps, the slowest way of entrance in the body, and this depends on a variety of factors that might increase its rate. These factors are the lipophilic character, the physical state of the compound, the solvent in which it is diluted and even the region of the body it is absorbed. (Vale, 1998)

It should be noted, however, that since most OP intoxications are of suicidal nature, the OPs enter the body by ingestion. Therefore, oral and digestive absorption assume high relevance. In these cases, also due to the high irrigation of these areas and lipophilic character of OPs, these are easily absorbed to the blood stream.

3.4.2. DISTRIBUTION, METABOLISM AND EXCRETION

After absorption, organophosphorous insecticides accumulate in the body fat, liver, kidneys and saliva. The lipophilic character of the compound will determine its higher or lower storage rate in fat; furthermore, its storage depends on the whether or not biological activation is needed. Biotransformation of these insecticides occurs mainly at the cytochrome P450. Nevertheless, other systems exist that possess identical ability and outcome; those are flavin-containing mono-oxygenase enzymes, N-oxidation and S-oxidation. Not in all cases the transformation in the cytochrome P450 leads to the activation of the biologically active compounds; indeed, in some cases this transformation facilitates the excretion of the compound. Some of these previously mentioned transformations are oxidative dealkylation and dearylation, ring hydrolation, thioether oxidation, deamination, alkyl and N-hydroxylation, N-oxide formation and N-dealkylation. (Vale, 1998)

In the majority of cases, the cytochrome P450 biotransformation makes the compounds water soluble for urinary excretion, though some excretion also occurs in the feces and exhaled air, but to a much lesser extent, however. The window of detection of these compounds is variable due to the storage in fat tissue. (Vale, 1998)

3.5. TOXIC DOSES AND SYMPTOMS

The toxic doses of a given pesticide is intrinsically related to its toxicity. Table 3 displays the toxicity levels of the studied pesticides, according to their LD50 levels, according to the WHO categorization.

Table 3 – Pesticides' toxic doses (LD50) and WHO classification. Values of LD50 are related to oral ingestion. (Mars and Ballantyne, 2004, IUPAC, 2009)

PESTICIDES	LD50* (mg Kg^{-1}) (RAT)	WHO CLASSIFICATION	CHEMICAL GROUP
Omethoate	64.6	Ib - Highly hazardous	Organophophorous
Dimethoate	245	II - Moderately hazardous	Organophophorous
Diazinon	1139	II - Moderately hazardous	Organophophorous
Chlorpyrifos	66	II - Moderately hazardous	Organophophorous
Parathion	2	Ia - Extremely hazardous	Organophophorous
Chlorfenvinphos	12	Ib - Highly hazardous	Organophophorous
Quinalphos	71	II - Moderately hazardous	Organophophorous
Azinphos	12	Ib - Highly hazardous	Organophophorous

As mentioned above, the symptoms are consequence of the localization of ACh receptors and receptor type (Figure 3), and therefore these will be detailed accordingly. Due to their chemical structure, these insecticides are easily distributed throughout the body, presenting a broad spectrum of symptoms, all associated to the overstimulation and subsequent paralysis of cholinergic receptors.

Action Site	Signs and Symptoms
Central nervous system	Giddiness (a whirling, dizzy sensation), anxiety, CNS stimulation at low to moderate doses due to sparing of ACh from hydrolysis, depression at high doses, apathy, confusion, restlessness, headache, dizziness, anoxia, insomnia, ataxia, absence of reflexes, Cheyne-Stokes respiration, depression of respiratory and circulatory centers, electroencephalographic (EEG) changes, convulsion, and coma
Muscarinic receptor	
Sweat glands	Sweating
Salivation glands	Excessive salivation
Lacrimation glands	Lacrimation (tearing)
Pupils	Constricted pupils (pinpoint, miosis)
Ciliary body	Blurred vision
Bronchi	Wheezing and increased bronchial secretion, cough, pulmonary edema
Cardiovascular system	Bradycardia (slow heart beat), fall in blood pressure
Urinary bladder	Urinary incontinence
Gastrointestine	Abdominal pain, vomiting, diarrhea, fecal incontinence
Nicotinic receptors	
Neuromuscular junction	Fasciculations, cramps, weakness, muscle twitching, respiratory difficulty, tightness in chest tremor, paralysis, cyanosis, arrest
Sympathetic ganglia	Tachycardia, elevated blood pressure (since only few cholinergic synapses are present in the vasculature, they have no control over blood pressure and the predominant action is stimulation of the sympathetic ganglia, i.e., increased blood pressure)

Figure 3 – Symptoms of organophophorus pescitides toxicity. (Abou-Donia, 1992)

3.6. TOXIC DOSES AND TREATMENT

One of the most common treatments to cholinergic toxicity is the administration of atropine, a blocker of the ACh receptors (muscarinic receptors only, it is ineffective in the CNS and nicotinic receptors) which will help to reduce the repeated stimulation given by the excess of ACh on the synaptic cleft by the inhibition of AChE. Oximes, such as pralidoxime (2-PAM) and trimedoxime (TBM-4) are also administrated, supplementing the atropine treatment; this helps the reactivation of AChE inhibited by the insecticide, hydrolyzing the phosphorylated AChE (Abou-Donia, 1992).

Respiratory failure is a common aspect of the intoxication, and therefore measures are taken to aid the respiratory system, including clearing of the airways, giving oxygen and possibly artificial ventilation.

Other procedures are taken in order to prevent further intoxication, such as skin washing with water and alkaline soap to remove the compound from the skin and promote hydrolysis of the ester. Conventional treatment includes managing of symptoms, using diazepam, or other anticonvulsants, to prevent damage to the body during the convulsive phase (Abou-Donia, 1992).

3.7. ETIOLOGY OF PESTICIDE INTOXICATIONS

Etiology is by definition the study of cause, and the main causes of organophosphorous intoxication are:

- Accidental – related, in its majority, to the occupational environment, usually agricultural, with little knowledge about the dangers of the pesticide under use, sometimes complete ignorance of the compound due to bad labeling, bad storage location/conditions, etc;
- Suicidal – usually happens in rural areas, where there is an easy access to these compounds. Though due to little knowledge of the compound by the individual and prolonged death times which can last from 5 min to 24 h, ending up in a second suicidal attempt by hanging.

- Homicidal – these are one of the rarest, but not completely absent, cases. The compounds' organoleptic characteristics are extremely marked and can be easily detected by the victim. (Gallardo, 2005; INCHEM, 2009)

3.8. INTOXICATION CASUISTIC IN THE SOUTH REGION OF PORTUGAL

From 2003-2006, the National Institute of Legal Medicine, South Branch, on its delegated area, detected a total of 103 positive cases for pesticide poisoning. In most of these cases (86 in 103) an organophosphorous compound was involved, as shown in Figure 4.

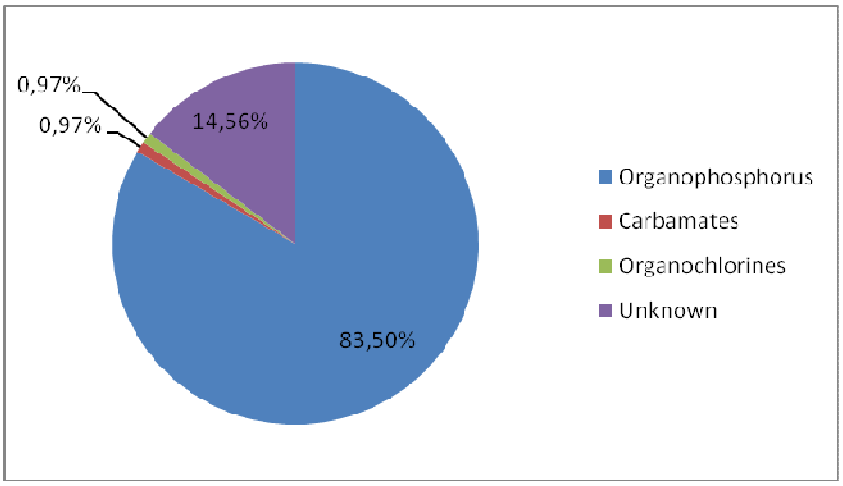


Figure 4 - INML statistics of pesticide intoxications between 2003 and 2006.

More than 100 different compounds are included in the organophosphorous family, representing a wide variety of chemically active substances. However, these positive cases were caused mainly by 9 insecticides, as shown in Figure 5.

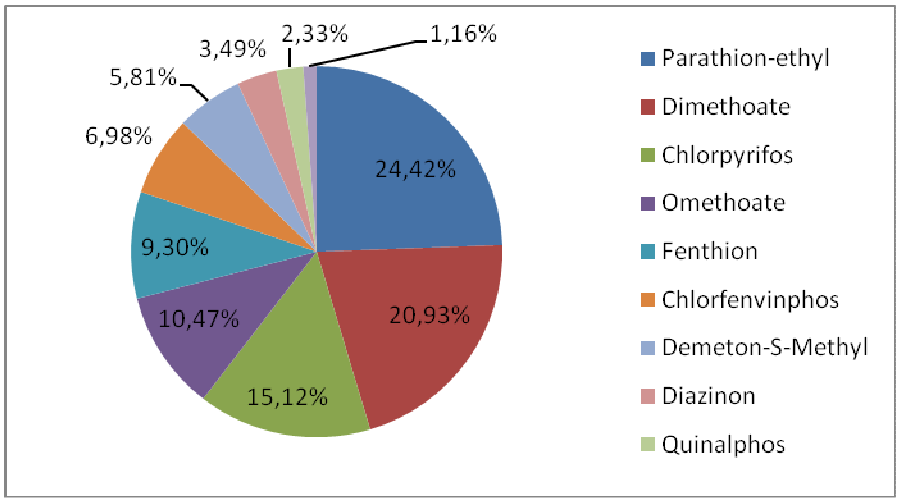


Figure 5 - Detailed statistics of organophosphorous casuistic

4. Legal Background of Pesticide Usage and Management in Portugal

Nowadays the political policy and environmental laws of pesticide usage and management in Portugal follow the European Union legislation and its directives, supporting the withdrawal from the market of those products that might be dangerous to the handling subjects, as well as to the consumers. In addition, all market entries of new products are controlled, as occurs with the residue levels in plant products. The national policies also protect the direct user by classifying pesticides, and regulating their labeling and packaging in order to avoid possible misusages. Table 4 to Table 9 resume the different legislations that exist concerning organophosphorous pesticides.

4.1. EUROPEAN LEGISLATION

European Union directives have been the source of harmonization of local policies and environmental laws throughout the European countries, and the backbone of the vast majority of law decrees in Portugal. Table 4 illustrates the importance of such directives.

Table 4 - European Legislation of plant protection products usage and management.

EUROPEAN DIRECTIVES' CODE AND DATE	EUROPEAN DIRECTIVES' SUMMARY
Council Directive 67/548/EEC of 27th of June 1967	The approximation of laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances
Council Directive 76/769/EEC of 27th of July 1976	On the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations
Directive 1999/45/EC of the European Parliament and of the Council of 31th of May 1999	Concerning the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labeling of dangerous preparations
Commission Directive 2001/60/EC of 7th August 2001 – adapting the following	– Directive 1999/45/EC of the European Parliament and of the Council of 31th of May 1999 – Commission Directive 98/98/EC – Council Directive 67/548/EEC – Directive 2000/33/EC – Directive 1999/45/EC – 2001/59/EC – Directive 67/548/EEC
Council Directive 91/689/EEC 12th of December of 1991	On hazardous waste
Directive 2006/12/EC of the European Parliament and Council on the 5th of April of 2006	Related to waste
Council Regulation (EEC) No 793/93 of 23th of March 1993	Evaluation and control of the risks of existing substances
Council Directive 79/409/EEC of 2nd of April 1979	Natura 2000 - On the conservation of wild birds. Pesticide sensitive areas
Council Directive 92/43/EEC of 21th of May 1992 – To complement Directive 79/409/EEC	Natura 2000 - on the conservation of natural habitats and of wild fauna and flora. Pesticide sensitive areas
Council Directive 98/24/EC of 7th of April of 1998	On the protection of the health and safety of workers from the risks related to chemical agents at work (fourteenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC)
Directive 2004/37/EC of the European Parliament and Council 29th of April 2004	On the protection of workers from the risks related to exposure to carcinogens or mutagens at work (Sixth individual Directive within the meaning of Article 16(1) of Council Directive 89/391/EEC)

Council Directive 89/391/EEC of 12th of June 1989	On the introduction of measures to encourage improvements in the safety and health of workers at work
Directive 2006/42/EC of the European Parliament and Council on the 17th of May of 2006	On machinery, and amending Directive 95/16/EC (recast)
European Parliament and Council Directive 95/16/EC of 29th June 1995	On the approximation of the laws of the Member States relating to lifts

4.2. PORTUGUESE LEGISLATION

4.2.1. MARKET INTRODUCTION

Market entry of new pesticide formulae, or withdrawal of a determined pesticide, are discriminated in Table 5.

Table 5 - Portuguese Legislation of market introduction of plant protection products. (Gallard, 2005; Diario daRepublica (DRE); V|lex; Apambiente; BDJUR; dgadr;APN)

LEGISLATION'S CODE AND DATE	SUMMARY	TYPE OF LEGISLATION
Decree-Law No. 94/98, of the 15th of April. D.R. No. 88, Series I-A	Adopts the technical standards of performance for the placing of plant protection products on the market	National Legislation - Decree-Law
Decree-Law No 341/98 of the 4th of November. D.R. No. 255, Series I-A	Establishes the uniform principles related to the evaluation and authorization of plant protection products and their placement on the market.	National Legislation - Decree-Law
Decree-Law No 284/94 of the 11th of November. D.R. No. 261, Series I-A	Transposing into national law Directive No. 91/414/EEC of the Council of the 15th of July concerning the placing of plant protection products on the market	National Legislation - Decree-Law
Decree-Law No 101/2002 of the 12th of April. D.R. No. 86, Series I-A	Making the inclusion of nine active substances in the Annex I to Decree-Law n. º 94/98, of April 15th, adopting technical implementing standards for the placement of plant protection products on the market, by transposing the Directives No 2001/21 / EC and 2001/87/EC, of the Commission, 5 March and 12 October respectively.	National Legislation - Decree-Law
Decree-Law No 121/2002 of the 3rd of May. D.R. No. 102, Series I-A	Establishes the legal regime on the marketing of biocidal products, transposing Directive No. 98/8/EC of the European Parliament and the Council of the 16th of February.	National Legislation - Decree-Law
Decree-Law No 131/97 of the 30th of May 1997	It attributes to the Direcção-Geral de Protecção das Culturas the power to grant permits for the sale of pesticides in processed wood preservatives.	National Legislation - Decree-Law
Decree-Law n.º 72-H/2003, of the 14th of April of 2003	Transposing into national law the Directive No 2001/103/EC, 2002/18/EC, 2002/37/EC, 2002/48/EC, 2002/64/EC and 2002/81/EC, all of the Commission, respectively of the 28th of November, 22nd of February, 3rd of May, 30th of May, 15th of July and 10th of October, for the inclusion of various substances in the Community Positive List. Amending the	National Legislation - Decree-Law

	Decree-Law No 94/98 of the 15th of April, that approved the standard norms for the implementation, relative to of applicable regimen, of plant protection products.	
Decree-Law nº 22/2001 of the 30th of January of 2001	N/A	National Legislation - Decree-Law
Decree-Law nº 238/2001 of the 30th of August of 2001	The present diploma transposes the Directives No 2000/80/CE, of the Commission, of the 4th of December, and 2001/28/CE, of the Commission, of the 20th of April, thus determining the substitution of Annex I of the Decree-Law No. 94/98, of the 15th of April, for the annex of the present diploma, of which it is integrant part.	National Legislation - Decree-Law
Decree-Law n.º 28/2002 of the 14th of February de 2002	1 - The present diploma transposes for the internal jurisprudence the Directivas No. 2001/47/CE and 2001/49/CE, of the Commission, respectively of the 25th and 28th of June, for the inclusion of Paecilomyces fumosoroseus active substances (of the strain Apopka 97, PFR 97 or CG 170, ATCC20874), afterwards named Paecilomyces fumosoroseus, and DPX KE 459 (flupyrsulfuron-methyl), afterward named flupyrsulfuron-methyl, in the Community Positive List. 2 - In Annex I of Decree-Law No. 94/98 of April 15th, as last amended by Decree-Law No 238/2001 of the 30th of August, are added No. 18 and 19, in the terms of the annex to the present diploma, of which it is integrant part.	National Legislation - Decree-Law
Decree-Law n.º 160/2002, of the 9th of July of 2002	Transposes for the internal jurisprudence the Directive No. 2001/36/CE, of the Commission, of the 16th of May, introducing changes to annexes II and III of the Decree-Law No. 94/98, of the 15th of April, for the placement of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 198/2002, of the 25th of September of 2002	Proceeds to the inclusion of two active substances in the Annex I to the Decree-Law No 94/98, of the 15th of April, that adopts standard norms of implementation referring to the placement of plant protection products on the market, transposing Directive No. 2001/99 / EC, of the Commission, of the 20th of November.	National Legislation - Decree-Law
Decree-Law n.º 215/2003, of the 18th of September of 2003	Transposes for the national jurisprudence the Directive No. 2003/23/CE, of the Commission, of the 25th of March, for the inclusion of active substances imazamox, oxasulfuron, etoxisulfuron, foramsulfuron, oxadiargyl and ciazofamide in the Community Positive List, modifying the Decree-Law No 94/98, of the 15th of April.	National Legislation - Decree-Law
Decree-Law n.º 22/2004, of the 22th of January of 2004	Transposes for the national jurisprudence the Directive No. 2003/82/CE, of the Commission, of the 11th of September, that modifies the Directive No. 91/414/CEE, of the Council, regarding the phrases type related to the special risks and to the phrases type related on precautions to take for plant protection products, adding the Annexes V and VI of Decree-Law No 94/98 of the 15th April concerning the placement of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 39/2004, of the 27th of February of 2004	Transposes for the national jurisprudence the Directives No. 2003/5/CE, 2003/31/CE, 2003/68/CE, 2003/79/CE and 2003/84/CE, of the Commission, of the 10th of January, 11th of April, 11th of July, 13th of August and 25th of September respectively, concerning the inclusion of active substances deltamethrin, 2,4-DB, beta-cyfluthrin, cyfluthrin, iprodione, linuron, maleic hydrazide, pendimethalin, trifloxystrobin,	National Legislation - Decree-Law

	carfentrazone-ethyl, mesotrione, fenamidone, isoxaflutole, Coniothyrium minitans, flurtamone, flufenacet, iodosulfuron, dimethenamid-P, picoxystrobin, fosthiazate and silthiofam, in the Community Positive List.	
Decree-Law n.º 22/2005, of the 26th of January of 2005	Transposes for the internal jurisprudence the Directives No. 2003/39/CE, of the 15th of May, 2003/70/CE, of the 17th of July, 2003/81/CE, of the 5th of September, 2003/112/CE, of the 1st of December, 2003/119/CE, of the 5th of December, 2004/30/CE, of the 10th of March, 2004/60/CE, of the 23rd of April, 2004/62/CE, of the 26th of April, and 2004/71/CE, of the 28th of April, of the Commission, including new active substances in the Annex I of the Decree-Law No. 94/98, of the 15th of April, the Directive No. 2004/97/CE, of the 27th of September, that modifies the Directive No. 2004/60/CE, regarding deadlines, as well as the Directives No. 2004/64/CE, of the 26th of April, and 2004/65/CE, of the 26th of April, introducing changes to the Decree-Law No. 39/2004, of the 27th of February.	National Legislation - Decree-Law
Decree-Law n.º 128/2005, of the 9th of April of 2005	Transposes for the internal jurisprudence the Directives No. 2004/20/CE, of the 2nd of March, 2004/58/CE, of the 23rd of April, 2004/99/CE, of the 1st of October, 2005/2/CE, of the 19th of January, and 2005/3/CE, of the 19th of January, of the Commission, including new active substances of plant protection products in the Annex I of the Decree-Law No. 94/98, of the 15th of April.	National Legislation - Decree-Law
Decree-Law n.º 154/2005, of the 6th of September of 2005	Updates the plant health regime that creates and defines measures of plant protection to prevent the introduction and spread within national and EU territory, including protected areas, harmful organisms to plants and plant products in whatever origin or source.	National Legislation - Decree-Law
Decree-Law nº 173/2005, of the 21st of October of 2005 (changed by the Decree-Law nº 187/2006, of the 19th of September of 2006)	1 - The present diploma regulates the activities of distribution, sales, provision of services of application of plant protection products and their application by end users. 2 - The plant protection products of low risk are not covered by the present diploma, with the exception of the applicable norms to the residues of packages and surpluses of these plant protection products.	National Legislation - Decree-Law
Decree-Law nº 187/2006, of the 19th of September of 2006	It establishes the conditions and procedures of security, in the scope of management systems of residues of packages and residues of surpluses of plant protection products and changes the Decree-Law No. 173/2005, of the 21st of October.	National Legislation - Decree-Law
Decree-Law n.º 19/2006, of the 31st of January of 2006	Transposes for the national jurisprudence the Directive No. 2005/25/CE, of the Council, of the 14th of March, and the Directive No. 2005/34/CE, of the Commission, of the 17th of May, introducing changes to annexes I and IV of the Decree-Law No. 94/98, of the 15th of April, concerning the placing of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 87/2006, of the 23rd of May of 2006	Transposes for the national jurisprudence the Directives No. 2005/53/CE, of the 16th of September, 2005/54/CE, of the 19th of September, and 2005/58/CE, of the 21st of September, of the Commission, introducing changes to the Annex I of the Decree-Law No. 94/98, of the 15th of April, concerning the placing of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 234/2006, of the 29th of November of 2006	Transposes for the internal jurisprudence the Directives No. 2005/57/CE, of the 21st of September, 2005/72/CE, of the 21st of October, 2006/10/CE, of the 27th of January, 2006/16/CE, of the 7th of February, 2006/19/CE, of the 14th	National Legislation - Decree-Law

	of February, 2006/45/CE, of the 16th of May, and 2006/76/CE, of 22nd of September, of the Commission, introducing changes to the Annex I of the Decree-Law No. 94/98, 15 of April, concerning the placing of plant protection products on the market.	
Decree-Law n.º 111/2007, of the 16th of April of 2007	Amending the Decree-Law No. 94/98, of April 15th, adopting technical norms of implementation concerning the placement of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 206/2007, of the 28th of May of 2007	Transposes for the internal jurisprudence the Directives No. 2006/5/CE, of the 17th of January, 2006/6/CE, of the 17th of January, 2006/41/CE, of the 7th of July, and 2006/75/CE, of the 11th of September, of the Commission, introducing changes to the Annex I of the Decree-Law No. 94/98, of the 15th of April, concerning the placing of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 334/2007, of the 10th of October of 2007	Transposes for the internal jurisprudence the Directives No. 2006/39/CE, of the 12nd of April, 2006/64/CE, of the 18th of July, 2006/74/CE, of the 21st of August, 2006/131/CE, of the 11th of December, 2006/132/CE, of the 11th of December, 2006/133/CE, of the 11th of December, 2006/134/CE, of the 11th of December, 2006/135/CE, of the 11th of December, 2006/136/CE, of the 11th of December, 2007/6/CE, of the 14th of February, and 2007/21/CE, of the 10th of April, of the Commission, introducing changes to the Annex I of the Decree-Law No. 94/98, of the 15th of April, concerning the placing of plant protection products on the market.	National Legislation - Decree-Law
Decree-Law n.º 61/2008, of the 28th of March of 2008	Making the 22nd Amendment of the Decree-Law No 94/98 of 15 April concerning the placing of plant protection products on the market, transposing for the internal jurisprudence the Directive No 2006/85/EC of the 23rd October, 2007/5/EC of the 7th February, 2007/25/CE of the 23rd April, 2007/31/CE of May 31th, 2007/50/CE, from August 2nd and 2007 / 52/CE, of the 16th of August, of the Commission.	National Legislation - Decree-Law
Decree-Law n.º 63/2008, of the 2nd of April of 2008	Making the 1st amendment of the Decree-Law No 82/2003 of 23 April approving the Regulations for the Classification, Packaging, Labeling and Safety Data Sheets of dangerous preparations, transposing for the internal jurisprudence the Directives No 2004/66/EC, of the 26th April, 2006/8/EC, of the 23rd January, and 2006/96/EC, of the 20nd November.	National Legislation - Decree-Law
Decree-Law n.º 244/2008, of the 18th of December of 2008	Making the 24th amendment of the Decree-Law No 94/98 of the 15th of April, concerning the placement of plant protection products on the market, transposing for the internal jurisprudence the Directive No. 2008/44/CE of the Commission of 4 April, amending Directive No. 91/414/EEC of the Council, to include the active substances benthiavalicarb, boscalid, carvone, fluoxastrobin, Paecilomyces lilacinus and prothioconazole and Directive No. 2008 / 45/CE, the Commission of April 4th, amending Directive No. 91/414/EEC of the Council regarding the extension of use of the active substance metconazole.	National Legislation - Decree-Law

4.2.2. MAXIMUM RESIDUE LEVELS

In order to protect the final consumer of the plant products, the residue levels of pesticides in these are controlled. Table 6 illustrates the established maximum for these levels.

Table 6 - Portuguese Legislation of maximum residue levels of plant protection products. (Gallardo, 2005; Diario da Republica (DRE); V|lex; Apambiente; BDJUR; dgadr;APN)

LEGISLATION'S CODE AND DATE	SUMMARY	TYPE OF LEGISLATION
Decree-Law n.º 215/2001, of the 2nd of August. D.R. n.º 178, Série I-A	Approves new maximum residue levels for plant protection products allowed within and on the surface area of cereals, fruits and vegetables. It establishes the regulations of the maximum limits of residues allowed for plant protection products in agricultural	National Legislation - Decree-Law
Decree-Law n.º 144/2003, of the 2nd of July of 2003 (only 10th and 11th article)	products of plant origin intended for human consumption, or even occasionally, animal feed, as well as agricultural products dried or transformed, or still after incorporated in compound feed, in that it may contain residues of plant protection products.	National Legislation - Decree-Law
Decree-Law n.º 27/2000, of the 3rd of March of 2000	Amending certain maximum residue levels for plant protection products on the surface and inside of fruits, vegetables and cereals, proceeding to the transposition for the internal jurisprudence of paragraphs Directives No. 97/71/EC and 98/82/EC, of the Commission, of December 15th and October 27th respectively.	National Legislation - Decree-Law
Decree-Law n.º 21/2001, of the 30th of January of 2001	Approves the list of maximum residue levels for plant protection products allowed inside and on the surface area of cereals, fruits and vegetables. Transposing Directives Nos 1999/71/EC, of July 14th and 2000/24/EC, of April 28th.	National Legislation - Decree-Law
Decree-Law n.º 256/2001, of the 22nd of September of 2001	Transposes for the domestic law the Directive No 2001/35/EC of 11 May amending the MRLs for plant protection products allowed inside of cereals, fruits and vegetables.	National Legislation - Decree-Law
Decree-Law n.º 31/2002, of the 19th of February de 2002	Amending and approving certain maximum residue levels for plant protection products allowed in agricultural products of plant origin, including fruit, vegetables and cereals.	National Legislation - Decree-Law
Decree-Law n.º 245/2002, of the 8th of November of 2002	Amending and approving certain maximum residue levels for active substances of plant protection products allowed in agricultural products of plant origin, including fruit, vegetables and cereals, and transposing for the national jurisprudence the Commission Directives 2002/5/EC paragraphs, and 2002 / 23/CE, of January 30th and February 26th, respectively.	National Legislation - Decree-Law
Decree-Law n.º 68/2003, of the 8th of April of 2003	Amending and approving certain maximum residue levels for active substances of plant protection products allowed in agricultural products of plant origin, including fruit, vegetables and cereals, and transposing for the national jurisprudence the Directives No. 2002/42/CE, 2002/66/CE, 2002/71/CE, 2002/76/CE and 2002/79/CE, of the Commission, of May 17th, of July 17th, of August 19th, of	National Legislation - Decree-Law

	September 6th and of October 2nd, respectively.	
Decree-Law n.º 156/2003, of the 18th of July of 2003	Amending and approving certain maximum residue levels for active substances of plant protection products allowed in agricultural products of plant origin, including fruit, vegetables and cereals, transposing for the national jurisprudence the Directive No 2002/97/EC of the Commission of December 16th, in part relating to agricultural products of plant origin, and Directive No 2002/100/EC, of the Commission of December 20th.	National Legislation - Decree-Law
Decree-Law n.º 300/2003, of the 4th of December of 2003	Amended and adopted maximum residue levels for active substances of plant protection products allowed in agricultural products of plant origin, transposing for the national jurisprudence the Directive No 2003/60/EC, of the Commission of June 18th in the part concerning the agricultural products of plant origin, paragraphs and Directives 2003/62/EC, of the Commission of June 20th, and 2003/69/EC, of the Commission of July 11th.	National Legislation - Decree-Law
Decree-Law n.º 39/2009, of the 10th of February of 2009	Ensures the implementation and guarantees compliance in the internal jurisprudence, the obligations under Regulation (EC) No 396/2005 of the European Parliament and Council of February 23rd on maximum residue levels of pesticides within and on the surface of food and feed of plant and animal origin.	National Legislation - Decree-Law

4.2.3. CLASSIFICATION, LABELING AND PACKAGING

Table 7 - Portuguese Legislation of classification, labeling and packaging of plant protection products. (Gallardo, 2005; Diario da Republica (DRE); V|lex; Apambiente; BDJUR; dgadr;APN)

LEGISLATION'S CODE AND DATE	SUMMARY	TYPE OF LEGISLATION
Decree-Law n.º 294/88, of the 24th of August of 1988	Establishes standards for classification, labeling and packaging of pesticides and adjuvants.	National Legislation - Decree-Law
Decree-Law n.º 82/2003, of the 23rd of April of 2003	Approves the Regulations for the Classification, Packaging, Labeling and Safety Data Sheet of Dangerous Preparations.	National Legislation - Decree-Law

4.2.4. SEEDS TREATED WITH PLANT PROTECTION PRODUCTS.

Fairly recently this decree-law was released to control the pesticides used to coat seeds, in order to maintain control throughout the production chain (Table 8).

Table 8 - Portuguese Legislation of management of seeds dealt with plant protection products. (Gallardo, 2005; Diario da Republica (DRE); V|lex; Apambiente; BDJUR; dgadr;APN)

LEGISLATION'S CODE AND DATE	SUMMARY	TYPE OF LEGISLATION
Decree-Law n.º 38/2009, of the 10th of February of 2009	Making the third amendment to Decree-Law No 144/2005 of 26 August, which regulates the production, testing, certification and marketing of seeds of species of agricultural and vegetable species, and transposing for the national jurisprudence the Directive No . º 2007/72/CE, the Commission of 13 December on the inclusion of forage species *Galega orientalis Lam*	National Legislation - Decree-Law

4.2.5. CONDITIONS OF COMMERCIALIZATION AND APPLICATION OF PLANT PROTECTION PRODUCTS.

The condition in which the pesticide products are commercialized were found to be an hazard point in the pesticide chain of usage, therefore a point that must be under surveillance and control, and Table 9 shows these control measures.

Table 9 - Portuguese Legislation in relation to conditions of commercialization and application of plant protection products. (Gallard, 2005; Diario da Republica (DRE); V|lex; Apambiente; BDJUR; dgadr;APN)

LEGISLATION'S CODE AND DATE	SUMMARY	TYPE OF LEGISLATION
Decree-Law n.º 173/2005, of the 21st of October of 2005	Regulates the activities of distribution, sales, provision of services of application of plant protection products and their application by end users.	National Legislation - Decree-Law
Decree-Law n.º 187/2006, of the 19th of September of 2006	It establishes the conditions and procedures of security, in the scope of management systems of residues of packages and residues of surpluses of plant protection products and changes the Decree-Law No. 173/2005, of the 21st of October.	National Legislation - Decree-Law

5. METHODOLOGY FOR THE DETERMINATION OF PESTICIDES

It is clear that over the years the methodology for pesticide determination and quantification has evolved drastically from the simple colorimetric tests to more complex chromatographic systems coupled with sophisticated and elaborated detection devices.

Several methods used in different technologies have been developed over time to better analyze pesticides in a variety of matrices, some more complex than others in cases needing previous separation and purification in order not to overload the now more sensitive detection.

In the following table (Table 10) are shown the methods used in the last 10 years to determine and quantify organophosphorous, organochlorine and carbamate pesticides in biological matrices. This research illustrated has been the back bone of this study, giving insight of the different approaches to the detection and determination of pesticides in complex matrices.

Table 10 - Table of methods used to determine pesticides over the last 10 years.

Pesticide	Category	Species	Sample	Extraction	Chromatography	Detection	LOD	LOQ	Reference
Chlorpyrifos and Dimethoate	Organophosphorous	Human	Plasma	N/A	High Performance Liquid Chromatography (HPLC)	UV detector	N/A	0.1 and 1.0 nmol/mL for chlorpyrifos and dimethoate respectively	Eddleston et al. (2009)
dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP) and diethyl phosphate (DEP)	Organophosphorous' Metabolites	Human	Hair	Decontamination step, solid-liquid extraction, followed by liquid-liquid extraction, pentafluorobenzyl bromide derivatization, clean-up on Florisil/PSA column	Gas Chromatography (GC)	Mass Spectrometry (MS)	ranged from 0.02 to 0.10 ng / mg	N/A	Margariti and Tsatsakis (2009)
Pirimicarb	Carbamates	Human	Stomach fluid, Urine and Plasma	N/A	Gas Chromatography (GC)	Mass Spectrometry (MS)	< 10 ng/mL	20 ng/mL	Hoffman et al. (2008)
PCBs, Chlordanes, Toxaphenes, HCHs, DDTs, and HCB, as well as PBDEs	Organochlorine	Polar Bears	Adipose Tissue (Subcutaneous) Blood (Femoral vein or artery)	Dichloromethane (DCM) - Gel Permeation Column (GPC) (Hexane: DCM (1:1) as elution solvent)	High Resolution Capillary Gas Chromatograph (GC)	Electron Capture Detector(ECD) and Electron Capture Negative Ion Mass Spectrometry (ECNIMS)	N/A	N/A	Bentzen, Muir, Amstrup and O'Hara (2008)
PCBs	Organochlorine	Human	Stomach Content	Soxhlet and liquid-liquid extraction	Gas Chromatography (GC) and Silica-SFE	Mass Spectrometry (MS)	N/A	N/A	Adenugba, McMartin and Beck (2008)
OCPs, nitro musks and PCBs	Organochlorine	Human	Milk	Micro Glass Column and eluted with n-hexane/acetone (2:1, v/v) and gel permeation chromatography (GPC)	High Resolution Gas Chromatography (HRGC)	Electron Capture Detector (ECD)	N/A	N/A	Raab et al (2008)
Chlorpyrifos, diazinon, malathion and parathion.	Organophosphorous	Human	Blood	Solid-Phase Extraction (SPE) (Oasis HLB™ cartridge)	Gas Chromatography (GC)	Mass Spectrometry (MS)	0.04 to 0.09 mg/L	0.13 to 0.17 mg/L	Park et al (2008)

(acephate, methidathion, dichlorvos, fenthion, EPN, diazinon, phenthoate, malathion, fenitrothion, and cyanophos	Organophosphorous	Human	Serum	Deproteinization by Acetonitrile	High Performance Liquid Chromatography (HPLC) (Xterra MS C18 stainless steel cartridge column equipped with an Xterra MS C18 guard column at 50°C susing 10mM ammonium formate-methanol as mobile phase)	Mass Spectrometry (MS) (triple quadrupole with APCI interface)	0.125µg/mL to 1 µg/mL	0.25µg/mL to 1.25µg/mL	Inoue et al. (2007)
PBDEs, PCBs, DDTs, HCB, Chlordane related pesticides, HCH and Toxaphene	Organochlorine	Ringed Seal	Blubber, Liver, Kidney and Muscle Tissue	Soxhlet extracted with n-hexane:acetone (4:1)	Gas Chromatography (GC)	Electron Capture Detector (ECD), Mass Spectrometry (MS) and Electron Capture Negative Ionization (ECNI)	N/A	N/A	Vorkamp et al (2007)
PBB and PCB	Organochlorine	Human	Serum	Ether-Ethyl or Hexane-Ether - Florisil or Florisil and silica gel column	Gas Chromatography (GC)	Electron Capture Detector (ECD)	N/A	N/A	Small et al (2007)
11 hydroxy metabolites of PCBs	Organochlorine	Rat	Plasma and Organ Tissues	Silica Column and 10 mL of n-hexane/dichloromethane (4:6, v/v) as an eluent	Gas Chromatography (GC)	Mass Spectrometry (MS)	N/A	N/A	Hong et al (2007)
Propoxur and Cypermethrin	Carbamates and Pyrethroids	Human	Meconium	Solid-Phase Extraction (SPE)	Gas Chromatography (GC)	Mass Spectrometry (MS)	N/A	N/A	LaFiura et al.(2007)
Karbutilate	Carbamates	Human	Urine	Solid-Phase Extraction (SPE) - Cartridge Bond Elut C18 - Elution: 10% acetonitrile	N/A	Photo-induced Chemiluminescence	10 µg/L	20µg/L	Amorim et al. (2007)
HCB, b -HCH, c-HCH, Heptachlor, Aldrine, Heptachlor epoxide, Dieldrine, o,p'- DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT and p,p'-DDT.	Organochlorine	Human	Milk	20 mL of n-hexane, 5 mL of acetonitrile and 1 mL of ethanol	Capillary Column Gas Chromatography	Electron Capture Detector (ECD)	N/A	N/A	Ennaceur et al (2007)
PCB congeners, p; p0-DDPCB congeners, p; p0-DDE, and HCB	Organochlorine	Human	Serum	N/A	Gas Chromatography (GC)	Electron Capture Detector (ECD)	N/A	N/A	Meeker et al. (2007)

Methylsulfonyl PCB and DDE metabolites	Organochlorine	Human	Adipose Tissues	Liquid–liquid extraction (n-hexane:acetone 2:1 v/v), Gel Permeation Chromatography (GPC) fractionation and adsorption chromatographic clean-up (33% KOH/silica gel).	Gas Chromatography (GC)	Electron Capture Detector (ECD) and Mass Spectrometry (MS)	N/A	0.1 - 0.4 ng/g of lipids	Karasek et al (2006)
Carbaryl	Carbamates	Rats	Plasma	Solid-Phase Extraction (SPE) cartridges with 50 mg polymer	N/A	In-line MIP (Molecular Imprinted Polymer)	10 µg/mL	10 µg/mL	Hantash et al (2006)
p,p0-DDT and congeners/metabolites, endosulphan and congeners/ metabolites, lindane, aldrin/dieldrin/endrin, hexachlorobenzene, methoxychlor and mirex	Organochlorine	Human	Placenta	Solid-Liquid Technique and purified by Preparative Liquid Chromatography	Gas Chromatography (GC)	Electron Capture Detector (ECD) and Mass Spectrometry (MS)	N/A	N/A	Lopez-Espinosa et al. (2006)
PCBs and PBDEs	Organochlorine	Human	Serum	solid-phase extraction (SPE) (Oasis® HLB cartridge) and the subsequent on-line fat elimination by directly dropping the eluate from the SPE cartridge onto a second cartridge containing layers of activated neutral silica gel and sulphuric acid modified silica gel	Gas Chromatography (GC)	Ion Trap Detector in the Tandem Mass Spectrometry Mode	N/A	N/A	Ramos et al (2006)
Propoxur, Cyfluthrin, Chlorpyrifos, Cypermethrin, Pretilachlor, Bioallethrin, Malathion, Diazinon, Lindane, DDT, Transfluthrinand a few metabolites	Carbamate, Organophosphate, Organochlorine, Pyrethroids and Chloroacetanilide	Human	Hair and Blood	Hair - Parent Pesticides - Solid-Liquid Extraction (2mL of Hexane) - Pesticide Metabolites - Derivatization (methanolic/hydrochloric acid methyl ester technique - 1mL of methanol and 1mL of 10N HCl added to the hair and heating the suspension at 80°C for 20min); Liquid-Liquid extraction (with 2mL of toluene) Blood - Parent Pesticides - Liquid-Liquid Extraction (with Hexane) - Pesticide Metabolites - Derivatization (same technique previously described)	Gas Chromatography (GC) (splitless mode)	Mass Spectrometry (MS)	Hair 0.18 to 5.88 µg/g Blood 3.10 to 98.00 ng/mL	Hair - Parent Pesticides - 0.25 to 62.50 µg/g - Pesticides Metabolites - 0.18 to 187µg/g Blood - Parent Pesticides - 0.10 to 25 µg/mL - Pesticides Metabolites - 0.13 to 33.33µg/mL	Ostrea et al. (2006)

Carbofuran, Carbaryl and their main metabolites	Carbamates	Human	Plasma	Mild Precipitation and Denaturation (with β-mercaptoethanol and ascorbic acid) - Solid-Phase Extraction (SPE) (Oasis HLB - Hydrophilic Lipophilic Balance) (Eluted: 2x 1mL Diethyl Ether; 2x Evaporated) - Derivatized 20µL of Trifluoroacetic Acid Anhydride and 10µL solution (0,02% Triethylamine in Tetrahydrofuran v/v)	Gas Chromatography (GC) (splitless mode)	Tandem Mass Spectrometry (MS/MS)	0.1 ng/mL	0.5 ng/mL	Petropoulou et al.(2006)
Carbaryl and metabolites	Carbamates	Rats	Plasmas	Liquid-Liquid Extraction (Acrtonitrile 300µL)	N/A	N/A	1.00ng/mL		Hantash et al (2006)
Polychlorinated biphenyls (PCB) congeners and 11 chlorinated pesticides and metabolites	Organochlorine	Human	Plasma	Solvent - 1:1:3 mixture of ammonium sulfate:ethanol:hexane Purification and Concentrator - Two Florosil columns	High Resolution Gas Chromatography (HRGC)	Electron Capture Detector (ECD)	N/A	Based on 3 times the average standart deviantion - 0.08 µg/L for p, p'-DDE, p, p'-DDT and β-HCH, and 0.04 µg/L for all other compounds.	Côté et al. (2006)
HCB, α-, β-, γ- HCH, p,p′-DDE and p,p′-DDT (expressed here as DDTs), trans- and cis-chlordane, oxychlordane (OxC) and trans-nonachlor (TN), PCBs and PBDEs	Organochlorine	Human	Maternal Serum, Umbilical Cord Serum and Human Milk	Empore™ SPE cartridges were washed with DCM and activated with MeOH and water (positive pressure of 2–4psi)	Gas Chromatography (GC)	Mass spectrometry (MS), detector operated in electron-capture negative ionization	N/A	ranged between 0.5 and 4 ng/g lw	Jaraczewska et al. (2006)
15 PCBs , a-, b-, c-HCH, HCB, p,p0-DDT and p,p0-DDE	Organochlorine	Human	Serum	Off-line solid phase extraction (SPE)	Gas Chromatography (GC)	microelectron capture detector	N/A	N/A	Petrik et al. (2006)
PCDDs, PCDFs, PCBs, and organochlorine pesticides	Organochlorine	Human	Serum	Turner et al.	High Resolution Gas Chromatography (HRGC)	High Resolution Mass Spectrometry (HRMS)	N/A	N/A	Lee et al. (2006)

Propoxur, Cyfluthrin, Chlorpyrifos, Cypermethrin, Pretilachlor, Bioallethrin, Malathion, Diazinon and Transfluthrin. Also lindane and DDT and some of their metabolites	Organochlorine, Organophosphorous, Pyrethroid and Carbamates	Human	Hair and Blood	Hair - collected with aluminium foil, pulverized into a fine powder, Fifty milligrams of powdered maternal hair and 2mL hexane was added. Solid–liquid extraction of the pesticides was conducted for 6 h using an IKA Vibrax VXR orbital shaker. The hexane extracts were separated by centrifugation at 2900g for 15 min. Blood - Tubes with EDTA - Parent pesticides were extracted from whole blood by liquid–liquid extraction - Pesticide metabolites, the compounds were derivatized and extracted through an HCl/methanolic methyl ester derivatization following the method described by Corrion et al. (2005).	Gas Chromatography (GC)	Mass Spectrometry (MS)	The limits of detection (LOD) for the individual parent pesticides and metabolites were determined using the empirical approach (Corrion et al., 2005).	N/A	Ostrea Jr. Et al. (2006)
DMP, DMTP, DMDTP, DEP, DETP, and DEDTP	Organophophorous' Metabolites	Human	Urine	Solid-phase Extraction (SPE)(conditioned with acetonitrile - 4 ml followed by 0.1MHCl - 4 ml, sample, dried at ~30 psi for 5 min, washed with 0.1M HCl - 1 ml,Elution was accomplished with acetonitrile - 7 ml), Post-Extraction Derivatization (1-chloro-3-iodopropane)	Gas Chromatography (GC)	Tandem Mass Spectrometry (MS/MS)	0.05 to 0.17 ng/mL	N/A	Hemakanthi De Alwis et al. (2006)
DMP, DMTP, DMDTP, DEP, DETP, and DEDTP	Organophosphorous' Metabolites	Human	Urine	Derivatization (benzyltolytriazine reagent) and Liquid-Liquid Extraction (LLE) with cyclohexane	Gas Chromatography (GC)	Flame Photometric Detector (FPD)	N/A	N/A	Yucra et al. (2006)

MCA and DCA, DMDTP, DMTP, DMP, DEP, DETP and IMPY, acephate and methamidophos	Organophosphorous and Organophosphorous' Metabolites	Human	Urine	MCA and DCA - Acidified, Solid-phase Extraction (SPE) (C_{18} micro-columns) and Derivatized (diazomethane) DMDTP, DMTP, DMP, DEP, DETP and IMPY- Derivatized (pentafluorobenzyl bromide at 70 C for 2 h) and Liquid-Liquid Extraction (hexane and methylene chloride) acephate and methamidophos -	Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC)	Mass Spectrometry (MS) (electron capture negative ionization source operating in single ion monitoring mode), Mass Spectrometry (MS) (electron impact ionization source operating in single ion monitoring mode) and Tandem Mass Spectrometry (MS/MS)	0.2 µg/L	N/A	Bouchard et al. (2006)
Quinalphos	Organophosphorous	Human	Urine and Blood	Solid-phase Micro Extraction (SPME) (coated 100 m Polydimethylsiloxane (PDMS) and 65 µm CarbowaxTM/Divinylbenzene (CW/DVB) - Direct immersion)	Gas Chromatography (GC)	Mass Spectrometry (MS) (electron impact (EI) mode, selected ion monitoring (SIM) mode)	Blood - 10ng/mL Urine - 2ng/mL	Blood - 50ng/mL Urine - 10ng/mL	Gallardo et al (2006)
malathion, parathion, methyl parathion and diazinon	Organophsphorus	Human	whole blood, blood plasma, urine, cerebrospinal fluid, liver and kidney.	Solid-phase Micro Extraction (SPME) (Polyacrylate (PA, 85 µm) and polydimethylsiloxane (PDMS, 100 µm) - Headspace)	Gas Chromatography (GC)	Nitrogen Phosphorus Detector (NPD)	2 to 55 ng/mL	0.02 to 0.5µg/mL	Tsoukali et al. (2005)
36 noncoplanar PCB congeners, 4 coplanar PCBs and 13 organochlorine pesticides or pesticide metabolites	Organochlorine	Human	Plasma	Organochlorines in plasma were measured by the Dioxin and Persistent Organic Pollutants Laboratory of the Centers for Disease Control and Prevention (CDC) in Atlanta, GA	N/A	N/A	N/A	N/A	De Roos (2005)
PCBs,HCB, α -,β -, γ - HCH, p,p' -DDT, p,p' - DDE	Organochlorine	Human	Serum	SPE column (1 g/6 Ml Alltech Extract-Clean High Capacity C_{18} endcapped, Alltech Associates Inc., Lokeren, Belgium)	Gas Chromatography (GC)	Micro Electron Capture Detector (µECD)	N/A	0.01–0.02 for PCBs and 0.01 − 0.16 ng/ml serum for OCPs.	Čonka et al (2005)

propoxur, diazinon, lindane, transfluthrin, malathion, chlorpyrifos, p,p' - DDT, bioallethrin, pretilachlor, cyfluthrin, cypermethrin	Organochlorine, Organophophorous, Pyrethroid and Carbamates	Human	Maternal and Cord Whole Blood	The pesticides were extracted by adding 3.1mL of hexane to all unknown samples and the negative control, while 3mL of hexane was added to the spiked positive controls	Gas Chromatography (GC)	Mass Spectrometry (MS)	LOD from <10 to 1,56 µg/mL	N/A	Corrion et al. (2005)
Propoxur, Predilachlor, p,p'-DDT, Lindane, Chlorpyrifos, Diazinon, Malathion,Bioalletrin, Cyfluthrin, Cypermethrin, Transfluthrin and 7 metabolites	Carbamates, Chloroacetanilide, Organochlorines, Organophosphates, Pyrethroids	Human	Whole Blood	For Parent Pesticide Analysis - Liquid-Liquid Extraction (3mL of Hexane); For Metabolite Analysis - Derivatization (Methanolic/Hydrochloric Acid Methyl Ester); Liquid-Liquid Extraction (2mL of Toluene)	Gas Chromatography (GC) (splitless mode)	Mass Spectrometry (MS)	0.10 µg/mL	0.10 µg/mL	Corrion et al. (2005)
Carbaryl, 1-naphthol, 2-naphthol, and Carbofuran, 3-hydroxycarbofuran, 7-phenol, carbofuran-3-keto, 3-hydroxycarbofuranphenol	Carbamates	Human	Urine	Enzymic Hydrolysis (50µL β-glucuronidase); Solid-Phase Extraction (Oasis HLB cartridges) (Eluted: 2x 1mL Diethyl Ether); Derivatized (20µL of Trifluoroacetic Acid Anhydride and 10µL solution (0,02% Triethylamine in Tetrahydrofuran v/v))	Gas Chromatography (GC) (splitless mode)	Tandem Mass Spectrometry (MS/MS)	0.03 ng/mL - 0.08 ng/mL	0.1 ng/mL - 0.2 ng/mL	Petropoulou et al.(2005)
ethylene-bis-dithiocarbamate (EBDC) and Ethylenethiourea (ETU)	Carbamates	Human	Urine	Solid-Phase Extraction (SPE) (Diatomaceous earth column with dichloromethane and derivatized mixture of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide and tert-butyldimethyilsilyl chloride)	Gas Chromatography (GC)	Mass Spectrometry (MS)	0.5 µg/g of creatinine	0.5 µg/g of creatinine	Colosio et al. (2005)
Bromoxynil ,2,4-D, dicamba, Fenoxaprop, MCPA, Ethalfluralin, Triallate, and Trifluralin	Herbicide	Human	Blood and Plasma	N/A	Gas Chromatography (GC)	Mass Spectrometry (MS)	1 to 100 µg/L		Semchuk et al (2004)

(dimethylphosfate (DMP), dimethylthiophosfate (DMTP), dimethyldithiophosfate (DMDTP), diethylphosfate (DEP), diethylthiophosfate (DEDP), and diethyldithiophosfate (DEDTP)); 3,5,6-trichloro-2-pyridinol (TCP), the main metabolite of chlorpyrifos; 3-phenoxybenzoic acid (3-PBA), a metabolite of pyrethroid insecticides; ethylenethiourea (ETU) a metabolite of ethylenebisdithiocarbamates; methamidophos (METH), an organophophorous insecticide.	Organophophorous' Metabolites, pyretroid's metabolite, Carbamate's metabolite and organophophorous	Human	Urine	Alkylphosphates - Derivatization (pentafluorobenzylbromide) TCP - Derivatization (bis(trimethylsylyl)-acetamide) Methamidophos - Liquid-Liquid Extraction (dichloromethane)	Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC)	Flame Photometric Detector (FPD), Mass Spectrometry (MS) and Spectrophotometer detector	2.5 to 50 nmol/L	N/A	Saieva et al. (2004)
4-nitrophenol (PNP) and 3-methyl-4-nitrophenol (3-Me-PNP)	Organophosphorous' Metabolites	Human	Urine	Direct injection (Dilution three-fold with 0.5% HCOOH solution) and after Hydrolyzed overnight (β-d-glucuronidase/sulphatase)	Coupled Column Liquid Chromatography (LC-LC) (1st - mobile phase consisting of acetonitrile–0.01% HCOOH in water, 2nd mobile phase consisting of acetonitrile–water)	Tandem Mass Spectrometry (MS/MS)	0.1 to 0.2 µg/L	1µg/L	Hernández et al. (2004)
N/A	Organophosphorous' Metabolites, pyretroid's metabolite, herbicides or metabolites	Human	Urine	Solid-Phase Extraction (SPE)	High Performance Liquid Chromatography (HPLC)	Tandem Mass Spectrometry (MS/MS) (Two spectrometers - Atmospheric Pressure Chemical Ionization (APCI) - Turbo Ion Spray atmospheric pressure ionization (TIS)	0.1 to 1.5 ng/mL	N/A	Olsson et al. (2004)

O,O-dimethylphosphate (DMP), O,O-diethylphosphate (DEP), O,O-dimethylthiophosphate (DMTP), O,O-diethylthiophosphate (DETP), O,O-dimethyldithiophosphate (DMDTP), and O,O-diethyldithiophosphate (DEDTP)	Organophosphorous' Metabolites	Human	Urine	Dried (under azeotropic conditions with isopropanol and nitrogen).Converted into their corresponding benzyl esters (benzyl bromide and diazotoluene) Solid-Phase Extraction (silica columns)	Gas Chromatography (GC)	Mass Spectrometry (MS)	3 to 6 ng/mL	N/A	Kupfermann et al. (2004)
dialkylphosphate (DAP) metabolites	Organophosphorous' Metabolites	Human	Urine	Lyophilized. Liquid-Liquid Extraction (2ml acetonitrile and 2 ml ethyl ether)	Gas Chromatography (GC)	Tandem Mass Spectrometry (MS/MS)	0.1 to 0.6 µg/L	N/A	Bravo et al. (2004)
dimethylphosphate (DMP), DEP, dimethylthiophosphate (DMTP), DETP, dimethyldithiophosphate (DMDTP), and diethyldithiophosphate (DEDTP)	Organophosphorous' Metabolites	Human	Urine	Concentrated to dryness (azeotropic codistillation with acetonitrile), Derivatized (1-chloro-3-iodopropane and potassium carbonate)	Gas Chromatography (GC)	Tandem Mass Spectrometry (MS/MS) (positive chemical ionization)	0.05 µg/L to 0.58 µg/L	N/A	Barr et al. (2004)
dimethyl-phosphate (DMP), diethyl-phosphate (DEP), dimethyl-thiophosphate (DMTP), diethyl-thiophosphate (DETP), dimethyl-dithiophosphate (DMDTP) and diethyl-dithiophosphate (DEDTP)) and cis-3-(2,2-dibromo-vinyl)-2,2-dimethyl-cyclopropane carboxylic acid (Br2CA), cis-3-(2,2-dichloro-vinyl)-2,2-dimethyl-cyclopropane carboxylic acid (cis-Cl2-CA), trans-3-(2,2-dichloro-vinyl)-2,2-dimethyl-	Organophosphorous' Metabolites and pyretroid's metabolites	Human	Urine	DMP, DEP, DMTP,DETP, DMDTP and DEDTP - Liquid-Liquid Extraction (acetonitrile/diethylether), Derivatization. Br2CA, cis-Cl2CA, trans-Cl2-CA and F-PBA - Solid-Phase Extraction (SPE), Methylation.	Gas Chromatography (GC)	Mass Spectrometry (MS)	DMP, DEP, DMTP,DETP, DMDTP and DEDTP -1 µg/L to 5µg/L Br2CA, cis-Cl2CA, trans-Cl2-CA and F-PBA - 0.1 to 0.2 µg/L	N/A	Heudorf et al. (2004)

cyclopropane carboxylic acid (trans-Cl2-CA) and 4-fluoro-3-phenoxy-benzoic acid (F-PBA)									
Acephate (AP), methamidophos (MMP), IMPY, DEAMPY, CIT, BTA, MDA, PNP, CMHC, $TCPY^{20}$,$TCPY^{25}$,$TCPY^{30}$ and $TCPY^{ms}$.	Organophosphorous' Metabolites	Human	Urine	Hyfrolysis (β-glucuronidase), Solid-Phase Extraction (SPE) (Oasis HLB 3cc cartridge) (preconditioned:1 mL of methanol + 1 mL of 5% methanol in 1% acetic acid. Sample. Washed: methanol/acid solution (0.8 mL). Elution:2 mL methanol) Fractions divided - Fraction 1 (Sample load + Wash):Liquid-Liquid Extraction cartridge (Chem Elute 3 mL, Varian) Fraction 2 (MeOH).	High Performance Liquid Chromatography (HPLC)(mobile phase: 30% acetonitrile in water with 0.15% acetic acid, flow rate: 40 µL/min, injection volume: 5 µL)	Tandem Mass Spectrometry (MS/MS) (Fraction 1 - positive ionization mode Fraction 2 - positive and negative mode)	0.1 to 8 ng/mL	N/A	Olsson et al. (2003)
PCB and p,p´-DDE	Organochlorine	Human	Serum	Procedures developed by the Centers for Disease Control (Needham 1981)	Gas Chromatography (GC)	Electron Capture Detector (ECD)	N/A	3.1 - 64.2 ng/g lipids	Hauser et al. (2003)
chlorpyrifos, diazinon, ethion, fenitrothion, malathion, methidathion, methyl parathion,phosmet, HCB, lindane, β-HCH, α- and β-endosulfan and its ether and sulfate metabolites, p,p'-DDT, p,p'-DDD and p,p'-DDE	Organophosphorous and Organochlorine	Human	Serum	Solid-Phase Extracted (SPE) (C18 cartridges)	Gas Chromatography (GC)	Tandem Mass Spectrometry (MS/MS)	0.05 to 0.5 ng/mL	0.2 to 9.0 ng/mL	Pitarch et al. (2003)

acephate, omethoate, phorate-oxon, phorate, dimethoate, propetamphos, terbufos, diazinon, paraoxon-methyl, disulfoton, parathion-methyl, malaoxon, paraoxon, ronnel, fenitrothion, pirymiphos, malathion, fenthion, chlorpyriphos, parathion-ethyl, ethion, carbophenothion, ENP, oxo-azinphos-methyl, phosalone, azinphos-methyl, azinphos-ethyl, Co-ral-o and Co-ral (Coumaphos)	Organophosphorous	Human	Tissues (liver, kidney, adipose)	Liquid-Liquid Extraction (2% ethanol in ethyl acetate)	Capillary Gas Chromatography (GC)	Mass Spectrometry (MS)	0.01 and 0.09 ng/mL	1 to 3 pg/µL	Russo et al. (2002)
2-methyl-3-phenylbenzoic acid (MPA) and 3-phenoxybenzoic acid (PBA)	Pyretroid's metabolites	Human	Urine	Liquid-Liquid Extraction (100 µl HCl (4 M) and 2 ml chloroform)	High Performance Liquid Chromatography (HPLC)	Ultraviolet (UV) Detector	2.5 ng/mL	N/A	Smith et al.(2002)
	Organochlorine and Organophosphorous	Human	Whole blood	Solid-phase MicroExtraction (headspace mode)	Gas Chromatography (GC)	Tandem Mass Spectrometry (MS/MS)	0.02-0.7 ng/mL	1 and 50 ng/mL	Hernández et al. (2002)
bromophos-ethyl, bromophos-methyl, chlorfenvinphos, chlorpyriphos, demethon-S-methylsulfon, diazinon, dichlorvos, dicrotophos, dimethoate, disulfoton, edifenphos, fenitrothion, fenthion, malathion, methidathion, mevinphos, monocrotophos, omethoate, parathion-ethyl, parathion-methyl, phosphamidon, and quinalphos	Organophosphorous	Human	Blood	Solid-phase MicroExtraction (headspace mode)	Gas Chromatography (GC)	Mass Spectrometry (MS)	0.01 and 0.3 µg/g	0.025 to 5.0 µg/g	Musshoff et al. (2002)

diethyl phosphate (DEP), diethylthiophosphate (DETP), dimethyldithiophosphate (DMDTP) and diethyldithiophosphate (DEDTP)	Organophosphorous[1] Metabolites	Human	Urine	Addiction of 40 mM tetrabutylammonium acetate	Liquid Chromatography (LC)	Tandem Mass Spectrometry (MS/MS)	1 to 2 µg/L	N/A	Hernández et al. (2002)
Furathiocarb and its metabolites (Carbofuran, 3-hydroxycarbofuran and 3-ketocarbofuran)	Carbamates	Rats	Plasma and Urine	Liquid–Liquid Extraction (0.7mL Ethyl Acetate/Hexane 75:25 (v/v))	High Performance Liquid Chromatography (HPLC) (post-column derivatization system)	Fluorescence detector	0,05 µg/ml furathiocarb, 0,025 µg/ml carbofuran, 0,025 µg/ml 3-hydroxycarbofuran and 0,05 µg/ml 3-ketocarbofuran	0.2 µg/mL	Liu et al (2002)
Ethylenethiourea (in urine indicator of Mancozeb exposure)	Carbamates	Human	Urine	Liquid-Liquid Extraction (Chem Elut CE120 Column, Eluted with 100mL of Dichloromethane); Evaporated; Reconstituted (2mL Dichloromethane); Gravity Column Chromatography -(Silica gel column (3mL); washed: (5mL) Dichloromethane, (1mL) Dichloromethane/Methanol (5:95 v/v); Eluted: (2mL) Dichloromethane/Methanol (5:95 v/v); Evaporated	High Performance Liquid Chromatography (HPLC) (reversed phase column)	Diode Array Detector (DAD/PDA)	0.5µg/g of creatinine	0.5µg/g of creatinine	Colosio et al. (2002)

Acetochlor, Alachlor, Atrazine, Bendiocarb, Carbofuran, Carbofuranphenol, Chlorothalonil, Chlorpyrifos, Chlorothal-dimethyl, Diazinon, Dichlorvos, Dicloran, Diethyltoluamide (DEET), Fonophos, 2-Isopropoxyphenol, Malathion, Metalaxyl, Methyl Parathion, Metolachlor, Parathion, cis-Permethrin, trans-Permethrin, Phorate, Phtalimide, Propoxur, Terbufos, Tetrahydrophthalimide, Trifluralin	Organophosphates, Carbamates, Chloroacetanilides, Pyrethroids, Triazines and Others	Human	Plasma and serum	Denaturation (4mL of Saturated Ammonium Sulfate); Solid-Phase Extraction (SPE) (OASIS and C18) (Eluted: 4mL Methylene Chloride, Dehydration: 1g Anhydrous Ammonium Sulfate, Transfered: 10µL of toluene,Re-Evaporated: to 10µL at room temperature);	Gas Chromatography (GC) (splitless mode)	Mass Spectrometry (MS)	0.5 - 20pg/g	0.25pg/mL	Barr et al . (2002)
Aldicarb	Carbamates	Human	Blood and Urine	N/A	High Performance Liquid Chromatography (HPLC)	N/A	N/A	N/A	Tracqui et al. (2001)
carbamates and related compounds (1-NAP), atrazine (AM), malathion (MDA), and chlorpyrifos and related compounds (TCPy)	Carbamates, Organophosphorous	Human	Urine	N/A	Capillary Gas Chromatography (GC) and Liquid Chromatography (LC)	Tandem Mass Spectrometry (MS/MS)	1.0 - 1.4 µg/L	N/A	Adgate et al. (2001)
29 organophosphates, 12 organochlorines, one phtalimide, one uracil, two triazines, one pyrethroid, 11 carbamates and three benzimidazoles	Organophosphate, Organochlorine, Phtalimide, Uracil, Carbamates and Benzimidazoles	Human	Serum	Solid-Phase Extraction (HLB OASIS® cartridges - Elution: 3mL Ethyl Acetate - GC/MS; and MCX OASIS® cartridges - 1st Elution:1mL MethanolWashed: 1mL 0.1N HCl, 2nd Elution: 1mL methanol + 1mL 5% ammoniated methanol - LC/MS)	Gas Chromatography (GC) (splitless mode) and Liquid Chromatography (LC) (ionspray® - Flow rate of 50µL/min using a gradient from 30% to 80% of acetonitrile in 2mM,pH 3 ammonium formate)	Mass Spectrometry (MS)	2.5 to 50 ng/mL	5 to 100 ng/mL	Lacassie et al. (2001)

3,5,6-trichloro-2-pyridinol (TCPyr)	Organophosphorous' Metabolites	Human	Urine	Hydrolised in acidic media. Automatic steam distillation. Solid-Phase Extraction (SPE) (polystyrene-divinylbenzene copolymer). Derivatisation (N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA))	Capillary Gas Chromatography (GC)	Mass Spectrometry (MS)	0.05 µg/L	0.1 µg/L	Koch et al. (2001)
demeton-S-methyl, phosphamidone, paraoxon ethyl, dialifos, fonofos, isofenphos, heptenophos, etrimfos, monocrotophos, triazophos, sulfotep, pyrazophos, pirimiphos, parathion ethyl, parathion methyl, azinphos ethyl, azinphos methyl, bromophos ethyl, bromophos methyl, chlorfenvinphos, fenthion, dichlorvos, dimethoate, terbufos and mevinphos (cis and trans)	Organophosphorous	Human	Urine, blood and serum	Liquid-Liquid Extraction (1 ml toluene)	Gas Chromatography (GC)	Phosphorus-Nitrogen sensitive Detector (PND) and Mass Spectrometry (MS)	0.01 mg/L	N/A	Tarbah et al.(2001)
3,5,6-trichloro-2-pyridinol (TCPyr)	Organophosphorous' Metabolites	Human	Urine	Hydrolised in acidic media. Automatic steam distillation. Solid-Phase Extraction (SPE) (polystyrene-divinylbenzene copolymer). Derivatisation (N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA))	Capillary Gas Chromatography (GC)	Mass Spectrometry (MS)	0.05 µg/L	0.1 µg/L	Koch et al. (2001)

Vamidothion, dimethoate, ethoprophos, cadusaphos, mevinphos, phorate, terbuphos, fonophos, chlorpyriphos-methyl, chlorpyriphos-ethyl, fenithrothion, bromophos-methyl, isophenphos, malathion, parathion-methyl, fenthion, methidathion, parathion-ethyl, pirimiphos-methyl, pirimiphos-ethyl,quinalphos, phenamiphos, phosalone, ethion, phosmet, pyrazophos, azinphos-methyl, azinphos-ethyl and coumaphos	Organophosphorous	Human	Blood and Serum	Blood: Deproteinization by Acetonitrile. Blood and Serum: Solid-Phase Extraction (SPE) (Oasis HLB 3cc cartridges) (Elution: Ethyl Acetate 3mL)	Gas Chromatography (GC)	Mass Spectrometry (MS)	5 to 25 ng/mL	10 to 50 ng/mL	Lacassie et al. (2001)
DMP, DEP, DMTP, DMDTP, DETP and DEDT	Organophosphorous' Metabolites	Human	Urine	Lyophilization, Derivatization (pentafluorobenzyl bromide (PFBBr))	Gas Chromatography (GC)	Tandem Mass Spectrometry (MS/MS)	0.02 to 0.5µg/L	N/A	Oglobline et al. (2001)
Glufosinate, bialaphos and glyphosate	Herbicides	Human	Urine and serum	N/A	Anion-Exchange Chromatography (AEC)	Integrated Pulsed Amperometric Detector (IPAD)	glufosinate, bialaphos and glyphosate - 20, 65 and 50 ng/mL, respectively	0.1 to 0.3 µg/mL	Sato et al. (2001)
methylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), dimethyldithiophosphate (DMDTP), and diethyldithiophosphate (DEDTP)	Organophosphorous' Metabolites	Human	Urine	Liquid-Liquid Extraction (acetonitrile/diethylether). Derivatization	Gas Chromatography (GC)	Mass Spectrometry (MS)	1 to 5 µg/L	N/A	Heudorf et al. (2001)

diethylphosphate (DEP), diethylthiophosphate (DETP), diethyldithiophosphate (DEDTP), dimethylphosphate (DMP), dimethylthiophosphate (DMTP), and dimethyldithiophosphate (DMDTP)	Organophosphorous' Metabolites	Human	Meconium	Lyophilization. Solid-Liquid Extraction. Derivatization	Isotope Dilution Gas Chromatography (ID GC)	Tandem Mass Spectrometry (MS/MS)	N/A	0.5 µg/g	Whyatt and Barr (2001)
azinphos-methyl, chlorpyrifos, diazinon, dimethoate, fenitrothion, fenthion, malathion, methidathion, parathionmethyl, phosmet, aldrin, dieldrin, p,p′-DDD, ,p′-DDE, p,p′-DDT, α- and β-endosulfan (-ether, -lactone and -sulfate), endrin, α-, β-, γ- and δ-HCH, hexachlorobenzene, heptachlor, heptachlorepoxide, and methoxychlor	Organochlorine, Organophosphorous and their metabolites	Human	Urine and serum	Solid-phase extraction (SPE)(500 mg C18 cartridge) and Liquid–liquid microextraction (LLME)	Gas Chromatography (GC)	Electron-Capture (ECD) and Nitrogen-Phosphorus Detectors (NPD)	URINE: SPE - 0.5 to 2.0 ng/mL LLME - 0.6 to 6.0 ng/mL BLOOD: SPE 1 to 10ng/mL	N/A	Pitarch et al. (2001)
dimethylphosphate (DMP), diethylphosphate (DEP), O,O-dimethylthiophosphate (DMTP), O,O-diethylthiophosphate (DETP), O,O-dimethyldithiophosphate (DMDTP), and O,O-diethyldithiophosphate (DEDTP)	Organophosphorous' Metabolites	Human	Urine	Liquid-Liquid Extraction (diethylether and acetonitrile). Derivatization (pentafluorobenzylbromide). Liquid-Liquid Extraction.	Gas Chromatography (GC)	Mass Spectrometry (MS)	1 to 5 µg/L	N/A	Hardt and Angerer (2000)

Acephate, chlorpyrifos, cyanox, diazinon, dichlorvos (DDVP), dimethoate, disyston, edifenphos (EDDP), EPN, estox, fenitrothion (MEP), fenthion (MPP), ormothion, isofenphos, isoxathion, malathion, methidathion (DMTP), monocrotophos, naled (BRP), phenthoate (PAP), parathion, prothiophos, pyridaphenthion, salithion, tetrachlorvinphos (CVMP), trichlorfon (DEP)and vamidothion	Organophosphorous and metabolites	Human	Urine	Liquid-Liquid Extraction (Diethyl ether)	N/A	Spectrophotometer	0.10 to 10 µg/mL	N/A	Namera et al. (2000)
chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP)	Organophosphorous and metabolites	Human	Urine	Deproteinization by Acetonitrile	Coupled-column liquid chromatography/ electrospray (LC-LC-ES)	Tandem Mass Spectrometry (MS/MS)	1.5 ng/mL in serum, and 0.5 ng/mL in urine	N/A	Sancho et al. (2000)
N-methylcarbamates, aldicarb, aldicarb sulphoxide, aldicarb sulphone, carbofuran and 3-hydroxicarbofuran	Carbamates	Human	Urine	Solid-Phase Extraction with graphite carbon (Disposable 3-ml SPE cartridges containing 500 mg of graphite carbon obtained from Supelco - Cartridges were pre-conditioned with 10 ml of ethyl acetate, 15 ml of CH_3CN and 10 ml of Milli-Q water)	Reverse-Phase Liquid Chromatography - Liquid Chromatography (RPLC–LC) (A mixture of CH_3CN–H_2O (5:95, v/v) was used has first mobile phase)	UV detector	0.3 - 1 µg/l	1 - 3 µg/l	Parrilla Vázquez et al. (2000)

Bromacil,Terbacil, Norfluzaron, Pyrazon, Ametryn, Atrazine, Cyanazine, Prometon, Prometryn, Propazine, Simazine, Metribuzin, Fenobucarb, Isoprocarb, Xylylcarb, Metolcarb, Carbaryl, Propoxur, Macbal, Furadan, Lannate, Benfluralin, Ethalfluralin, Fluchloralin, Isopropalin, Nitralin, Pendimethalin, Prodiamine, Profluralin, alachlor and metolachlor	Carbamates and others	Human	Whole Blood, Plasma, Urine and Tissues	Diazines - Liquid–liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME)	Gas chromatography (GC) and High-performance liquid chromatography	Mass Spectrometry (MS)	Diazines from 0.11 to 0.14 µg/ml - Triazines from 6 ng/ml to 1.4 µg/ml - Carbamates from 0.5 ng/ml to 1 µg/ml - Dinitroanilines from 1.9 pmol/mol to 4.5 pmol/mol - Chloroacetanilides 3ng/ml	Diazines from 0.16 to 10 µg/ml - Triazines from 6.25 ng/ml to 400 ng/ml - Carbamates from 1 ng/ml to 6 µg/ml - Chloroacetanilides 1 ng/ml to 1000 ng/ml	Kumazawa and Suzuki (2000)
2-thiazolidinethione-4-carboxylic acid (metabolite of alkylene bisdithiocaramates)	Carbamates	Human	Urine	Transformation to uncharged form - 400µL of 2M Hydrochloric Acid; Liquid–Liquid Extraction (Ethyl acetate and Hexane (75:25 v/v; 0,7ml); Evaporation of Solvent; Derivatization (300 µL of Diazoethane/Toluene solution)	Capillary Gas Chromatography (GC) (splitless mode)	Mass Spectrometry (MS)	0.7 µg/ l in urine	13 µg/L	Weiss et al. (1999)
Metabolites of Pirimicarb (DDHP, MDHP and ADHP)	Carbamates	Human	Urine	Liquid-Liquid Extraction (2x (5mL of Diethyl Ether/Acetonitrile 1:1 v/v) ; Derivatization (1.5mL Acetonitrile with 100µL of PFBBr(Pentafluorobenzyl bromide)-Acetonitrile (1:2 v/v) ; Liquid-Liquid Extraction (2x (1mL Heptane),	Gas Chromatography (GC) (splitless mode)	Mass Spectrometry (MS)	0.5 µg/ l (DDHP), 1 µg/ l (MDHP) and 4 µg/l (ADHP)	2 µg/l	Hardt and Angerer (1999)
Methomyl	Carbamates	Human	Blood	2x Precipitation; Solid-Phase Extraction (With a column packed with 5g of Extrelut powder; Eluted: Dichloromethane:ethyl acetate:chloroform (65:25:10, 15 ml); Derivatization (10µL methomyl or 20µL of MTBSTFA for tert-butyldimethylsilyl (tBDS) derivatization)	Gas Chromatography (GC) (splitless mode)	Mass Spectrometry (MS)	0.5ng/g	1 ng/g	Ito et al. (1998)

II– EXPERIMENTAL

1. INSTRUMENTATION

- Refrigerator –Biomedical Division (2-8°C);
- Freezer - Liebherr inferior a -15°C;
- Super Freezer - Isoterme Paineis Isotermicos Glacial;
- Rollers Roller Mixer SRT2/9 – Stuart Scientific ;
- Centrifuge – Megafuge 1.0 – Heracus Sepatech);
- Sample Concentrator – Techne – DRI-BLOCK® DB3;
- Ultrasonic bath – Grant XB14;
- Vortex – Velp Scientifica 2x3;
- Millipore Simplicity 185 SimpaKOD2;
- pH meter electrodes 827 pH Lab Ω Metrohm Swissmade;
- Calibrated Pipettes and Dispensers.

1.1. EXTRACTION SYSTEM

A vacuum manifold was used from Varian Inc. (Palo Alto, USA) for support of the solid-phase extraction Oasis® HLB cartridge 3cc/60mg 30µm that were obtained from Waters (Milford, MA, USA).

1.2. CHROMATOGRAPHIC AND DETECTION SYSTEMS

Chromatographic analysis was performed using an HP 6890 gas chromatograph equipped with a model 5972 mass selective detector (Hewlett-Packard, Waldbronn, Germany).

A capillary column (30 m × 0.25 mm I.D., 25 µm film thickness) packed with 5% phenylmethylsiloxane (HP5-MS), supplied by J & W Scientific (Folsom, CA, USA), was used.

2. MATERIAL

2.1. REAGENTS AND SOLVENTS

Reagents used, 2-propanol, ethyl acetate, acetic acid, methanol and formic acid, were analysis grade with the exception of the methanol in the reconstitution which was GC grade. All reagents were purchased from Merck (Darmstadt, Germany).

2.2. STANDARDS

All analytical standards omethoate, dimethoate, diazinon, chlorpyrifos, parathion-ethyl, chlorfenvinphos Z and E, quinalphos, azinphos-ethyl and ethion (IS) were purchased from Merck (Darmstadt, Germany). The purity of analytical were as follows: omethoate 98.3%, dimethoate 99.4%, diazinon 98.3%, chlorpyrifos 99.9%, parathion-ethyl 98.8, chlorfenvinphos Z and E 97.7%, quinalphos 99.3%, azinphos-ethyl 99.1% and ethion 97.9%.

2.3. BIOLOGICAL SAMPLES

Blank blood samples used in this work were obtained from the excess supplies of the Portuguese Institute of Blood (outdated transfusions), preserved with citrate phosphate

dextrose (1:7). Post-mortem samples used in the method were obtained from the Laboratory of Forensic Toxicology, South Branch, National Institute of Legal Medicine.

All samples were stored frozen until analysis.

2.4. WORKING SOLUTIONS

Stock standard solutions were prepared, from respective analytical standards, at a concentration of 10 mg/mL in methanol, with the exception of ethion (I.S.) which was prepared at 1 mg/mL in methanol. Subsequently four working solutions at 1 mg/mL, 100 µg/mL, 10 µg/ml and 1 µg.mL for proper addition pesticide concentration without overloading the blood sample with methanol, and at 10 µg/mL for ethion, were prepared by appropriate dilution of the stock solutions with methanol.

These solutions were stored protected from light at -15 °C.

2.5. BUFFER SOLUTIONS

Buffer solutions are remarkably resistant to pH changes caused by the addition of an acid of alkaline solution, providing more stability to the samples spiked with pesticides. The choice regarding the buffer was related to the pesticides' pKa, which is low, denoting the need of a rather acidic buffer solution. Several buffer solutions were tested, to study which of these acidic buffer solutions were best suited for the procedure. Acetic acid and ammonium acetate buffer solution provided the best results and was chosen for this work.

For the preparation of the acetic acid and ammonium acetate buffer solution 0.1M, 7.7g of ammonium acetate were weighted to a 1 L volumetric flask, 3.3 mL of acetic acid were added along with milliQ water until the volume was full.

3. CHROMATOGRAPHIC AND DETECTION CONDITIONS

Chromatographic conditions were as indicated in Figure 6. Initial oven temperature was 130 °C for 2 min, followed by an increase of 5 °C/min to 190 °C, raised by 10 °C/min to 240 °C, and and a third ramp of 15 °C/min to the final temperature of 270 °C, where it was kept constant for 7 min. Using this temperature program, a good separation of all compounds was achieved. The temperatures of the injection port and detector were set to 280 and 310 °C, respectively. Split injection mode (ratio 10:1) was adopted, and the carrier gas was helium at a constant flow rate of 1 mL/min. The mass spectrometer was operated with a filament current of 300 µA and an electron energy of 70 eV in the electron impact (EI) mode.

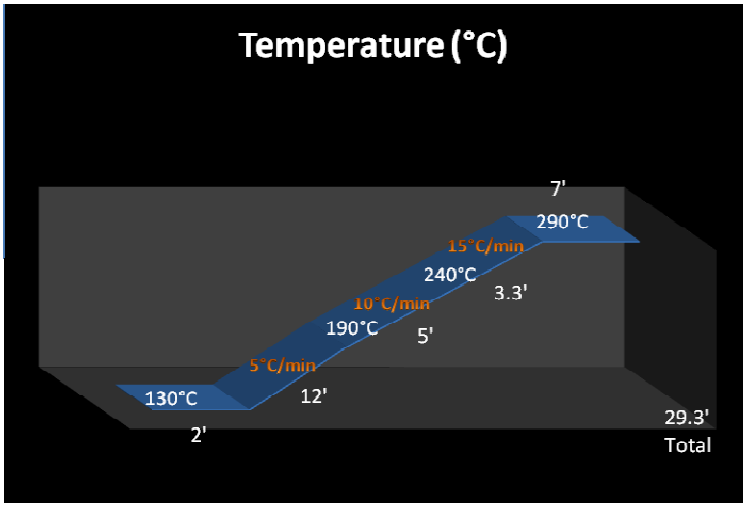


Figure 6 – Chromatographic conditions.

4. EXTRACTION PROCEDURE

A 500 µL whole blood sample was diluted with 5 mL of buffer solution 0.1M (acetic acid and ammonium acetate pH 4.88), and spiked with 50 µL of IS solution (at 10 µg/mL). The mixture was agitated for 15 min and centrifuged at 3500 rpm for 10min at room temperature. The supernatant was added to an Oasis® HLB extraction cartridge, previously and 2 mL of MilliQ water, the column was washed sequentially with 2mL of a 5% methanolic solution in distilled water, and dried under full vacuum for 15 min. The analytes were eluted with 2 mL of mixture methanol:isopropanol (1:1; v/v), which was afterwards evaporated to dryness at room temperature (to avoid pesticide evaporation) under a gentle stream of nitrogen. The dry extract was reconstituted in 65 µL of methanol, transferred to autosampler vials, and 2 µL was injected onto the GC.

5. RESULTS AND DISCUSSION

5.1. IDENTIFICATION OF COMPOUNDS

The compounds were identified by their retention time and mass spectrum. These were assessed via the injection of individual solutions of each pesticide in the full scan mode. Quantification was performed in the SIM mode, and therefore three ions were chosen for each pesticide, taking into account their relative abundance and the non-existence of the same ions in pesticides with close elution times. Indeed, the pairs chlorfenvinphos and quinalphos; and parathion and chlorpyrifos have close retention times, and therefore the selected ions were unique for each pesticide. Table 11 shows the retention times and selected ions.

Table 11 – Individual retention times and selected ions of the studied pesticides.

PESTICIDE	RETENTION TIME (MINUTES)	SELECTED IONS		
Omethoate	9,18	156*	110	79
Dimethoathe	11,88	125	93	87
Diazinon	13,34	137	199	304
Chlorpyrifos	16,47	197	199	314
Parathion	16,51	291	139	155
Chlorfenvinphos	17.59	323	295	267
Quinalphos	17,65	118	157	298
Ethion (IS)	19,84	231		
Azinphos	22,42	132	160	105

* Quantification ions are underlined

To extend the instrument's lifetime and avoid possible misinterpretations of chromatograms, the method's run time was widened while injecting a biological sample, in order to determine cholesterol's retention time and ensure that it will leave the column within the method's run time. Since cholesterol elutes within the method's runtime, there was no need to increase it.

5.2. OPTIMIZATION OF THE EXTRACTION PROCEDURE

Solid phase extraction is designed for separation/purification of analytes before instrumental analysis (usually chromatographic). This technique consists basically of a stationary phase to which the analytes adsorb depending on their affinity. Sample borne interferences are washed with disrupting solutions (though the strength of those solutions must be low in order not to lose analyte). Finally, the analytes are eluted with a solution of high affinity for them.

The main steps that could influence pesticide extraction and detection were optimized previously, in order to decrease matrix interferences and enhance the signal-to-noise ratio. Not all the studied pesticides' pKa values are known, but the majority of these are acidic. This characteristic has lead to the possibility of using MAX (Mixed-mode Anion-eXchange and reversed-phase sorbent for acids) SPE extraction cartridges employing an anionic interaction than a rather polar. Both MAX and HLB extraction cartridges were tested following the supplier's indications. Though pesticides denote more acidic characteristics due to their low pKa, better results were obtained using the HLB cartridges, as can be seen in Table 12.

Table 12 - Comparison between the extractions cartridges HLB and MAX.

Pesticide	Concentration µg/mL	HLB		MAX		HLB→MAX
		CV	Absolute Averages	CV	Absolute Averages	Average Decrease in Absolute Values
Omethoate	1,5	14,46%	57513	25,81%	2243	-95,62%
	3	35,74%	141605	17,07%	6656	-94,65%
	6	18,22%	405827	12,99%	15829	-96,11%
Dimethoate	1	6,46%	87118	20,58%	48217	-42,82%
	2	8,84%	170187	2,22%	129492	-23,45%
	4	17,00%	336464	11,35%	228173	-32,36%
Diazinon	1,3	10,89%	92095	8,91%	61831	-27,48%
	2,6	22,75%	154626	1,41%	153808	0,01%
	5,2	25,04%	248504	12,16%	265160	6,90%
Chlorpyrifos	1	7,58%	69597	7,36%	39842	-40,50%
	2	13,29%	125557	5,08%	94442	-24,80%
	4	23,77%	213157	12,48%	152462	-28,49%
Chlorfenvinfos	1,3	3,61%	108341	7,68%	70625	-32,78%
	2,6	8,49%	220601	2,97%	179860	-18,24%
	5,2	15,86%	404115	11,36%	310265	-23,35%
Parathion	1	7,64%	48102	16,22%	22011	-51,46%
	2	8,31%	103189	4,92%	71352	-30,64%
	4	21,29%	210722	14,23%	142618	-32,52%
Quinalphos	1	3,55%	40221	2,07%	27018	-30,73%
	2	7,68%	84790	3,96%	69478	-17,73%
	4	9,50%	152021	13,34%	130107	-14,45%
Azinphos	1	5,00%	139679	11,27%	71893	-46,76%
	2	7,72%	305930	3,96%	193008	-36,18%
	4	19,44%	563489	11,08%	331699	-41,23%

Several buffer solutions of different pH values were tested for the optimization of the extraction procedure (Table 13).

Table 13 - Buffer solutions tested

BUFFER SOLUTION	PH
H_2O	≈7.0
PBS	7.4
KH_2PO_4	4.5
HOAc	4.9
Na_2HPO_4	5.6

The best results were obtained with KH_2PO_4 and HOAc buffers. However, since these results were statistically undifferentiated, HOAc was selected since its preparation is easier.

One of the steps in which more effort and focus were given was the elution step, as the 2 mL of methanol instructed by the suppliers were found to lack elution strength. Several elution solutions were tested, and significant differences were found amongst them. These solutions are presented in Table 14.

Table 14 - Elution solutions tested and respective volume

VOLUME	ELUTION SOLUTION
2 mL	Methanol
4 mL (2 mL +2 mL)	Methanol + Methanol with 2% formic acid
4 mL (2 mL +2 mL)	Ethyl Acetate + Ethyl Acetate with 2% formic acid
4 mL (2 mL +2 mL)	Methanol + Ethyl Acetate
4 mL (2 mL +2 mL)	Methanol + 2-propanol
4 mL (2 mL +2 mL)	Methanol
2 mL	2-propanol
2 mL	Ethyl Acetate
2 mL	Methanol: 2-propanol (50:50 – v/v)

Ethyl Acetate and 2-propanol had very strong elution power when compared to methanol. Both solvents yielded similar results, but the volatility of ethyl acetate appeared to have a deleterious effect on some pesticides. Indeed, omethoate and dimethoate were significantly affected by this, as the obtained peak areas were extremely small.

Therefore, 2-propanol was preferred, but due to it's viscosity a mixture with methanol (50:50) was selected as the elution solvent, since fast elutions were obtained.

Two different solutions were tested for the washing step, an aqueous solution of 5% methanol, and an aqueous solution of 5% NH_4OH. The best results were obtained with the former solution, and therefore this was chosen for this work.

6. VALIDATION

After optimization the methodology was validated according to internationally accepted criteria (FDA, 2001). The studied parameters were selectivity, linearity, calibration curves, precision and accuracy, limits of detection and quantitation.

6.1. SELECTIVITY

Selectivity (sometimes called specificity) is the ability of the bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present. (Peters, et al, 2001)

To evaluate the selectivity of the method, being blood the essential matrix, forty samples of post-mortem blood were gathered in ten different pools of blood of approximately 10 mL each. These pools were extracted according to the previously described procedure, and injected with no addiction of pesticides or even internal standard (I.S.), to verify the absence of the signal. A second extraction and injection followed this first, this time the samples were spiked with pesticides and I.S. at a 1 μg/mL each.

The obtained chromatograms were compared (Figure 7-Figure 22). The peaks were well-separated, and no interferences were observed.

The ratios of the selected ions were compared to ensure their identity, and so were their relative retention times. The criteria of conformity are discriminated in Table 15, the margins of tolerance are calculated based on the percentage of peak area compared to the main peak area (relative peak area).

Table 15 - Tolerance margin of each relative peak area and retention times

TOLERANCE	
PEAK RELATIVE AREA	GC/MS
>50%	10%
25 até 50%	20%
<25%	5%
Retention Time	0,2
Relative Retention Time	1%

BLANK SAMPLES FROM POOL 6

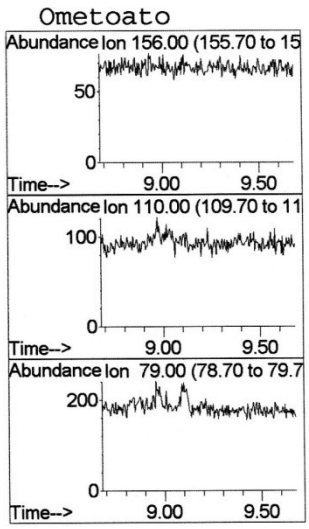


Figure 7 - Selectivity omethoate (blank sample).

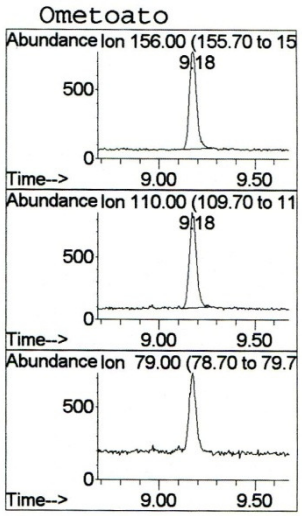


Figure 8 - Selectivity omethoate (spiked sample).

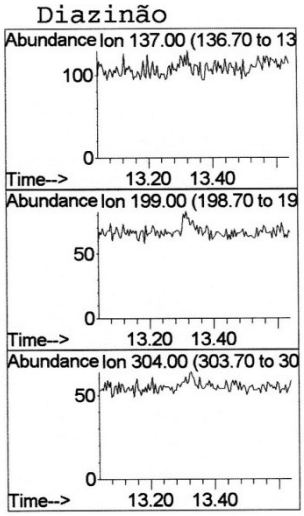


Figure 9 - Selectivity diazinon (blank sample).

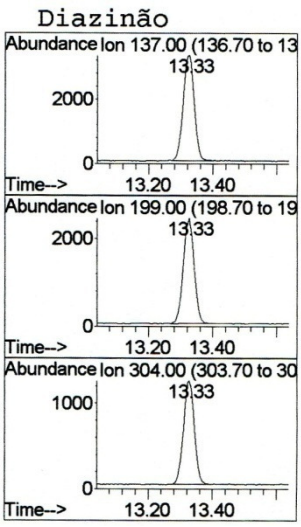


Figure 10- Selectivity diazinon (spiked sample).

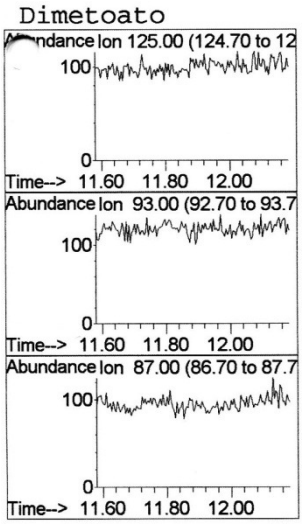


Figure 11 - Selectivity dimethoate (blank sample).

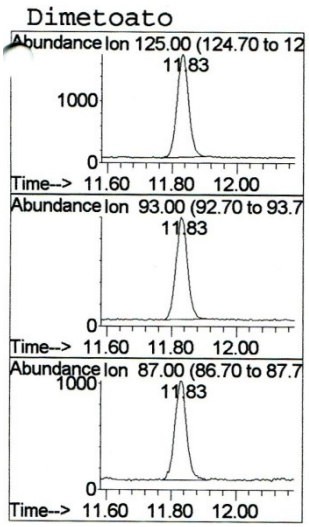


Figure 12 - Selectivity dimethoate (spiked sample).

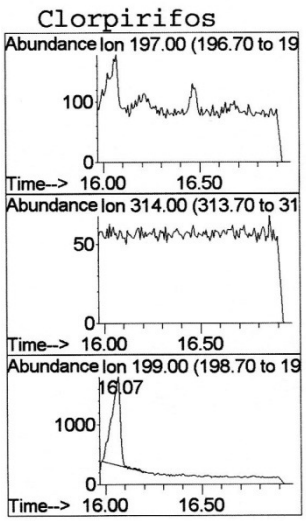


Figure 13 - Selectivity chlorpyrifos (blank sample).

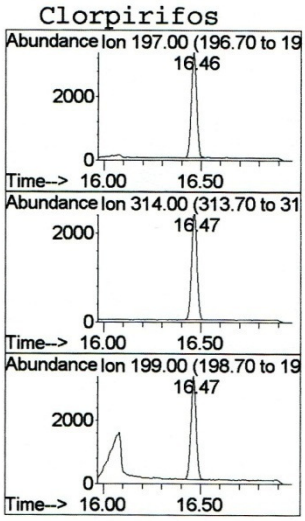


Figure 14 - Selectivity chlorpyrifos (spiked sample).

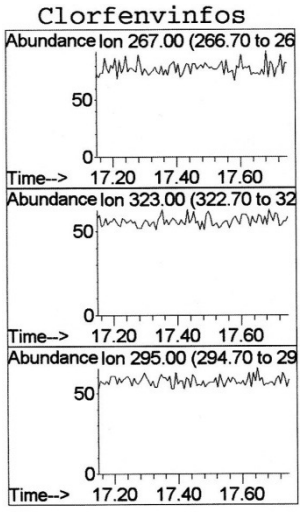


Figure 15 - Selectivity chlorfenvinphos (blank sample).

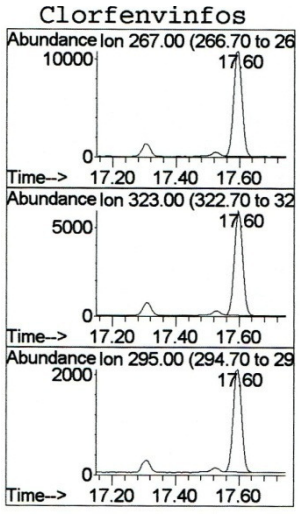


Figure 16 - Selectivity chlorfenvinphos (spiked sample).

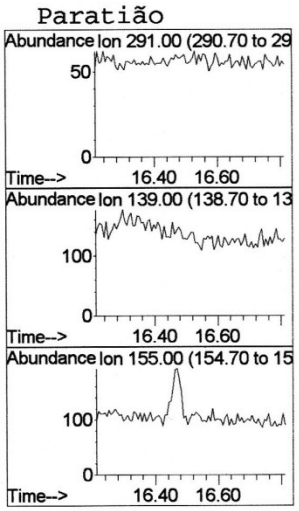


Figure 17 - Selectivity parathion (blank sample).

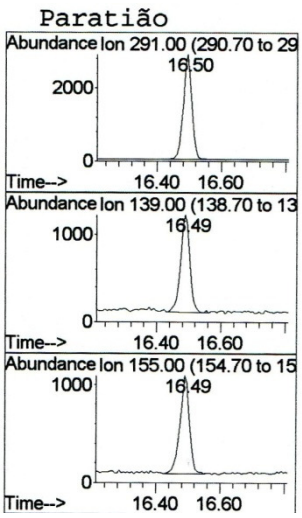


Figure 18 - Selectivity parathion (spiked sample).

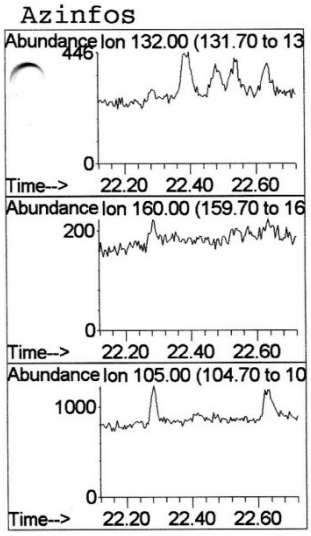


Figure 19 - Selectivity azinphos (blank sample).

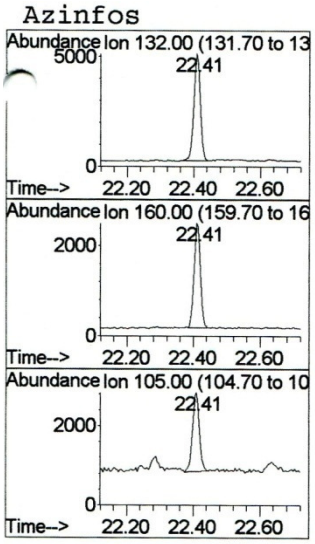


Figure 20 - Selectivity azinphos (spiked sample).

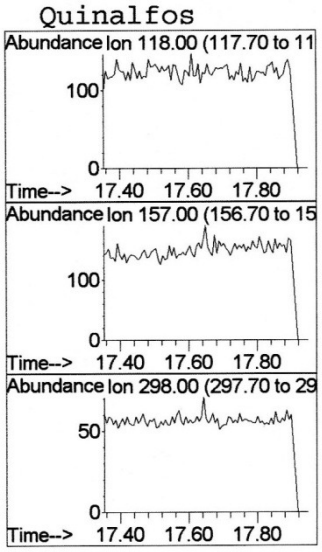


Figure 21 - Selectivity quinalphos (blank sample).

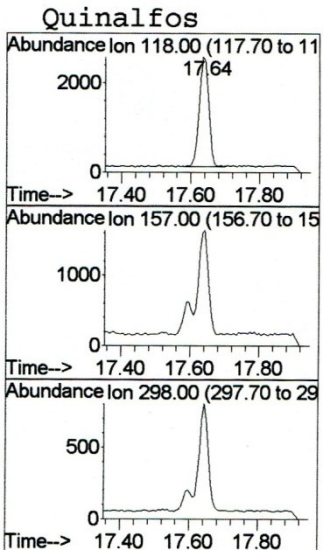


Figure 22 - Selectivity quinalphos (spiked sample).

6.2. LINEARITY

Linearity of a method is its ability to maintain proportional linear responses, to increasing concentrations of the analyte(s) within a certain range. So it can be ascertained a concentration based on a given response. (Peters et al., 2001, FDA, 2001)

Linearity of the method for all pesticides was established on spiked blood samples prepared and analyzed using the described extraction procedure in the range of 0.05 to 25.00 μg/mL, with a total of 15 calibrators.

The linearity obtained for each pesticide and the one-way ANOVA and linear regression results are presented in figures 23 to 30 and tables 16 to 23.

OMETHOATE

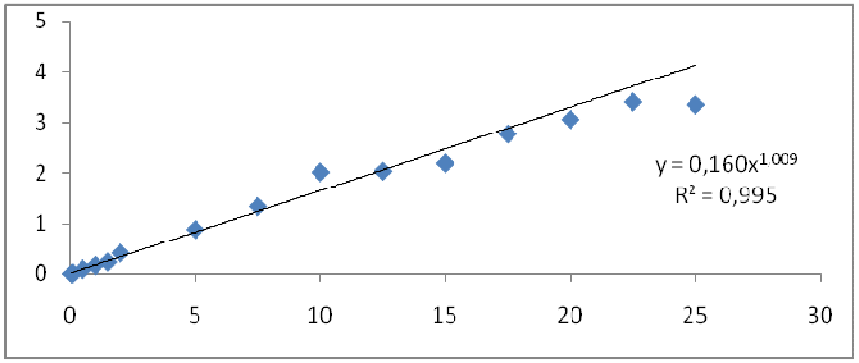


Figure 23 - Omethoate Non-Linear Curve

Table 16 - Omethoate regression table

Regression Statistics	
Multiple R	0,990009167
R Square	0,980118151
Adjusted R Square	0,978588778
Standard Error	0,188885931
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	22,8646331	22,8646331	640,8627302	1,90708E-12
Residual	13	0,463812633	0,035677895		
Total	14	23,32844573			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0,1130009	0,07221515	1,564781065	0,141642874	-0,043010447	0,269012246	-0,043010447	0,269012246
X Variable 1	0,144301944	0,005700194	25,31526674	1,90708E-12	0,131987422	0,156616465	0,131987422	0,156616465

DIMETHOATE

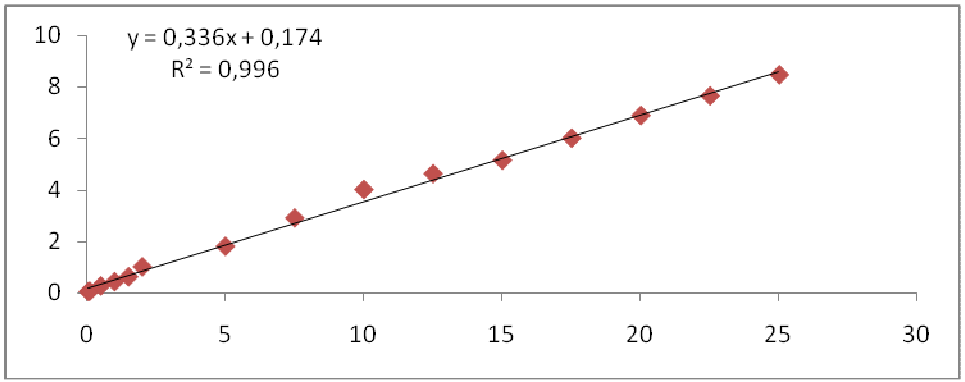


Figure 24 - Dimethoate Linear Curve

Table 17- Dimethoate regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,998226389
R Square	0,996455924
Adjusted R Square	0,996183303
Standard Error	0,18435545
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	124,2253905	124,2253905	3655,0928	2,56485E-17
Residual	13	0,441830116	0,033986932		
Total	14	124,6672206			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0,174018253	0,07048305	2,468937611	0,028191005	0,021748881	0,326287625	0,021748881	0,326287625
X Variable 1	0,336352955	0,005563474	60,45736349	2,56485E-17	0,324333801	0,34837211	0,324333801	0,34837211

DIAZINON

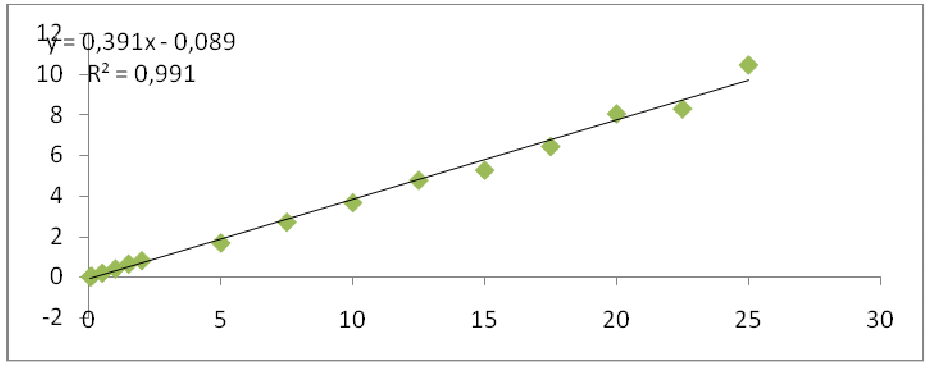


Figure 25 - Diazinon Linear Curve

Table 18 – Diazinon regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,995942287
R Square	0,991901039
Adjusted R Square	0,991278042
Standard Error	0,325046082
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	168,2179153	168,2179153	1592,144118	5,5328E-15
Residual	13	1,373514417	0,105654955		
Total	14	169,5914298			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0,088992362	0,124272102	-0,716108935	0,486589422	-0,357465915	0,179481191	-0,357465915	0,179481191
X Variable 1	0,391404835	0,009809232	39,90168064	5,5328E-15	0,370213278	0,412596392	0,370213278	0,412596392

CHLORPYRIFOS

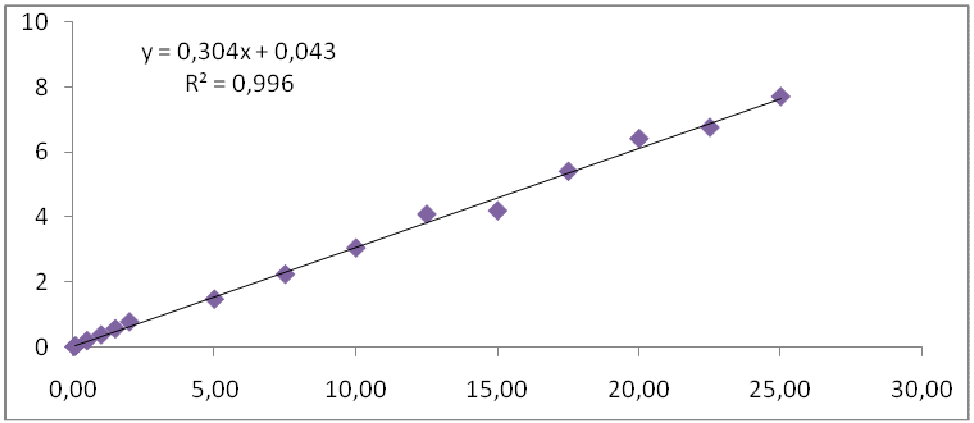


Figure 26 - Chlorpyrifos Linear Curve

Table 19 - Chlorpyrifos regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,998185498
R Square	0,996374289
Adjusted R Square	0,996095388
Standard Error	0,168819051
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	101,8159047	101,8159047	3572,50394	2,97416E-17
Residual	13	0,370498335	0,028499872		
Total	14	102,186403			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0,043045253	0,064543151	0,666922089	0,516485028	-0,096391747	0,182482253	-0,096391747	0,182482253
X Variable 1	0,304507417	0,005094617	59,77042697	2,97416E-17	0,293501166	0,315513667	0,293501166	0,315513667

PARATHION

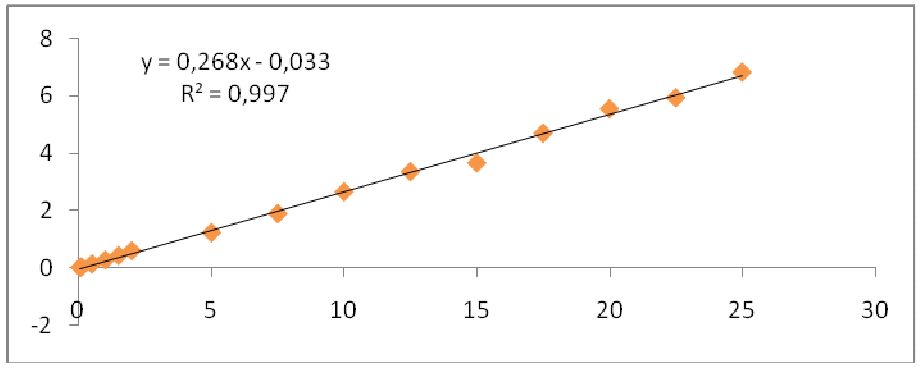


Figure 27 - Parathion Linear Curve

Table 20 - Parathion regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,998641523
R Square	0,997284891
Adjusted R Square	0,997076037
Standard Error	0,128788241
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	79,20046899	79,20046899	4775,021469	4,53684E-18
Residual	13	0,215623344	0,016586411		
Total	14	79,41609234			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0,033017969	0,049238512	-0,670572033	0,514230488	-0,139391307	0,073355369	-0,139391307	0,073355369
X Variable 1	0,268567798	0,003886568	69,10153015	4,53684E-18	0,260171379	0,276964218	0,260171379	0,276964218

CHLORFENVINPHOS

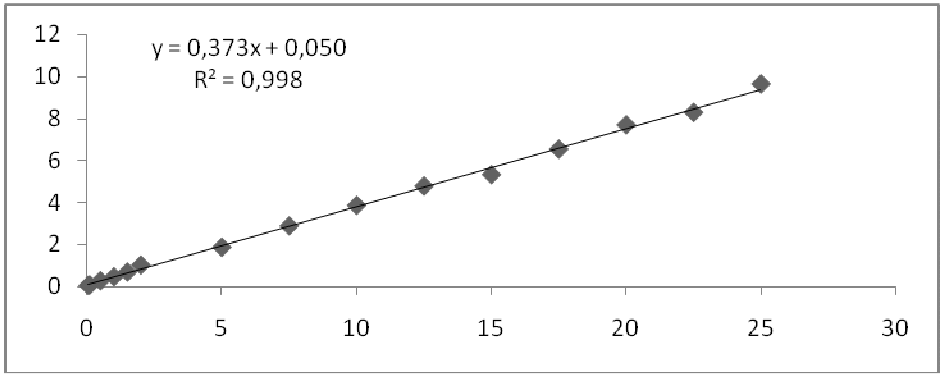


Figure 28 - Chlorfenvinphos Linear Curve

Table 21- Chlorfenvinphos regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,999023994
R Square	0,99804894
Adjusted R Square	0,997898858
Standard Error	0,151653221
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	152,9423572	152,9423572	6650,043778	5,29361E-19
Residual	13	0,298983091	0,022998699		
Total	14	153,2413403			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0,05084546	0,057980285	0,876943947	0,396433739	-0,07441333	0,17610425	-0,07441333	0,17610425
X Variable 1	0,373210585	0,004576587	81,54780057	5,29361E-19	0,36332347	0,383097699	0,36332347	0,383097699

QUINALPHOS

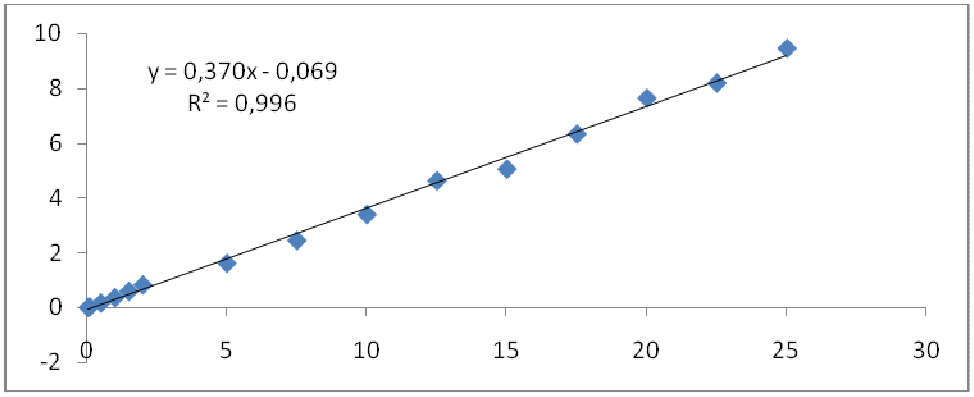


Figure 29 - Quinalphos Linear curve

Table 22 - Quinalphos regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,998137319
R Square	0,996278107
Adjusted R Square	0,995991808
Standard Error	0,208195674
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	150,8354686	150,8354686	3479,846393	3,52607E-17
Residual	13	0,563490703	0,043345439		
Total	14	151,3989593			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0,069281861	0,07959768	-0,870400503	0,399868902	-0,241242193	0,102678472	-0,241242193	0,102678472
X Variable 1	0,370631051	0,006282923	58,99022286	3,52607E-17	0,35705762	0,384204481	0,35705762	0,384204481

AZINPHOS

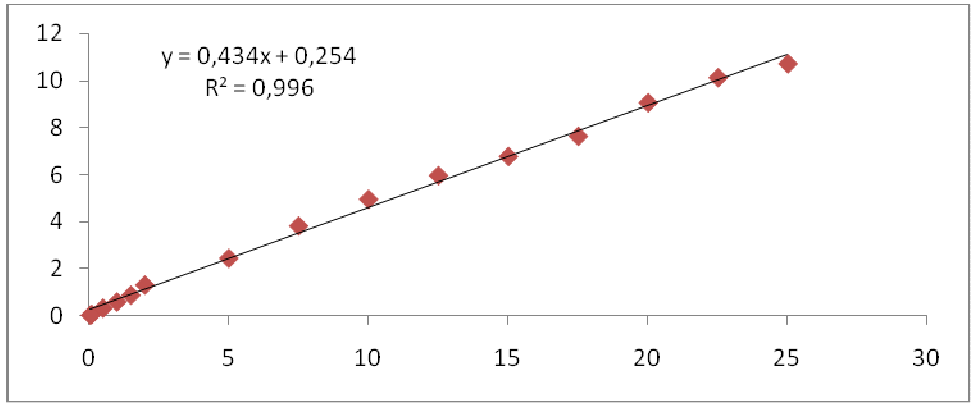


Figure 30 - Azinphos Linear Curve

Table 23 - Azinphos regression table

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,998285506
R Square	0,996573952
Adjusted R Square	0,99631041
Standard Error	0,234378371
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	207,7277389	207,7277389	3781,4593	2,05792E-17
Residual	13	0,714131871	0,054933221		
Total	14	208,4418708			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0,254411712	0,089607888	2,83916649	0,013943514	0,06082564	0,447997784	0,06082564	0,447997784
X Variable 1	0,434947971	0,007073064	61,49357121	2,05792E-17	0,419667545	0,450228397	0,419667545	0,450228397

6.3. CALIBRATION CURVES

After it has been established that a pesticide has linear, or non-linear but mathematical predictable, behavior within a certain range, calibration curves with wider gaps between concentrations can be conducted in order to ascertain the intraday and also intermediate precision of a method. The number of concentrations levels chosen were eight has shown in Table 24. The number of Quality Controls (QC) was two (low at 0.50 µg/mL and high at 17.50 µg/mL) with three replicates each. For Repeatability or Intraday Precision a total of four concentrations (two low due to limits of quantification, one medium and one high – shown in Table 25 were chosen with five replicates.

Table 24 - Repeatability concentration data

REPEATABILITY	
	CONCENTRATION (µg/mL)
Low	0.05
Low	0.10
Medium	5.00
High	25.00

Table 25 - Calibration curve concentration data

CALIBRATION DATA	
	CONCENTRATION (µg/mL)
1	0.05
2	0.10
3	1.00
4	5.00
5	10.00
6	15.00
7	20.00
8	25.00

6.4. LIMITS OF DETECTION AND QUANTIFICATION

The limit of quantitation (LOQ) was defined as the lowest pesticide concentration that could be measured with adequate precision (coefficient of variation of less than 20%) and accuracy (within ± 20% of the nominal concentration). The limits of detection (LOD), defined as the lowest tested concentration yielding a signal-to-noise ratio higher than 3 (FDA,2001).

The LOQ was determined to be 0.05 µg/mL (LLOQ – lower limit of quantification). This applies to all pesticides in this study except for omethoate, for which the LLOQ was 0.10 µg/mL (Table 26). The calibration curves were extracted and analyzed over a period of 30 days. All quantified values were within a ±15% range of the theoretical value given by the curve. Not too much attention was given the LOD, because all values are quantified and the LOQ is quite low, taking into account the blood values normally seen in intoxications.

Calibration data is shown in Table 26.

Table 26 - Calibration data

PESTICIDE	CURVE TYPE	WEIGHING FACTOR	CALIBRATION RANGE (µg/mL)	SLOPE	INTERCEPT	R^2	LOQ (µg/mL)
Omethoate	Power	Power	0.10 – 25.00	$0.3571 \pm 0.2304 \times^{(1.3042 \pm 0.1696)}$		0.9942 ± 0.0059	0.1
Dimethoate	Linear	1/x	0.05 – 25.00	1.4344 ± 0.48605	-0.03109 ± 0.03880	0.9973 ± 0.0018	0.05
Diazinon	Linear	1/x2	0.05 – 25.00	0.7237 ± 0.17058	0.02064 ± 0.00676	0.9944 ± 0.0024	0.05
Chlorpyrifos	Linear	1/x2	0.05 – 25.00	1.1048 ± 0.24881	0.05191 ± 0.01617	0.9948 ± 0.0030	0.05
Parathion	Linear	1/x2	0.05 – 25.00	1.0070 ± 0.22702	-0.02510 ± 0.01711	0.9961 ± 0.0008	0.05
Chlorfenvinphos	Linear	1/x2	0.05 – 25.00	1.2080 ± 0.32883	0.00436 ± 0.01477	0.9935 ± 0.0020	0.05
Quinalphos	Linear	1/x2	0.05 – 25.00	0.7343 ± 0.19453	0.00966 ± 0.00956	0.9946 ± 0.0022	0.05
Azinphos	Linear	1/x2	0.05 – 25.00	2.1625 ± 0.56415	0.09693 ± 0.03488	0.9949 ± 0.0022	0.05

6.5. INTERMEDIATE PRECISION

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. (FDA,2001)

Intermediate precision, in addiction to the previous definition, refers to the precision within several days, reporting the variation of individual measures over a period of 30 days.

The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20%. (FDA,2001)

Concerning the acceptance criteria, at least 67% (4 out of 6) of the QC (quality control) samples should be within 15% of their respective nominal value, 33% of the QC samples (not all replicates at the same concentration) may be outside 15% of nominal value. In certain situations, wider acceptance criteria may be justified. (FDA,2001)

Table 27 shows the average values of concentration found, bias and CV.

Taking into account the acceptance criteria of the FDA – Bioanalytical Method Validation, the results of the QC were quite good, presenting low relative errors (BIAS), in most cases below 10%, except for parathion, with 17.29% below the nominal value. However, taking into account that these compounds are pesticides and their presence indicates a situation of intoxication, this value is not so big. Omethoate's mean CV is quite high, but considering that the average BIAS for this compound falls within 10% of nominal value, this CV only a poorer precision, but quantification is still possible.

Table 27 - Quality controls average values

Pesticide	Spiked Concentration (µg/mL)		Concentration Found (µg/mL)	Bias* (%)	C.V (%)
Omethoate	Low	0.50	0.46	-8.69%	40.42%
	High	17.50	18.44	5.39%	12.28%
Dimethoate	Low	0.50	0.46	-7.88%	11.54%
	High	17.50	19.18	9.60%	5.32%
Diazinon	Low	0.50	0.52	3.26%	7.78%
	High	17.50	18.46	5.50%	5.32%
Chlorpyrifos	Low	0.50	0.54	8.05%	6.28%
	High	17.50	18.13	3.58%	5.54%
Parathion	Low	0.50	0.41	-17.29%	14.21%
	High	17.50	19.45	11.14%	3.67%
Chlorfenvinphos	Low	0.50	0.52	3.91%	7.20%
	High	17.50	18.55	5.98%	5.66%
Quinalphos	Low	0.50	0.52	3.02%	6.52%
	High	17.50	18.45	5.43%	5.84%
Azinphos	Low	0.50	0.51	1.05%	7.27%
	High	17.50	19.04	8.83%	6.10%

* Mean relative error (bias) between measured and spiked concentrations

6.6. Repeatability or Intraday Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. (FDA,2001)

Intraday precision reports the changes of individual measures within the same day.

With the exception of omethoate, all pesticides' concentrations were within a ±15% interval of their expected value, and the CV's were below 15% (Table 28).

Concerning intraday repeatability, the pesticides demonstrate good results and reveal ed adequate reproducibility. The intraday variation between aliquots was within the 15% range for all pesticides, except for omethoate, which presented a different behavior, having a few outliers

that shifted the average out of the margin. This deviation occurred in the medium concentrations, and not at the edges.

Table 28 - Repeatability data

Pesticide	Spiked Concentration		Concentration Found	Bias	C.V
Omethoate	Low	0.10	0,10	0,05%	11,65%
	Medium	5.00	3,79	-24,15%	34,27%
	High	25.00	23,83	-4,70%	11,79%
Dimethoate	Low	0.05	0,05	-0,60%	6,65%
	Low	0.10	0,10	2,17%	7,00%
	Medium	5.00	4,43	-11,36%	5,86%
	High	25.00	27,34	9,37%	4,20%
Diazinon	Low	0.05	0,05	-8,15%	1,93%
	Low	0.10	0,1	-1,98%	6,26%
	Medium	5.00	4,45	-10,99%	3,05%
	High	25.00	28,12	12,50%	1,44%
Chlorpyrifos	Low	0.05	0,05	-3,31%	1,68%
	Low	0.10	0,11	7,27%	3,26%
	Medium	5.00	4,65	-6,90%	5,95%
	High	25.00	27,23	8,91%	2,56%
Parathion	Low	0.05	0,05	8,57%	10,01%
	Low	0.10	0,09	-6,80%	5,21%
	Medium	5.00	4,38	-12,44%	2,23%
	High	25.00	28,30	13,20%	2,75%
Chlorfenvinphos	Low	0.05	0,04	-11,83%	3,07%
	Low	0.10	0,10	3,36%	6,05%
	Medium	5.00	4,65	-6,92%	6,54%
	High	25.00	26,41	5,63%	4,59%
Quinalphos	Low	0.05	0,05	-4,00%	5,29%
	Low	0.10	0,10	5,27%	5,26%
	Medium	5.00	4,57	-8,53%	5,08%
	High	25.00	28,20	12,79%	1,28%
Azinphos	Low	0.05	0,05	-4,10%	2,25%
	Low	0.10	0,10	1,20%	3,93%
	Medium	5.00	4,59	-8,16%	5,75%
	High	25.00	28,36	13,46%	4,30%

III – CONCLUSIONS

The developed method was considered validated and adequate for the qualitative and quantitative determination of organophosphorous pesticides in human blood. It also denotes significant sensitivity, allowing the detection of pesticide amounts as low as 50 ng/mL (100 ng/mL for omethoate) utilizing only 0.5 mL of sample. The use of this small amount of sample is important in forensic situations, especially where there is little sample availability and several analyses and procedures are needed.

The studied compounds presented in general good behavior throughout the whole procedure, being omethoate the most limitating compound. In fact, some of the optimized parameters were limited by this compound's instability.

In addition, several other parameters were not determined, and these would have been helpful for the method's characterization. For instance, it would have been important to calculate the method's absolute recovery, to assess whether or not there is loss of analytes during sample preparation; as well as the analytes' instability in both stored and processed samples. The method's recovery has been optimized previously, but its neat value was not determined. This parameter may be overcome, provided that precision and accuracy are adequate. However, analyte stability is perhaps the most important parameter in method validation. Indeed, if an analyte is not stable during sample storage, the whole procedure will be biased, despite of the adequate precision and accuracy. Unfortunately, this parameter is often not studied during method validation.

Despite of these issues, the developed method is simple and does not consume too much time, since sample preparation can be easily done within a few hours. Four these reasons, this procedure was considered adequate for application in routine toxicological analysis.

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