Francisca Chinemerem Ezeigwe

Licenciada em Química

Identification of pesticides in forensic samples by mass spectrometry

Dissertação para obtenção do Grau de Mestre em Química Bioorgânica

Orientador: Alexandra Maria Moita Antunes, Professora, Instituto Superior Técnico

Co-orientador: Maria João Caldeira, Doutora,Laboratório de Polícia Científica

Júri:

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Março 2021

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ABSTRACT

The illegal use of pesticide formulations for the intentional/non-intentional poisoning of domestic/wild animals is, unfortunately, very common. This is a non-selective form of killing that affectsboth target and non-target species, endangers the environment and constitutes a risk in terms of publichealth.

This study was undergone under the scope of a protocol established between the Laboratório de PolíciaCientífica da Polícia Judiciária (LPC/PJ) and Instituto Superior Técnico (IST) with the ultimate goal of establishing analytical protocols based on liquid chromatography low resolution tandem mass spectrometry (LC-MS/MS) analysis suitable for the identification of pesticides in forensic samples suspected of being involved in criminal animal poisoning. Towards this end, a data base of LC-MS/MS data of distinct pesticide standards was first constructed. The choice of pesticides included in the data base was based on the previous knowledge of their common use for illegal killing of domestic and wild animals in Portugal and Spain. Several samples provided by LPC/PJ were then analysed under the same conditions used for pesticide standards. This enabled the identification of pesticides in 10 forensic samples. Therefore, the methodology developed revealed to be suitable for the identification of pesticides in forensic samples.

Keywords: pesticides identification; mass spectrometry; LC-MS analysis; animal poisoning; forensic samples.

RESUMO

O uso ilegal de formulações de pesticidas para o envenenamento intencional ou não intencional deanimais domésticosou selvagens é, infelizmente, frequente. Esta é uma forma não seletiva de matar que afeta não só as espécies alvo como outras espécies. Esta prática põe em risco o meio ambiente econstitui um perigo em termos de saúde pública..

Este trabalho foi efetuado no âmbito de um protocolo estabelecido entre Laboratório de Polícia Científica da Polícia Judiciária (LPC/PJ) e o Instituto Superior Técnico (IST) e tem como objetivo estabelecer protocolos analíticos baseados na técnica de cromatografia líquida acoplada à espectrometria de massa de baixa resolução de tandem (LC-MS/MS) para a identificação de pesticidasem amostras forenses, de casos suspeitos de uso de pesticidas para o envenenamento de animais. Para tal, foi primeiro construída uma base de dados de LC-MS/MS com vários pesticidas padrão. A escolha dos pesticidas a incluir nesta base de dados teve como base o conhecimento prévio de seu uso comum num contexto de intoxicação de animais domésticos e selvagens em Portugal e Espanha. Várias amostras cedidas pelo LPC/PJ, que foram obtidas de casos onde havia a suspeita da utilizaçãode pesticidas para a intoxicação de animais, foram depois analisadas nas mesmas condições experimentais utilizadas para as amostras padrão. Esta estratégia revelou ser adequada para identificação de alguns pesticidas em amostras forenses, tendo-se identificado pesticidas em 10 das amostras forenses analisadas.

Palavras-chave: identificação de pesticidas; espectrometria de massa; análise LC-MS;envenenamento de animais; amostras forenses.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APCI	Atmospheric-pressure chemical ionization
API	Atmospheric-pressure ionization
ARs	Anticoagulant rodenticides
ESI	Electrospray ionization
ESI (+)	Electrospray ionization in positive mode
ESI (-)	Electrospray ionization in negative mode
FGARs	First-generation anticoagulant rodenticides
GPC-30g biobased S-X_3	Gel permeation chromatography-30g biobased
HPLC	High performance liquid chromatography
IST	Instituto Superior Técnico
IP-AG11-HC	Ionpac-AG11-High capacity Column
LPC/PJ	Laboratório de Polícia Científica da Polícia Judiciária
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled with mass spectrometry
LC-HRMS-QTOF	Liquid chromatography–high resolution mass spectrometry coupled toquadrupole-time of flight
LC-HRMS	Liquid chromatography–high resolution mass spectrometry
LLE	Liquid-liquid extraction
MS	Mass spectrometry
m/z	Mass-to-charge ratio.
µm	Micromole
Ml	Mililitre
mM	Milimolar
m/m	mass/mass
MRM	Multiple reaction monitoring
Ops	Organophosphate
APA	Portuguese Environment Agency
Rt	Retention time
RP	Reverse phase column
SGARs	Second-generation anticoagulant rodenticides
SIM	Selected ion monitoring
SPE	Slid phase extraction
MS/MS	Tandem mass spectrum
TOF-MS	Time-of-flight mass spectrometry
v/v	volume per volume

1. INTRODUCTION:

1.1 USE OF PESTICIDES IN THE CRIMINAL CONTEXT FOR THE KILLING OF ANIMALS

The illegal use of commercial formulations of pesticides is a non-selective and massive form ofanimal killing as it might result in the death of target and non-target animals. This continues to happen all over Europe. Thus, in recent times, the illegal/ intentional killing of animals is considered an act of animal cruelty and a criminal offence, therefore is punishable by the European law. An European convention on the protection of pet animals prohibits the killing of animals through the use of poisonoussubstance or drugs. This convention forbids the cause of unnecessary pain, suffering, or distress on animals.[1] Most importantly, socio-cultural factors, or a society's level of tolerance to abuse of animals as well as its willingness to the conservation of life and the environment, constitute a major factor in thecontrol of this heinous act.

Exposures leading to the death of animals can result from accidents (resulting from the use, misuse) or deliberate abuse of these toxic compounds. Generally, the deliberate poisoning of domestic animals (such as dogs and cats) or wild animals, considered harmful to human activities, with pesticidecan be attributed to their degree of toxicity, type of agricultural management of a society, availability in local market or neighbouring countries among many other factors[2] In fact, the rate at which a pesticideis applied in the illegal killing of animals is irrespective of the various restrictions or bans on the use of these toxic compounds. It rather depends on the toxicity of commercial formulation as well as its commercial availability.[3] For example, carbamate insecticides such as aldicarb and carbofuran (Fig1.1),which were banned in the EU, are still reported in poisoning of domestic animals. This, therefore, meansthere are still loopholes that allow access to these chemicals. Hence, in addition to ban placed on the purchase or use of these toxic compounds, stricter laws and restriction must be put in place for authorized or professional users.

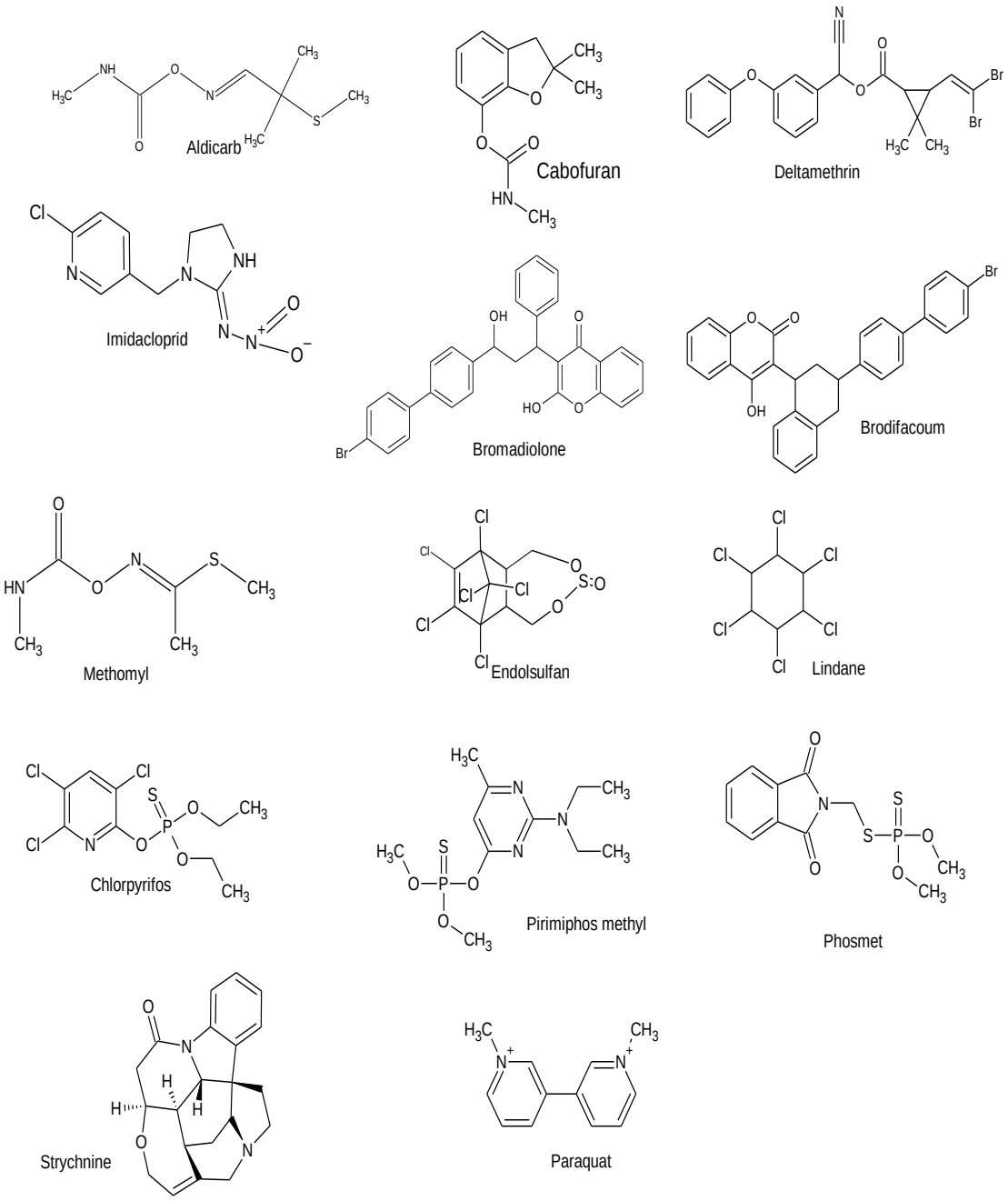


Fig 1.1: Structures of the pesticides: aldicarb, carbofuran, deltamethrin, imidacloprid, bromadiolone, brodifacoum, methomyl, endosulfan, lindane, chloropyrifos, pirimiphos methyl, phosmet, strychnine, paraquat.

Summarily, many reasons have been proposed to explain why pesticides are illegally used against animals. Analysing the causes, it is found to range from sociocultural factors to less concern orthe reluctance of government in the different regions as pertain conservation of the environment. The easy accessibility to these toxic pesticides and as well as the lack of control of the possession of prohibited compounds, constitute additional causes for their illegal use for intoxication of animals.

1.2 Classes of Compounds Mostly Used for the Purpose of KillingAnimals

The death of domestic animals, as a result of the illegal poisoning are mainly through oral intakeof the poisons. The type of pesticides most used to kill animals varies from country to country. Nonetheless, rodenticides, are the primary cause of domestic animal poisoning, with about 47.7% of recorded cases. Additionally, insecticides, such as deltamethrin (Fig1.1) have also been referenced to be a major cause of poisoning. Anticholinesterase insecticides such as carbamates and organophosphates were found to be the second most cause of intoxication in domestic animals. While the neonicotinoids insecticides, such as the imidacloprid (Fig1.1) and organochlorines, accounted for lesser number of poisoning cases[4] Carbamates especially aldicarb and carbofuran (Fig 1.1) have beenfound during post-mortem analysis to be common culprit for deliberate domestic animal poisoning**.**[5]

In Spain, poisoning episodes in domestic/pet animal are usually attributed to rodenticides and insecticides. This trend is followed by herbicides, molluscicides and fungicides which have been found to be the most common toxicants in the deliberate (illegal) or unintentional killing of domestic animals. Of these pesticides, anticoagulant rodenticides such as bromadiolone and brodifacoum (Fig 1.1), carbamate insecticides such as aldicarb, methomyl and carbofuran (Fig 1.1), are the most common toxicant used. While organochlorine insecticides such as endosulfan and lindane (Fig 1.1) are the most common of the class. Organophosphate (Ops) insecticides such as chlorpyrifos, pirimiphos methyl andphosmet (Fig 1.1) were also found to be among the most frequently involved in poisoning episodes. Whereas banned in Spain since 1994, the rodenticide strychnine (Fig 1.1)) was among one of the mostused pesticides for killing animals. There have also been occasional cases related to paraquat (Fig 1.1),poisoning in dogs**. O**ther pesticides found to be responsible for death of domestic animals were, fenazaquin, oxamyl, diazinon, fenthion, and difenacoum (Fig 1.2).

The list of banned pesticides in the European union by a program and a label for sustainable farming include the following among others; strychnine, lindane, carbofuran, bromadiolone, aldicarb, paraquat, brodifacoum, endosulfan (Fig 1.1), warfarin, chlorophacinone, phorate, , methamidophos, mevinphos, difenacoum (Fig 1.2) parathion, coumatetralyl, dichloride, flocoumafen, azinphos-ethyl andazinphos-methyl (Fig 1.3).[6] In Portugal, where this study is carried out, pesticides such as parathion, glyphosate, chloropyrifos have been banned. Nonetheless, despite the ban of these toxic compounds, some of them have been found to be continually used in some parts of the country (centre of Portugal). Of these pesticides, organophosphorus insecticides (azinphos-ethyl, azinphos methyl, parathion and quinalphos (Fig1.3) constitute the most important class, followed by carbamates and herbicide such as paraquat (Fig 1.1). Others commonly

involved in poisoning include, chloropyrifos, deltamethrin (Fig 1.1), glyphosate (Fig 1.3).[7] While, poisoning related to diquat, difenacoum (Fig 1.2), lindane and strychnine (Fig 1.1) have also been recorded in the country.[8] In the European Union, thirty three (33) organophosphate pesticides have been banned, but chlorpyrifos remains on the market, with consistentuse in Spain, Portugal, and France.[9]

As expected, Portugal with the closest border to Spain, most of the toxic compounds that have been found to be the common culprit in the illegal killing of animals is Spain have also been found to becommonly used in Portugal for the same heinous purpose. The Portuguese Environment Agency APA,argued that regarding the presence of banned substances in the analysis carried out in the different poisoning episodes, that they must have been brought by Spanish farmers and that individuals can always buy what they want over the internet**.**[9] It should be stressed that in Portugal a law has been passed since 1995 against the disaearane or that contributes decisively to the disappearance of one or more animal or plant specie by an individual is punishable by the law (Decreto-lei nº 48/95 artigo 278)[10]

In other European countries such as Belgium, the Czech Republic, Greece and Italy, carbamates were found to be responsible for most acute dog poisoning incidents. A study carried out in France on various poisoning cases, revealed that carbamates accounted for 37%, organophosphate insecticides OPs 19%, pyrethroids 14% and organochlorines 3%, while carbofuran, aldicarb (Fig 1.1) and mevinphos (Fig 1.2) with 20% were recorded to be the source of animal poisoning. Methomyl (Fig 1.1) (carbamate insecticide) was also found to be one of the applied poisons in granular and concentrated formulations used against flies, but as usual, led to the death of non-target animals. In Northern Greece, insecticides have been also recorded to be the main source of animal poisoning. Withthe carbamates, methomyl and carbofuran (Fig 1.1) being the main cause of animal death, followed by organophosphate such as parathion (Fig 1.3), phorate and methamidophos (Fig 1.2). Same scenario was also observed in a research carried out in the central and northern Italy, as carbamates followed byorganophosphate insecticides, were again seen to be the common culprit in animal death.

The cholinesterase inhibitors were recorded as the main cause of animal poisoning and death. In the southern part of the country (Italy), anticoagulant rodenticides (ARs) and organophosphate insecticideswere found to be the common culprit. Reports again revealed that carbamates (aldicarb, carbofuran and methomyl, organochlorines (endosulfan, lindane (Fig 1.1) and organophosphate insecticides (chlorpyrifos, pirimiphos methyl and phosmet (Fig 1.1), were the insecticides that were a common sourceof animal poisoning**.**[5]

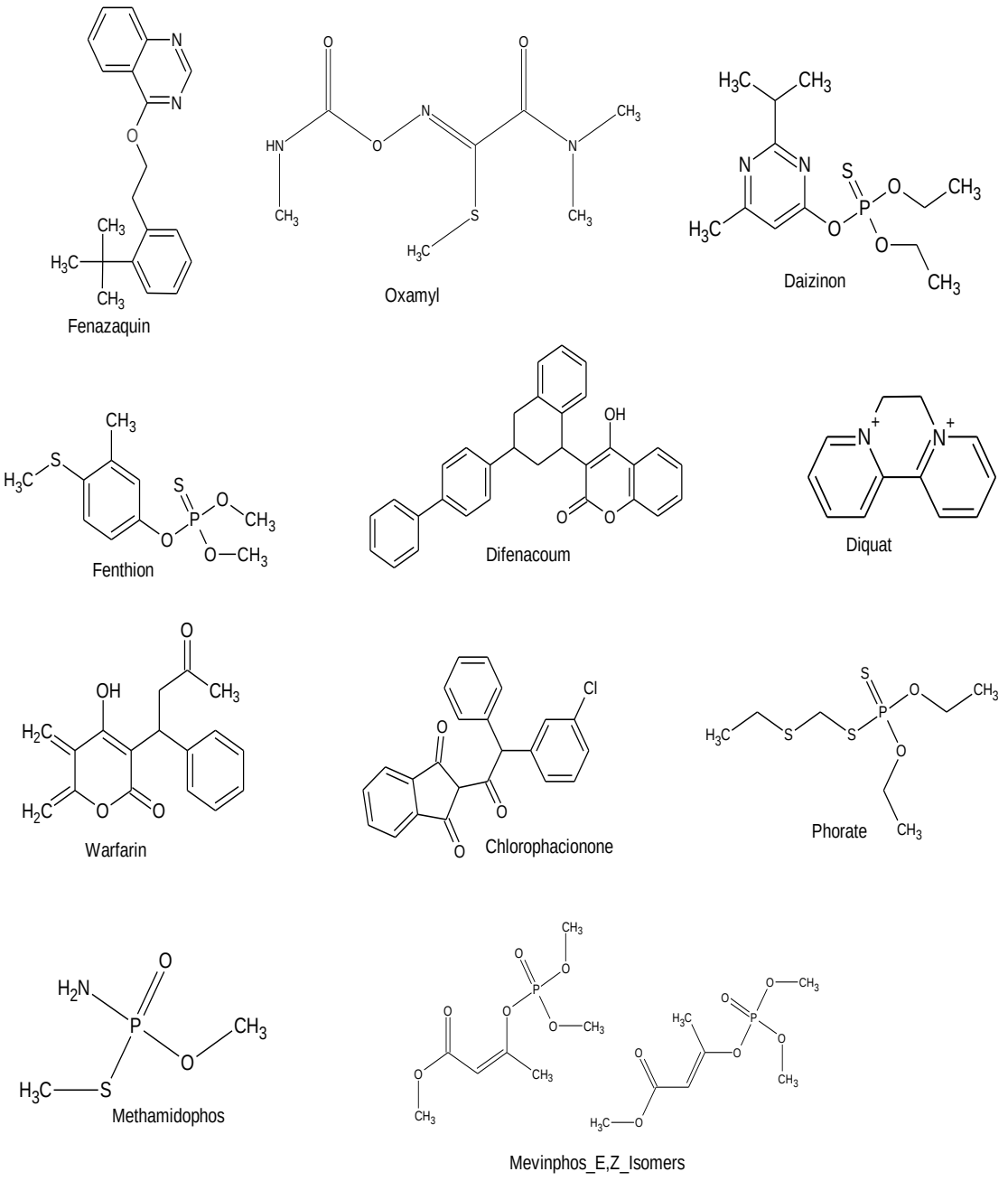


Fig 1.2: Structures of pesticides; fenazaquin, oxamyl, diazinon, fenthion, difenacoum, diquat,warfarin, chlorophacionone, phorate, methamidophos, mevinphos.

1.2.1. The use of Rodenticides for the Purpose of Killing Animals

Rodents are a major nuisance to humans as they not only interfere with the daily activities of humans but also harbour endemic diseases. Hence, their population control becomes imperative. This is mainly achieved using rodenticide, mainly the group of anticoagulant rodenticides (ARs). Due to the increased use of these toxic compounds, some persons employ them in the illegal killing of domestic animals. This happens largely because of their commercial availability and high-level toxicity, particularlythe second-generation anticoagulant rodenticide (for example, brodifacoum (Fig 1.1) played a major role in the illegal eradication of vertebrate pets).

Worldwide rodents and their associated threats to crops, infrastructure as well human health iscontrolled and or managed with rodenticides.[3,4] Rodenticides employed in the control of rodent population are classified into two groups: anticoagulant and non-anticoagulant. Based on their chemicalstructure, all anticoagulant rodenticides, may be grouped in two distinct categories: hydroxycoumarin orindandione-based rodenticides, which can be first-generation anticoagulants (FGARs) or second- generation anticoagulant rodenticides (SGARs). FGARs were the first rodenticide introduced in the 1940s for controlling rodent population; they require multiple feed or several doses for intoxication of target to occur. SGARs were introduced to overcome bait shyness (resistance to poison resulting from learning by association of symptoms by animals to bait consumed) experienced with the first-generationanticoagulants. They persist in the tissues and bio-accumulates in the tissues, hence are more potent than the first-generation anticoagulant rodenticides FGARs.[11]

FGARs such as warfarin, chlorophacinone (Fig 1.2), coumatetralyl , coumafuryl, and coumachlor (Fig 1.3), were developed during the 1940s and 1950s. Rodents poisoned by these toxic compounds usually die from internal bleeding, due to loss of blood clothing by the blood vessels as a result of damaged capillaries. The FGARs require multiple feeds to produce their effect, hence not verypotent. Over the years, rodents have developed resistance to these rodenticide as they were able to associate symptoms to baits eaten, this is known as “bait shyness”. This led to the development of the SGARs, known as the super-warfarin, in other to combat bait shyness. Examples of these class are: difenacoum (Fig 1.2), bromadiolone, brodifacoum (Fig 1.1), and other 72 agrochemicals. Due to their higher potency, the second-generation anticoagulant rodenticides SGARs are occasionally referred to as single feed anticoagulants.[12] The most common indanedione rodenticides are chlorophacinone (Fig 1.2), diphacionone, pindone, valone (iso-valeryl indanedione) (Fig 1.4).

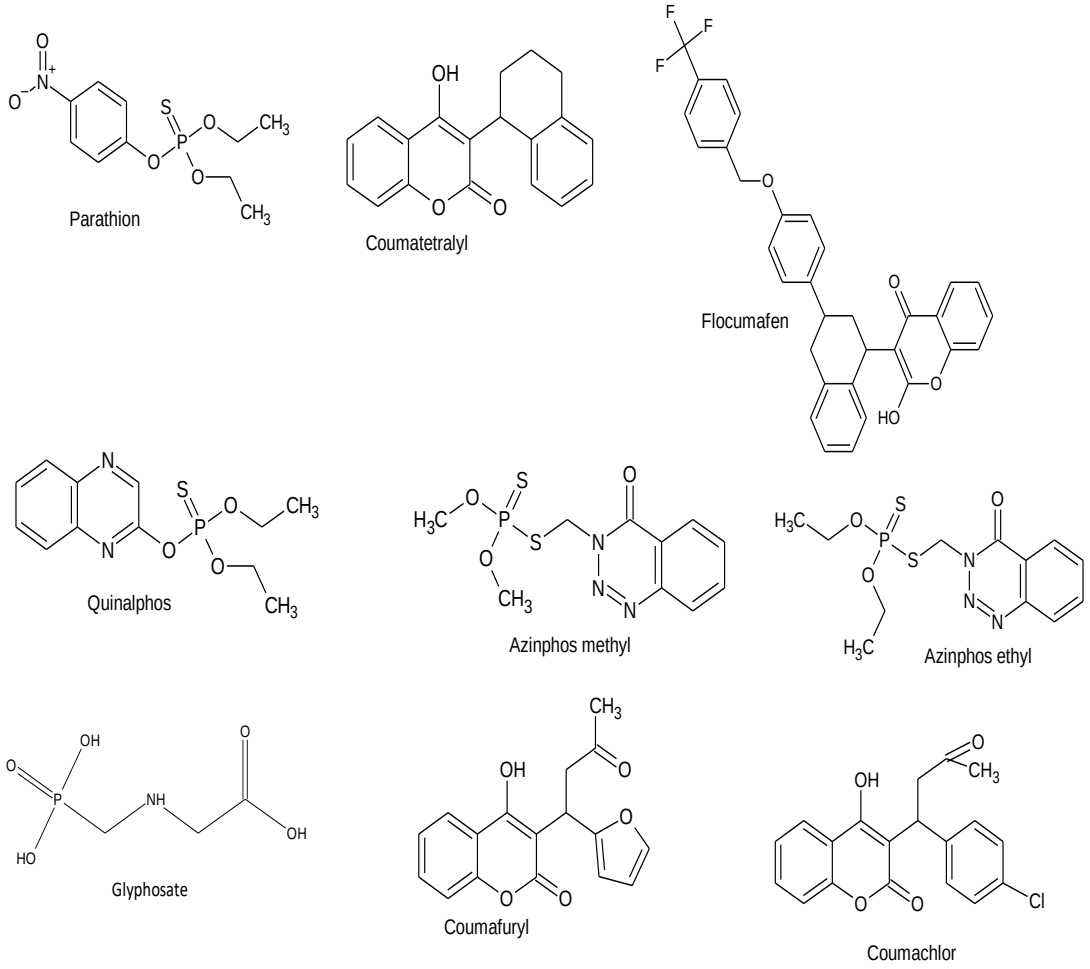


Fig 1. 3: Structure of the rodenticides; parathion, coumatetralyl, flocuomafen, azinphos ethyl,azinphos methyl, quinalphos, glyphosate, coumafuryl, coumachlor.

SGARs are more potent and have high acute toxicity generally than the first generations, hencetheir common use[13]. In fact, a research carried out in France, revealed that over 60% of poisoning cases, were as a result of poisoning with second generation anticoagulant rodenticides[5].

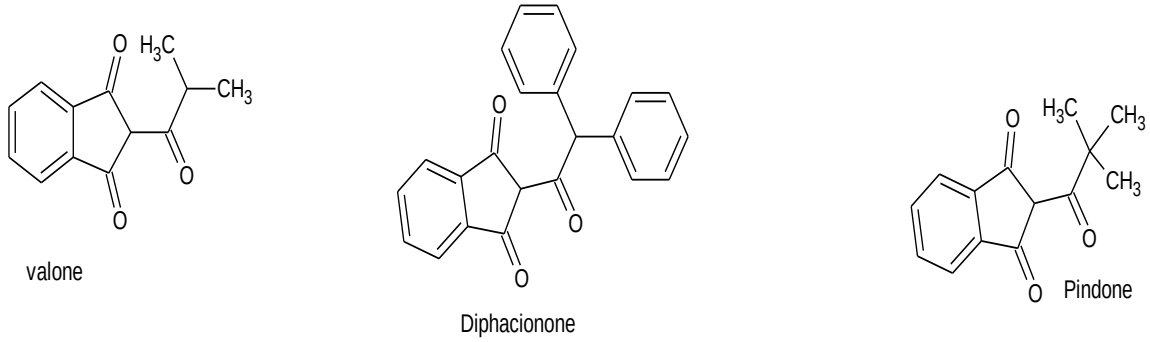


Fig 1. 4: Structures of the pesticides; diphacionone, pindone, valone (iso-valery indanedione).

1.3 Analytical Methods Used to Identify Pesticides

Chromatographic techniques allied with mass spectrometry detection (LC-MS) are inevitable in forensic toxicology field, as these techniques allow the separation of components of complex mixture and identification of wide range of chemical substances[14]

It is very difficult to have a general analytical methodology for all types of pesticides, due to their structural and chemical diversity. The methodology to be used for the identification of pesticides should,therefore, be carefully chosen according to the type of pesticide. This encompasses not only the methodology to be used for the extraction of compounds from the multiple matrixes but also the type ofstationary phase used for the chromatographic separation and the most suitable mode of ionization for the mass spectrometry (MS) detection.

The forensic samples to be analysed, in the present work, were collected in Portugal, very closeto the Spanish border. Therefore, we first investigated the analytical methodologies most suitable for the identification of pesticides most used for the purpose of killing animals, in these two countries. Based on the revised literature data, we established a method LC-MS for the determination of these pesticidesfound in the samples collected. (see section 1.2).

The table below shows literature methodologies used for the identification of pesticides that are going to be used in this work.

Table 1.1: LC-MS experimental conditions referenced in the literature for the identification of the pesticides that are going to be used in the current work

Compound	Mobile Phase/Column	Mode Of Analysis	Ref.
Bromadiolone	A: 5 mM ammoniumformate bufer pH 10.2 B: methanol, at 0.5 mL/min. / RP18	ESI+ MS/MS, MRM Mode	**[14]**
Carbofuran	A: ultrapure water as theaqueous phase B: methanol (HPLC –MSgrade), at 800 mL/min. / RP18	ESI-	**[3]**
Chlorophacionone	A: methanol +2 mM ammonium acetate in water (5 + 95, v/v) and B: methanol containing 2 mM ammonium acetate, at 0.35 mL /min. / RP18	ESI- MRM Mode	**[15]**
Azinphos ethyl and Azinphosmethyl	A: ultrapure water as theaqueous phase B: ACN / RP-18 column	ESI-	**[17]**
Aldicarb, Aldicarb sulfone and Aldicarb sulphoxide	A: 10 mM NH4OAc in water B: 10 mM NH4OAcin methanol at 500μL/min. / RP18	ESI+	**[16]**
Coumatetralyl	A: Ammonium acetate(5mM) B: methanol at 0.2mL/min. / RP18	ESI-, ESI+, SIM Mode	**[18]**
Deltamethrin	A: Acetonitrile B: 20 mM ammonium acetatebuffer at 0.31 mL/min. /RP18	ESI+	**[19]**
Difenacoum	A: 7.5 mM ammonium formate in ultrapure wateras the aqueous phase B: methanol (HPLC –MS grade) C: 2% formic acid. / Hydro-RP column	ESI+	**[3]**
Flocoumafen	A: methanol +2 mM ammonium acetate in water (5 + 95, v/v) and B: methanol containing 2 mM ammonium acetate, at 0.35 mL /min. / RP18	ESI- MRM Mode	**[15]**
Imidacloprid	(A) 7.5 mM ammonium formate in ultrapure water as the aqueous phase, (B) methanol (HPLC –MS grade) as the organic phase and (C) 2% formic acid /analytic Synergi Hydro-RP column (4.0 mm, 150 4.6 mm;	ESI-	**[3]**
Oxamyl	A: 10 mM NH4OAc in water B: 10 mM NH4OAc in methanol at 500μL/min. / RP18	ESI+	**[16]**
Strychnine	A: (0.5% isopropanol in 0.1% acetic acid in water) B: (5% isopropanol in ethanol) / C8	MRM-ESI+	**[21]**
Parathion	A: 10 mM NH4OAc in water B: 10 mM NH4OAc in methanol at 500μL/min. / RP18	ESI -	**[3]**
Glyphosate	A: H2O-citric acid B: NEt3 (g)/ Ion pac IP- AG11-HC	ESI- MRM	**[22]**
Warfarin	A: 0.2% acetic acid B: methanol/ C8	ESI-, MRM	**[18]**

1.4 Basics Of Liquid Chromatography Coupled With Mass Spectrometry

Over the years, liquid chromatography has been coupled to different mass analysers for the identification of compounds. Presently the use of low resolution mass spectrometric detectors such as triple-quadrupole and ion traps and high resolution detectors such as time-of-flight MS (TOF-MS), are amply used for the detection of pesticides both in aqueous and solid specimens.[14] Whereas the low resolution are less expensive and more accessible, the high resolution detection provides a much higher sensitivity and selectivity, but are not so accessible due to their high price.

Liquid chromatography with mass spectrometry detection (LC-MS) is a hyphenated technique that uses liquid chromatography (LC) for the separation and MS for detection/identification. MS is a powerful tool for both quantitative and qualitative analysis of organic molecules and biological macromolecules. It has no mass limitation and relies on the formation of gas-phase ions, positively or negatively charged, that can be isolated electrically or magnetically depending on their mass-to-charge ratio *m/z.* MS analysis provides important information regarding the structure and composition of an analyte. The sensitivity of MS depends greatly on the mass analyser used. A fundamental requirement of mass spectrometry is that molecules are ionized and analysed as gas phase ions which are characterized by their mass-to-charge ratio (*m/z*).

A MS experiment typically consists of five steps: (i) sample introduction, (ii) analyte ionization,
(iii) ion separation according to their *m/z*, (iv) ion detection, and (v) data processing. Mass spectrometers are usually comprised of three components (Fig1. 5); **1.** ion source or ionizer, that produces the ions to be analysed; **2.** Mass analyser; is the heart of the mass spectrometer and separates ions according to their mass-to-charge ratio m/z; and **3.** Detector; is responsible for counting the molecules at each mass-to-charge value reported by the mass analyser [24]

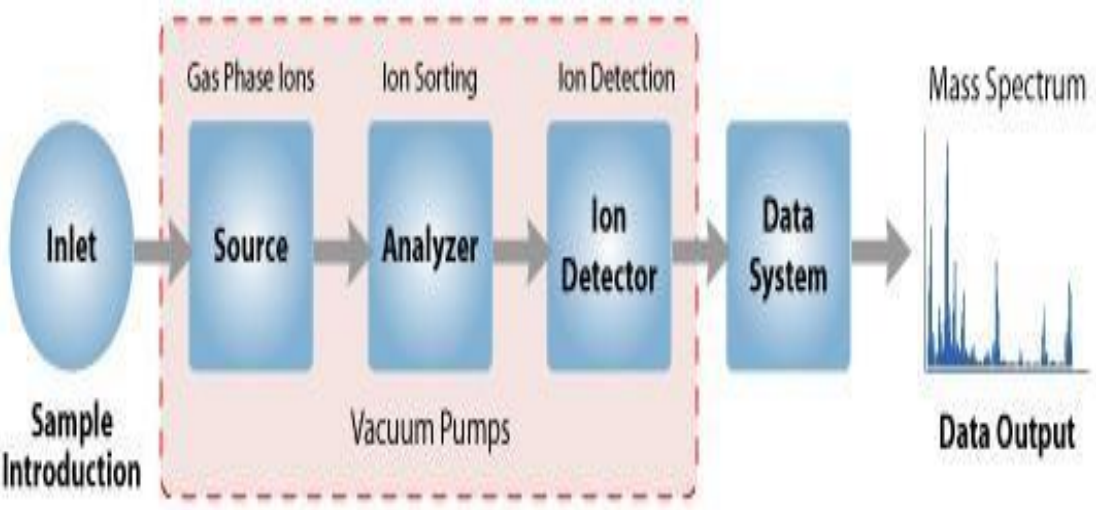


Fig 1.5. Principal components of a mass spectrometer [25]

The tandem mass spectrum (MS/MS) gives additional and valuable information about the structure of the precursor ion, by the isolation of a specific *m/z* (precursor ion) which is then subjected to dissociationleading to the production of fragments or product ions.

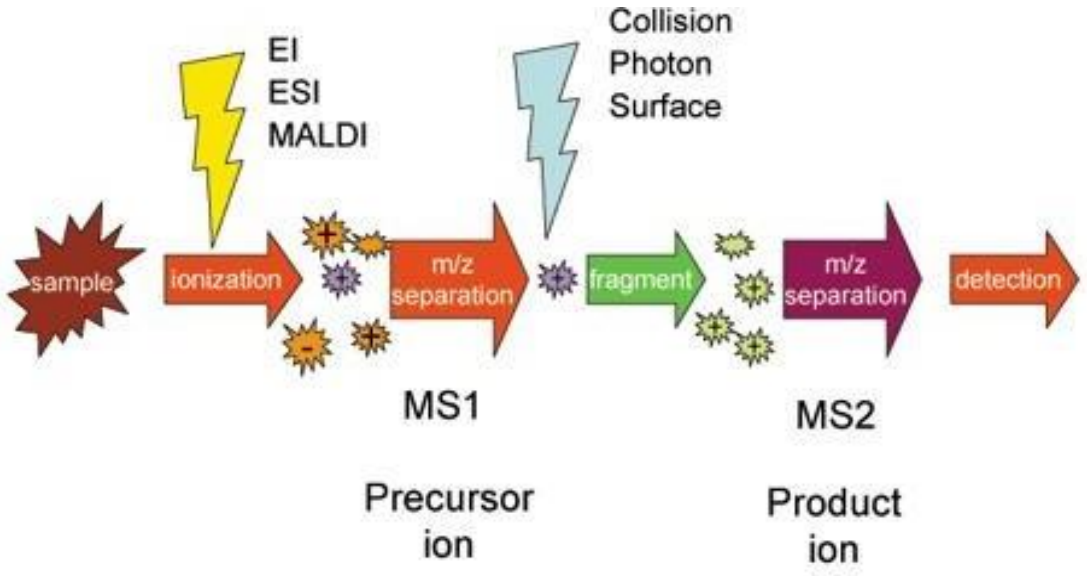


Fig1.6. Tandem mass spectrometry (MS/MS) scheme [26]

Electrospray ionization (ESI) and the atmospheric-pressure chemical ionization (APCI) are atmospheric-pressure ionization (API) soft ionization techniques which gained popularity in the late 1990s mainly due to their compatibility with the LC flow rate, simplicity and their robustness. The ESI and the APCI are currently used, not only for the identification of pesticides but also in the analysis of biomolecules and pharmaceuticals. APCI has the advantage of detecting more non-polar compounds than ESI. While ESI is most often more suitable for polar and chemically ionizable compounds.

ESI mode of action (positive or negative) is based on the production of ions in solution, which is a high voltage is applied to a liquid to create an aerosol. **T**he technique requires the dissolution of samples to be analysed in a buffer or polar solvent introduced in the mass spectrometer. This allows for its infusion into the ionization source under atmospheric pressure. Application of high electrical potential at the needle which introduces solvated samples in the ionizer results in nebulization (formation of highly charged droplets). The breakdown of the droplets to a very reduced size is usually achieved by vaporization, using a warm neutral gas, in most cases nitrogen. As a result, the coulombicforce action on the surface of the shrinking droplets eventually becomes so high that it exceeds the surface tension of the droplets leading to the formation of gaseous phase of the solvent. Generally, theprocess is favourable for solvents with low *m/z* values. The technique has the advantage of high sensitivity (ionization efficiency) and lower detection limit potential**.**[24]

1.5 Aim of the Thesis

This thesis was undergone under the scope of a protocol established between the "Laboratório de Polícia Científica da Polícia Judiciária (LPC/PJ) e o Instituto Superior Técnico (IST) aimed at the:

1. Development of a suitable low resolution-based LC-MS/MS-based protocol for the identification of pesticides in samples provided by the LPC/PJ. These samples were obtained from cases where the intoxication of animals was suspected.

2. Construction of a database composed from LC-MS/MS data of standard pesticides to be used to identifyor rule out the presence of these toxic compounds in forensic samples.

3. Compare the identification ability of the LC-MS/MS protocol developed with a LC-HRMS methodology previously used for the identification of pesticides in the same forensic samples.

2. RESULTS AND DISCUSSION

2.1 PREAMBLE

The first step of this work consisted of the analysis by LC-MS/MS with ESI of a group of distinctpesticides (Table 2.1), for the preparation of a data base, with the goal of being used for the identificationof pesticides present in forensic samples. The choice of the pesticides to be included in this first data base was based on the previous knowledge about their use in Portugal and Spain, for the purpose of illegal intoxication of animals.

Following this first step, the forensic samples were analysed under the same conditions used toanalyse the standards. The identification of pesticides present in the forensic samples was performed upon comparison of LC-MS data (retention time (Rt), *m/z* of protonated or deprotonated molecule and main MS/MS fragment ions) obtained for each forensic sample with the one present in the pesticide standards data base.

2.2 CONSTRUCTION OF LC-MS DATABASE OF PESTICIDESTANDARDS

The selected pesticides were analysed by LC-MS/MS, in the full scan and MS/MS mode, underthe experimental conditions stipulated for this work that consisted on: (1) a polar RP18 Kinetex (Phenomenex) column was used, since this type of stationary phase guarantees the elution of very polarcompounds, which in standard RP18 columns are not retained, thus offering the possibility of analysingwith a single column a greater number of compounds with very different polarity; [27] **(**2) LC-MS/MS analysis by both ESI (+) and ESI (-); and (3) each sample was analysed in two distinct LC runs, usinga solution of formic acid (0.1%) and 5mM ammonium acetate pH 6.5 as the aqueous eluent, to ensure the ionization conditions of a wide range of pesticides;

Following the analysis of each standard under the conditions previously described, a database(Table 2.1) was built containing the following information*: m/z* of the protonated or deprotonated molecule, retention time (Rt), preferential ionization mode - ESI (+) or ESI (-) and *m/z* values for the most abundant fragments (obtained in the MS/MS spectra).

Table 2.1 Database from LC-MS data of commercial pesticides used in the current work

Compound	Ionization mode[1]	Retention time Tr, (min)	Precursor ion *m/z*	Fragment 1 *m/z*	Fragment 2 *m/z*
Bromadiolone	ESI+ **ESI -**	11.1 11.1	509 525	251	
Carbofuran	**ESI+**	8.0	222	165	
Parathion	**ESI+**	10.5	292	236	
Glyphosate	ESI+ **ESI -**	0.9 0.9	170 168	88. 150	
Azinphos ethyl	**ESI+**	9.0	318	261	160
Azinphos ethyl	**ESI+**	10.1	346	289	
Flocoumafen	**ESI+** ESI -	not observed	-	-	-
Warfarin	ESI+ **ESI -**	9.2 9.2	309 307	163 161	
Aldicarb	**ESI+**	7.2	191	116	
Difenacoum	ESI+ ESI -	not observed	-	-	
Coumatetralyl	**ESI+** ESI -	9.8	293 291	175 247	
Fluconazole	**ESI+**	9.6	307	289	
Deltamethrin	**ESI+**	12.4	50 6	345	
Terramycin	**ESI+**	5.1	46 1	426	
Strychnine	**ESI+**	1.1	355	264	

[1]In bold is indicated the preferred mode of ionization.

A more detailed explanation of the MS data obtained for each pesticide standard is going to be providedin the following paragraphs.

2.2.1 Bromadiolone Standard

Bromadiolone elutes at 11.1 min, in the experimental conditions used. The full scan mass spectrum of the bromadiolone standard displays signals at *m/z* 509 and 525 in the ESI (+) and ESI (-), respectively. These signals exhibit the isotopic pattern expected for a mono-brominated compound, exhibiting two signals of equal intensity with a difference of two units, due to the presence of the two most abundant isotopes of bromine (^{79}Br and ^{81}Br) in the compound. Nonetheless, it should be stressedthat in the ESI (+), bromadiolone does not display the expected signal corresponding to the protonated molecule. Instead, it shows the fragment ion at *m/z* 509 $[M-H2O + H]^+$, stemming from the loss of a water molecule from the protonated molecule, corresponding to the structure displayed in Fig 2. 1. It should also be stressed that the extracted ion chromatogram at *m/z* 525 and 509, (Fig 2.1G and H) show that the bromadiolone is better detected in the ESI (-) than the ESI (+) mode.

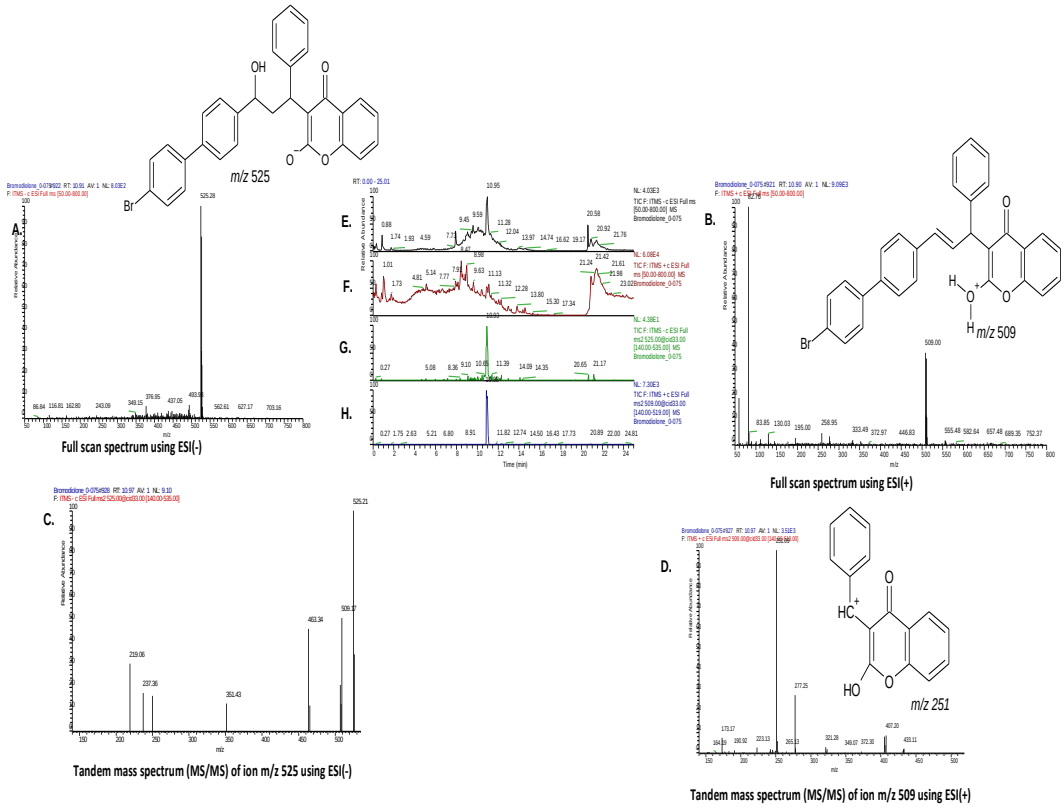


Fig 2.1. Bromadiolone standard: E. Total ion Chromatogram obtained in the full scan ESI (-) mode; F. Total ion Chromatogram obtained in the ESI (+) mode; G. Extracted ion chromatogramof ion at *m/z* 525, obtained in the ESI (-) mode; and H. Extracted ion chromatogram of ion at *m/z*509, obtained in the ESI (+) mode. Also shown are the full scan (A and B) and MS/MS (C and D) spectra along with the structures of the main fragments observed.

2.2.2 Carbofuran Standard

Carbofuran eluted at 8.0 min under the experimental conditions used. This pesticide is only observable when ionized in the ESI (+) mode and the full scan spectrum obtained displays the protonated molecule $[M+H]^+$ at *m/z* 222. The tandem mass spectrum of ion *m/z* 222, showed predominantly the fragment ion at *m/z* 165 $[C_{10}H_{12}O_2]^+$, corresponding to the structure shown in Fig 2.2B

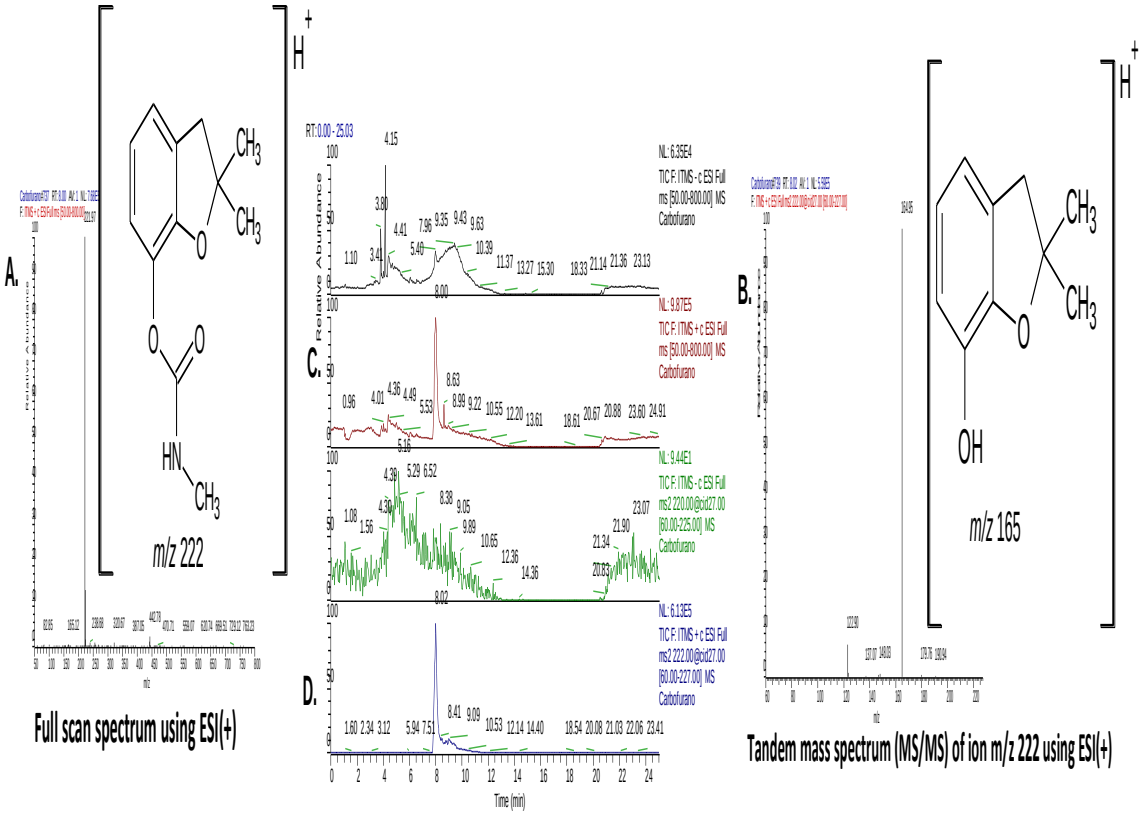


Fig 2.2. Carbofuran standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D. Extracted ion chromatogram of ion at *m/z* 222, obtained in the ESI (+) mode. Also shown arethe full scan spectra (A) and MS/MS spectra (B) along with the structures of the main fragmentsobserved.

2.2.3 Parathion Standard

Parathion standard is only observable by LC-MS in the ESI (+) mode, eluting at 10.5 min. The full scan mass spectrum of parathion exhibits a protonated molecule $[M + H]^+$ at *m/z* 292 (molecular mass of parathion is 291). The MS/MS of ion $[M+H]^+$ at *m/z* 292 exhibits the fragment ion at *m/z* 236 $[C_6H_7NO_5PS]^+$, which results from the loss of the diethyl group from the protonated molecule. the proposed structure for the major fragment ion is shown in Fig 2.3

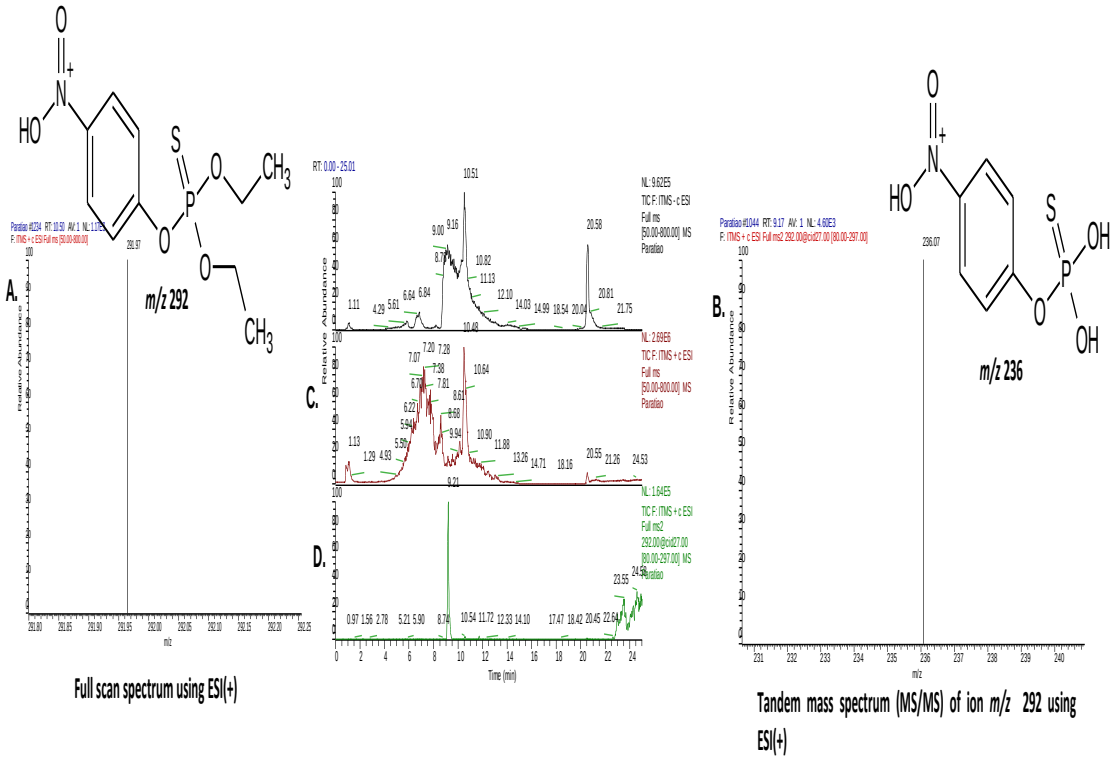


Fig 2.3 Parathion standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D.Extracted ion chromatogram of ion at *m/z* 292, obtained in the ESI (+) mode. Also shown are the full scan (A) and MS/MS (B) spectra along with the structures of the main fragments observed.

2.2.4 Glyphosate Standard

The highly polar molecule, glyphosate elutes at 0.9 min at the experimental conditions used andit is observable in both ESI (+) and ESI (-) modes. Nonetheless, a more intensive signal is obtained in the ESI (-) mode. The full scan spectra of this pesticide exhibited signals at *m/z* 170 $[M+H]^+$ and at *m/z* 168 $[M-H]^-$, in the ESI (+) and ESI (-) mode, respectively. The MS/MS spectrum from the precursor ion *m/z* 170 $[M+H]^+$, obtained in the ESI (+) mode, exhibited the fragment ion at *m/z* 88 $[C_3H_6NO_2]^+$ as the main fragment ion. While tandem mass spectrum obtained in the ESI (-) mode of the precursor ion at *m/z* 168 $[M-H]^-$ produced the fragment ion *m/z* 150 $[C_3H_5NO_4P]^-$ Fig 2.4 below depicts the proposed structures for the major fragment ions.

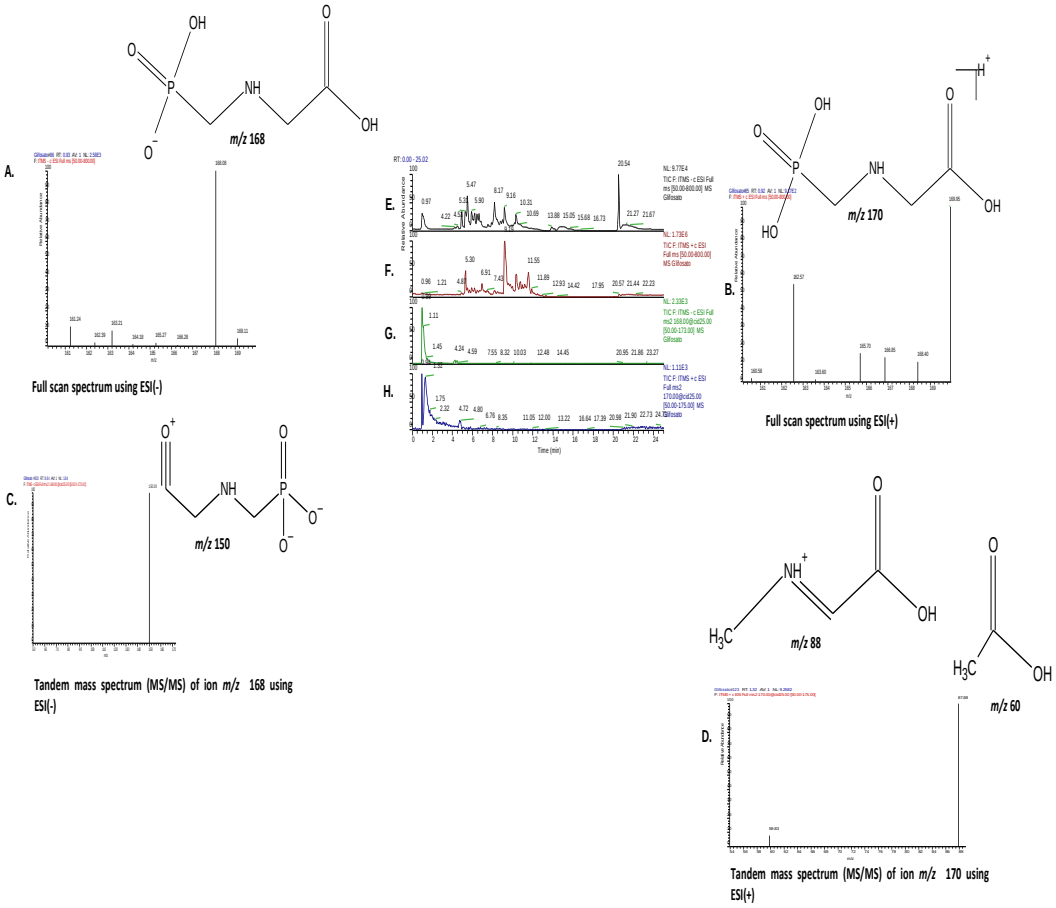


Fig 2.4. Glyphosate standard: E. Total ion chromatogram obtained in the full scan ESI (-) mode;F. Total ion chromatogram obtained in the full scan ESI (+) mode; G. Extracted ion chromatogram of ion at *m/z* 168, obtained in the ESI (-) mode and H. Extracted ion chromatogram of ion at *m/z*170, obtained in the ESI (+) mode. Also shown are the full scan (A and B) and MS/MS (C and D) spectra along with the structures of the main fragments observed.

2.2.5 Azinphos methyl standard

Azinphos methyl elutes at 9.0 min at the experimental conditions used. It is only identified by LC-MS when analyzed in the ESI (+) mode. The full scan spectrum exhibits the protonated molecule $[M+H]^+$ at *m/z* 318 as the base peak (molecular mass of azinphos methyl is 317). The tandem mass spectrum of this ion displays predominantly the fragment ion at *m/z* 261 $[C_6H_4N_3O_3PS_2]^+$ and the other minor fragment ion at *m/z* 160 $[C_8H_6N_3O]^+$. The proposed structure for these fragment ions are shown in Fig 2.5**.**

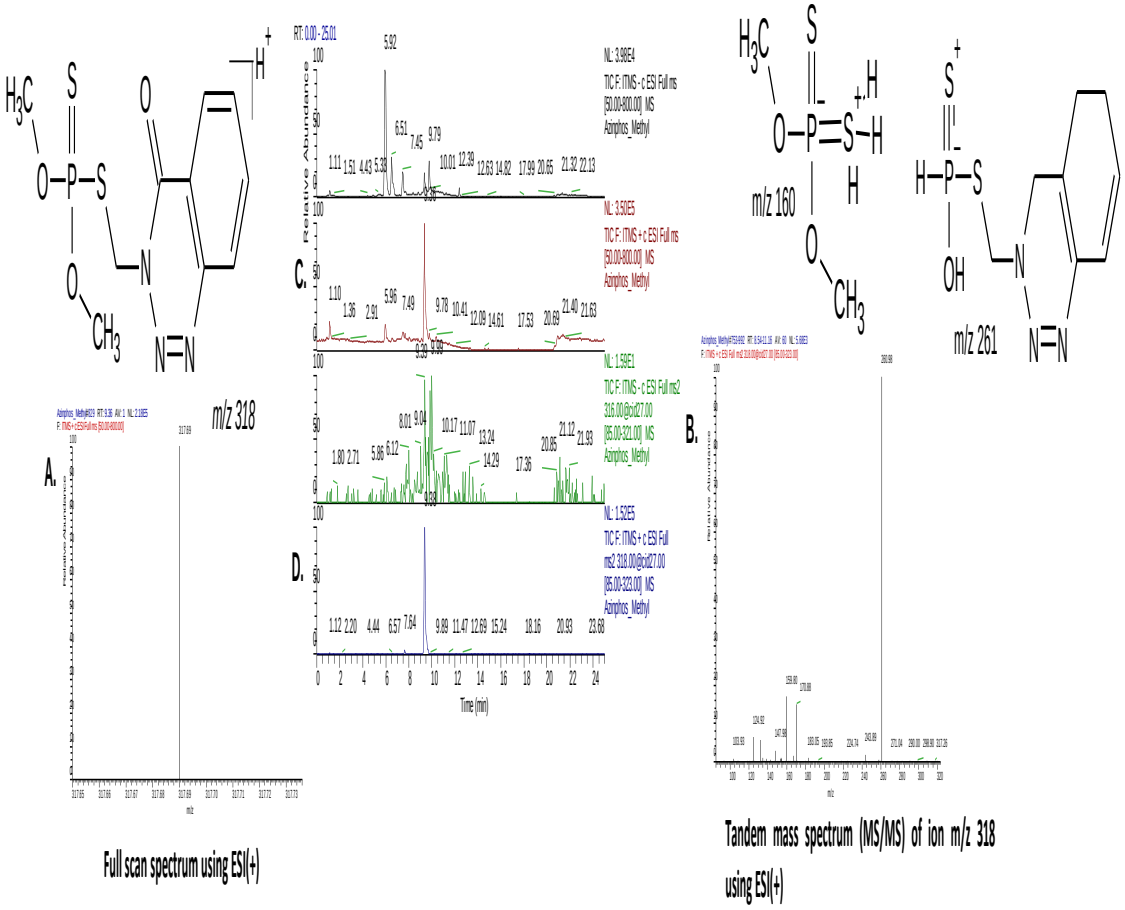


Fig 2.5. Azinphos methyl standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D. Extracted ion chromatogram of ion at *m/z* 318, obtained in the ESI (+) mode. Also shownare the full scan (A) and MS/MS (B) spectra along with the structures of the main fragments observed.

2.2.6. Azinphos ethyl standard

Azinphos ethyl elutes at 10.1 min at the experimental conditions used. It is only identified by LC-MS when analysed in the ESI (+) mode. The full scan spectrum gave the protonated molecule$[M+H]^+$ at *m/z* 346. The tandem mass spectrum of ion *m/z* 346, showed predominantly the fragment ion at *m/z* 289 $[C_8H_8N_3O_3PS_2]^+$. The proposed structure for the fragmentation mechanism is shown in Fig 2.6 below.

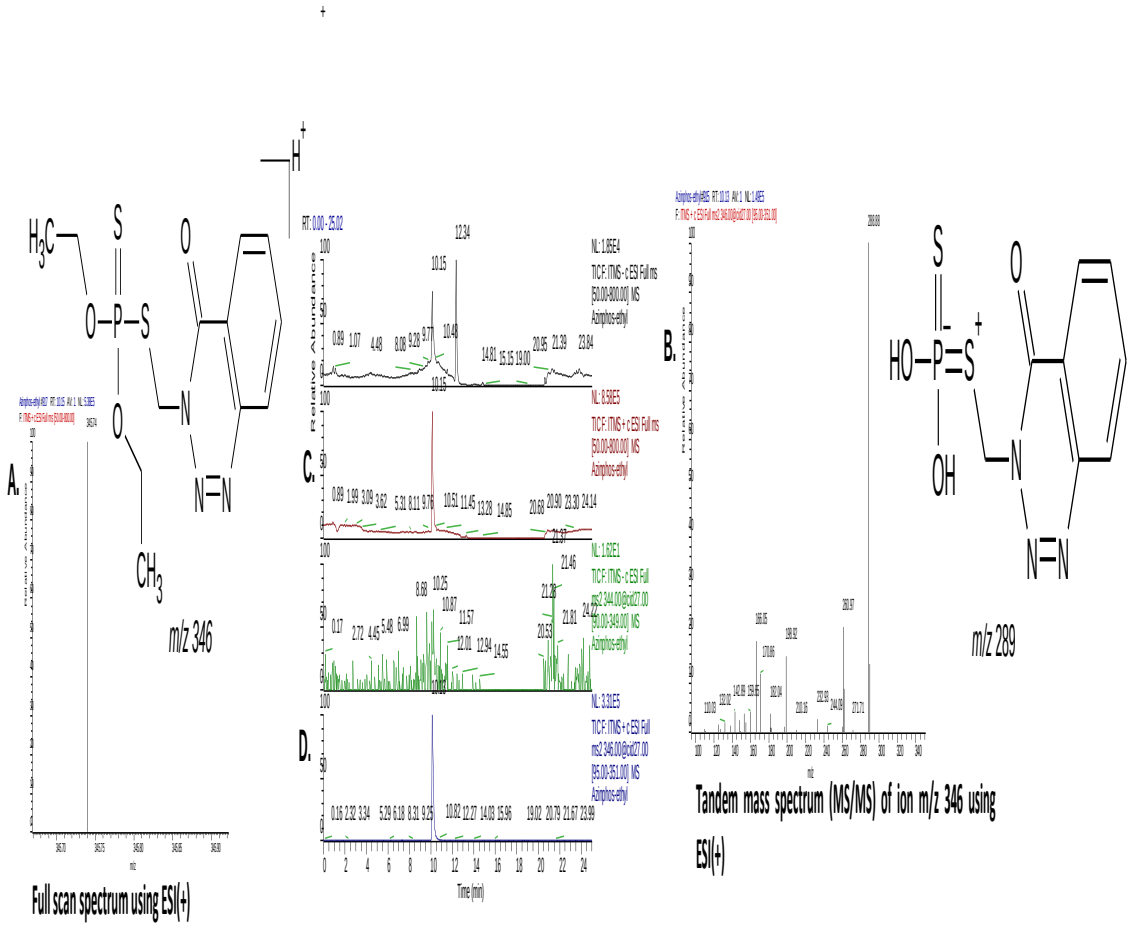


Fig 2.6 Azinphos ethyl standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D. Extracted ion chromatogram of ion at *m/z* 346, obtained in the ESI (+) mode. Also shown are the full scan (A) and MS/MS (B) spectra along with the structure of the main fragment observed.

2.2.7 Warfarin standard

Warfarin elutes at 9.2 min at the experimental conditions used, and it is observable in both ESI(+) and ESI (-) modes. Nonetheless, a more intensive signal is obtained in the ESI (-) mode. The full scan spectra of the pesticide exhibited signals at *m/z* 309 $[M + H]^+$ and at *m/z* 307 $[M-H]^-$ in the ESI (+) and ESI (-) modes, respectively. The tandem mass spectrum obtained in the ESI (+) mode of ion at *m/z*309 $[M+H]^+$, exhibited the fragment ion at *m/z* 163 $[C_9H_7O_3]^+$, as the base peak, corresponding to the loss of the 4-phenylbutan-2-one moiety. While the tandem mass spectrum obtained in the ESI (-) mode of the precursor ion at *m/z* 307 exhibited the fragment ion at *m/z* 161 $[C_9H_5O_3]^-$ as the major ion fragment. In Fig 2.7 are shown the proposed structures for the fragment ions obtained.

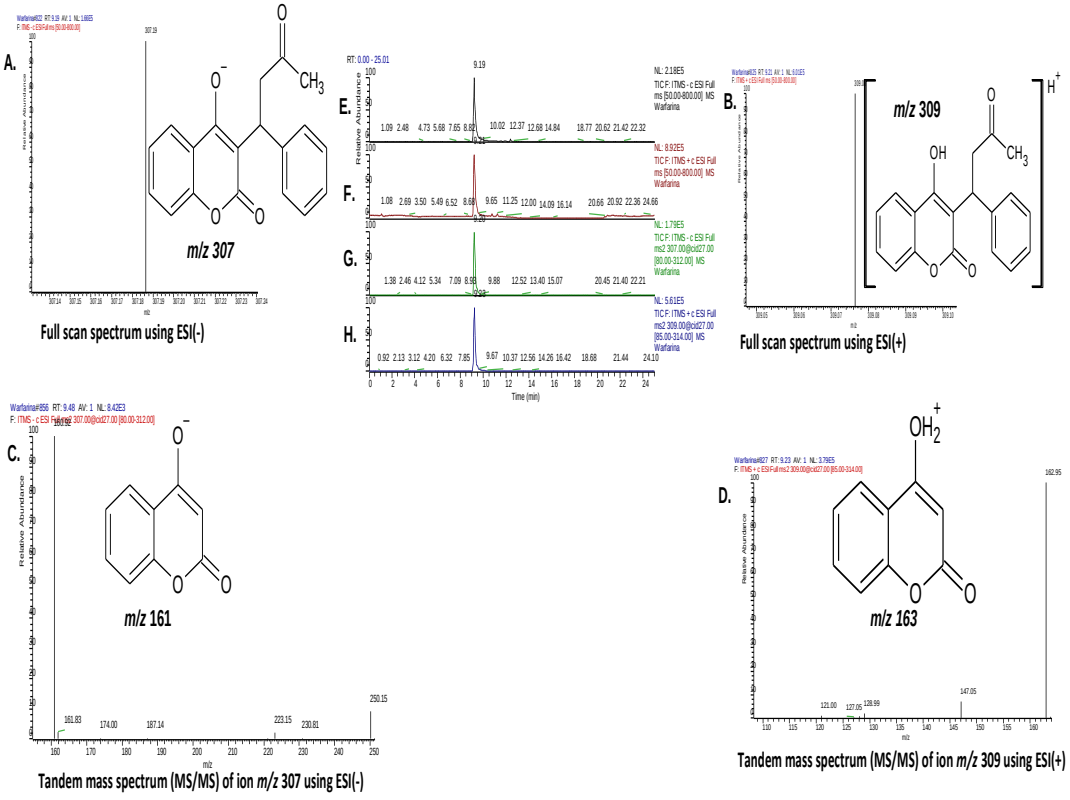


Fig 2.7 Warfarin standard: E. Total ion chromatogram obtained in the full scan ESI (-) mode; F. Total ion chromatogram obtained in the full scan ESI (+) mode; G. Extracted ion chromatogram of ion at *m/z* 307, obtained in the ESI (-) mode; and H. Extracted ion chromatogram of ion at *m/z*309, obtained in the ESI (+) mode. Also shown are the full scan (A and B) and MS/MS (C and D) spectra along with the structures of the main fragments observed.

2.2.8 Aldicarb standard

Aldicarb elutes at 7.2 min at the experimental conditions used, and it is only identified by LC- MS when analysed in the ESI (+) mode. The full scan spectrum gave the protonated molecule ion at *m/z* 191 $[M+H]^+$. The tandem mass spectrum of ion at *m/z* 191 $[M+H]^+$ exhibited predominantly an ion at *m/z* 116, which results from the loss of the group -$OCONHCH_3$ from the protonated molecule. Also, the ammonium ion adduct $[M+NH_4]$ at *m/z* 208 can be seen from the full scan spectrum. The proposed structure for the fragment ions obtained is shown in Fig 2.8

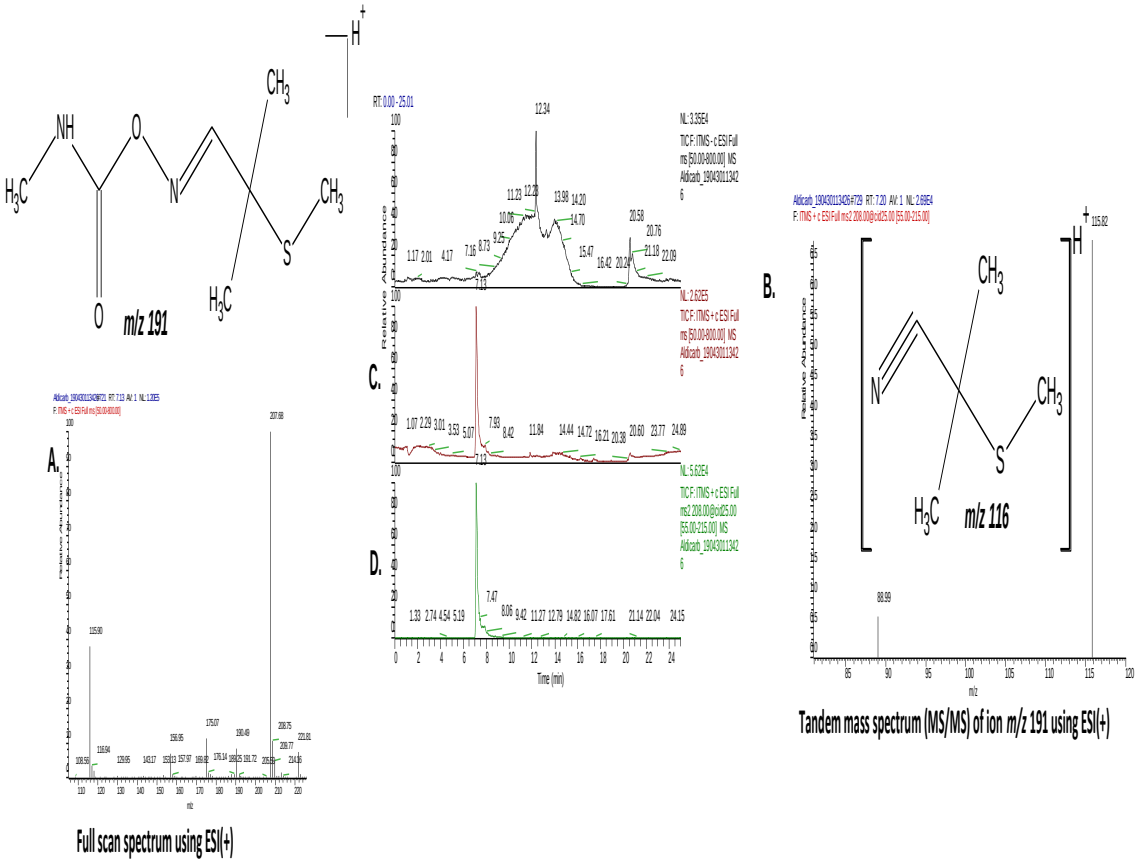


Fig 2.8. Aldicarb standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D.Extracted ion chromatogram of ion at *m/z* 191, obtained in the ESI (+) mode. Also shown are the full scan (A) and MS/MS (B) spectra along with the structures of the main fragments observed.

2.2.9 Coumatetralyl standard

Coumatetralyl elutes at 9.8 min at the experimental conditions used and it is observable in bothESI (+) and ESI (-) modes. Nonetheless, a more intensive signal is obtained in the ESI (+) mode. The full scan spectra of the pesticide exhibited signals at *m/z* 293 $[M + H]^+$ and at *m/z* 291 $[M-H]^-$ in the ESI (+) and ESI (-) mode, respectively. The tandem mass spectrum obtained in the ESI (+) mode of ion *m/z*293 $[M+H]^+$ exhibited predominantly the fragment ion at *m/z* 175 $[C_{10}H_7O_3]$.$^+$ While the tandem mass spectrum obtained in the ESI (-) mode of the precursor ion at *m/z* 291 exhibited the fragment ion at *m/z*247 $[C_{18}H_{15}O]^-$ as the major ion fragment. The fragmentation mechanisms are depicted in Fig 2.9.

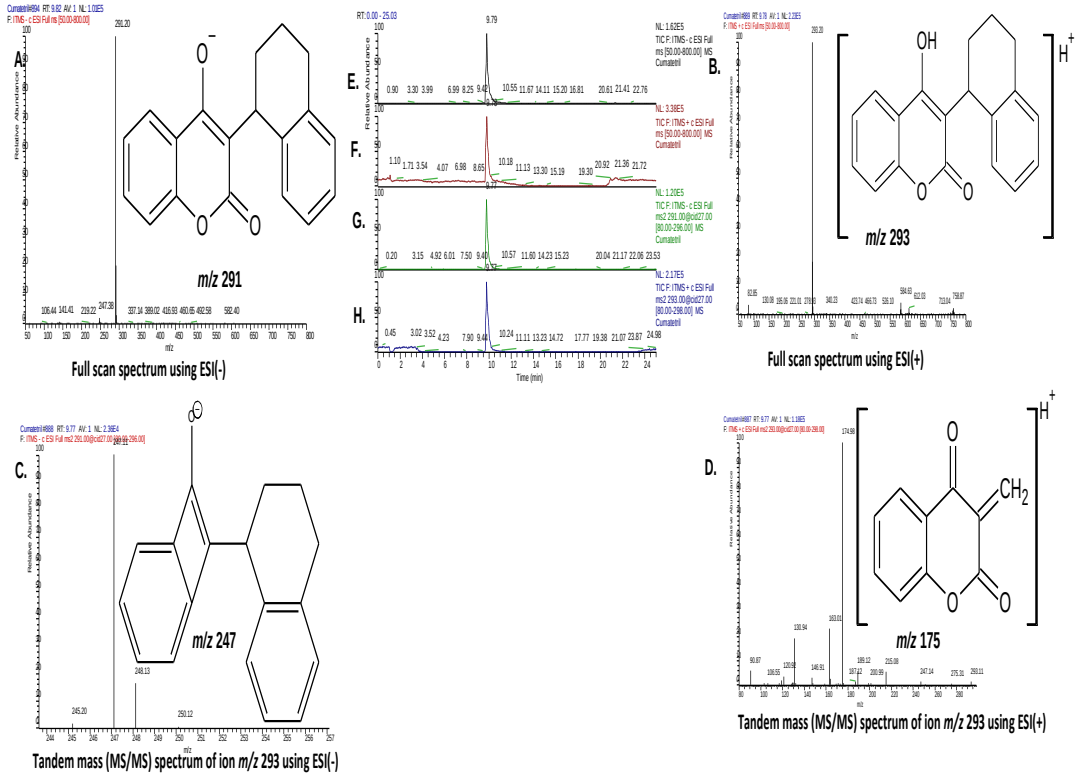


Fig 2.9 Coumatetralyl standard: E. Total ion chromatogram obtained in the full scan ESI (-) mode; F. Total ion chromatogram obtained in the full scan ESI (+) mode; G. Extracted ion chromatogram of ion at *m/z* 291, obtained in the ESI (-) mode and H. Extracted ion chromatogramof ion at *m/z* 293, obtained in the ESI (+) mode. Also shown are the full scan (A and B) and MS/MS(C and D) spectra along with the structures of the main fragments observed.

2.2.10 Fluconazole standard

Fluconazole elutes at 9.6 min at the experimental conditions used. It is only identified by LCMS when analysed in the ESI (+) mode. The full scan spectra of the pesticide exhibited signals at m/z 307 [M + H]+ in the ESI (+). The tandem mass spectrum obtained in the ESI (+) mode of ion m/z 307 [M+H]+ exhibited predominantly the fragment ion at m/z 289 [C13H11F2N6] + due to the loss of H2O (18 u) from the protonated molecule. The fragmentation mechanism is depicted in Fig 2.10 below.

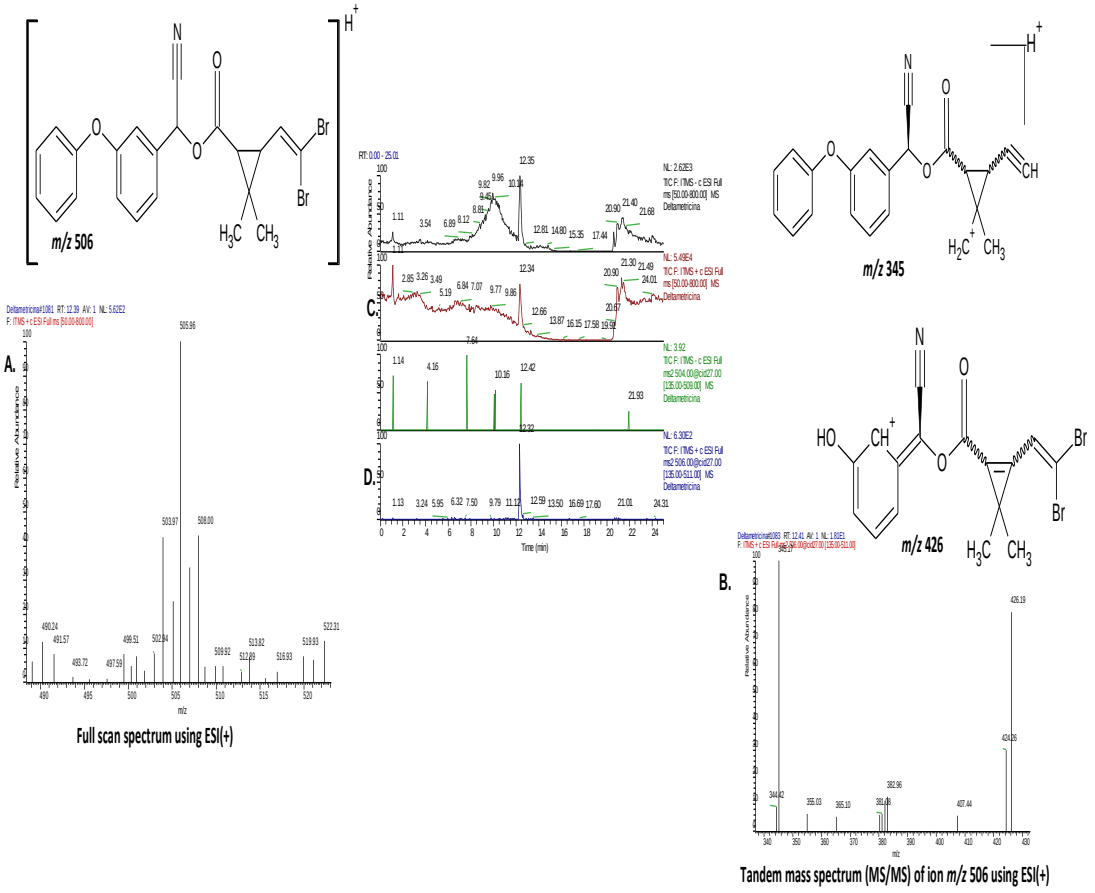


Fig 2.10. Fluconazole standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D. Extracted ion chromatogram of ion at *m/z* 307, obtained in the ESI (+) mode. Also shown are the full scan (A) and MS/MS (B) spectra along with the structure of the main fragment observed.

2.2.11 Deltamethrin standard

Deltamethrin elutes at 12.4 min at the experimental conditions used, and it is only identified by LC-MS when analyzed in the ESI (+) mode. The full scan spectrum depicts the protonated molecule ionat *m/z* 506 $[M+H]^+$. The MS/MS spectrum of this ion exhibited predominantly a signal at *m/z* 345 $[C_{22}H_{19}NO_3]^{2+}$ as the fragment ion. The fragmentation mechanism is shown in Fig 2.11 below.

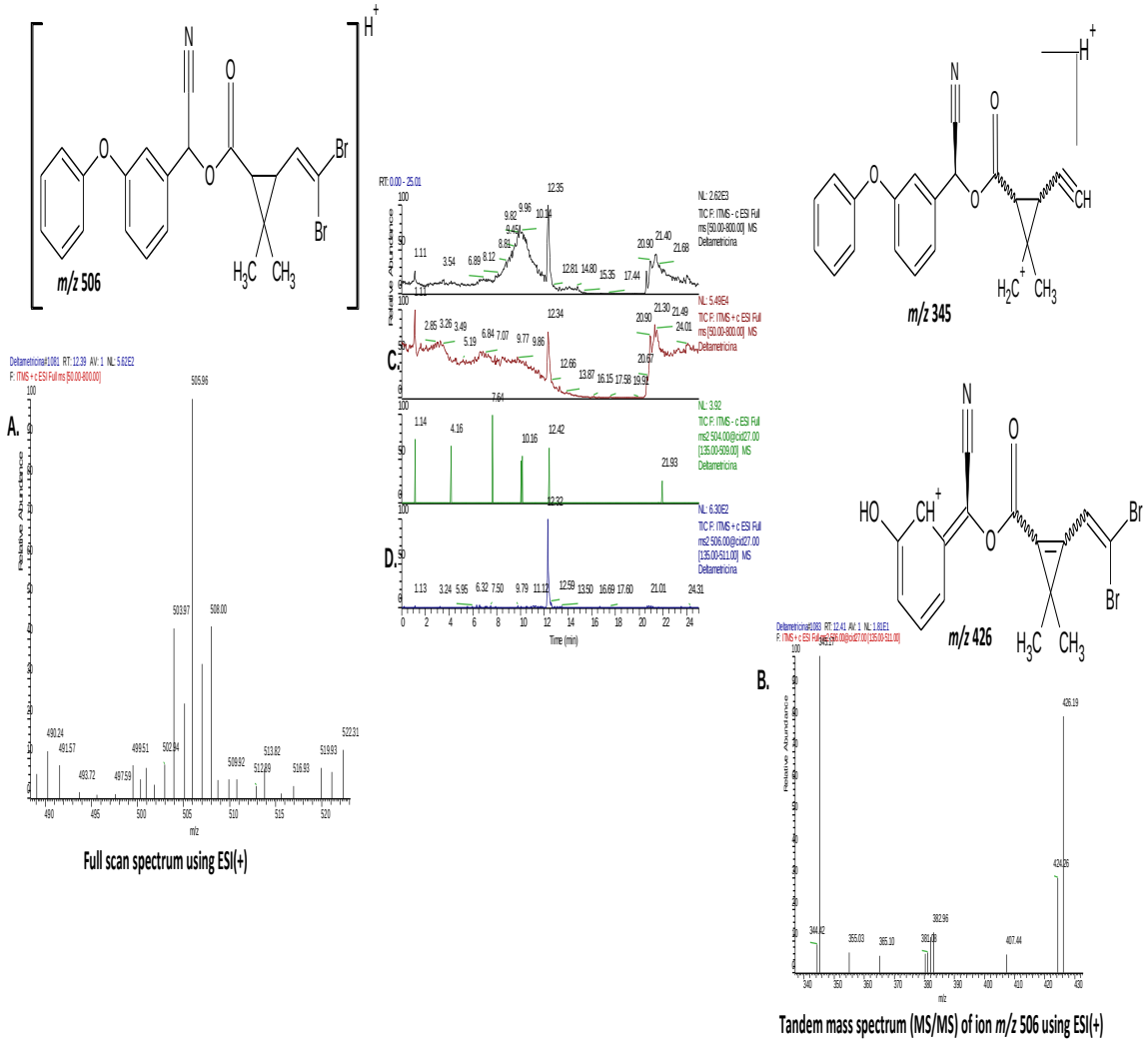


Fig 2.11. Deltamethrin standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D. Extracted ion chromatogram of ion at *m/z* 506, obtained in the ESI (+) mode. Also shown are the full scan (A) and MS/MS (B) spectra along with the structure of the main fragment observed.

2.2.12 Terramycin standard

Though terramycin is not a pesticide, it was included in the data base since we had the indication that this antibiotic was used, in at least one case, for the purpose of animals intoxication. Thebest sensitivity of terramycin was achieved on ESI (+) mode. The compound elutes at 5.1 min at the experimental condition used. The full scan spectrum obtained for terramycin under the selected LC–MS conditions is characterised by a protonated molecular ion at *m/z* 461 $[M+H]^+$ The MS/MS spectrum obtained for the protonated molecule of terramycin exhibited predominantly the fragment ion at *m/z* 426 $[M+H-NH_3-H_2O]^+$ The proposed structure for the fragment ion is depicted in Fig 2.12 below.

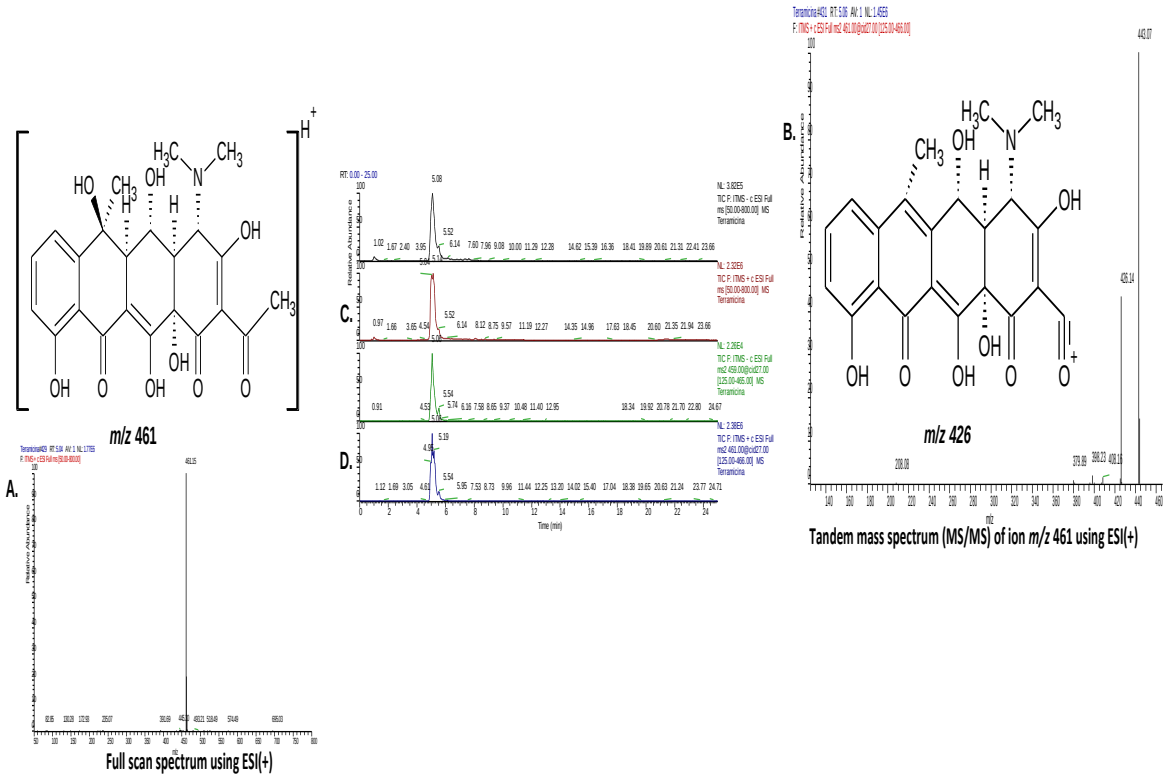


Fig 2.12. Terramycin standard: C. Total ion chromatogram obtained in the full scan ESI (+) mode;

D. Extracted ion chromatogram of ion at *m/z* 461, obtained in the ESI (+) mode.

Also shown arethe full scan (A) and MS/MS (B) spectra along with the structure of the main fragment observed.

2.2.13 Strychnine standard

The best sensitivity of strychnine was achieved by LC-MS ESI (+) mode. The highly toxic pesticide elutes at 1.1 min. The full scan spectrum exhibits the ion corresponding to the protonated molecule at *m/z* 335 $[M + H]^+$. The MS/MS spectrum of this ion showed the base fragment ion peak at *m/z* 264, resulting from the loss of C_3H_5NO from the protonated molecule of strychnine. The proposed fragmentation mechanism is shown in Fig 2.13 below.

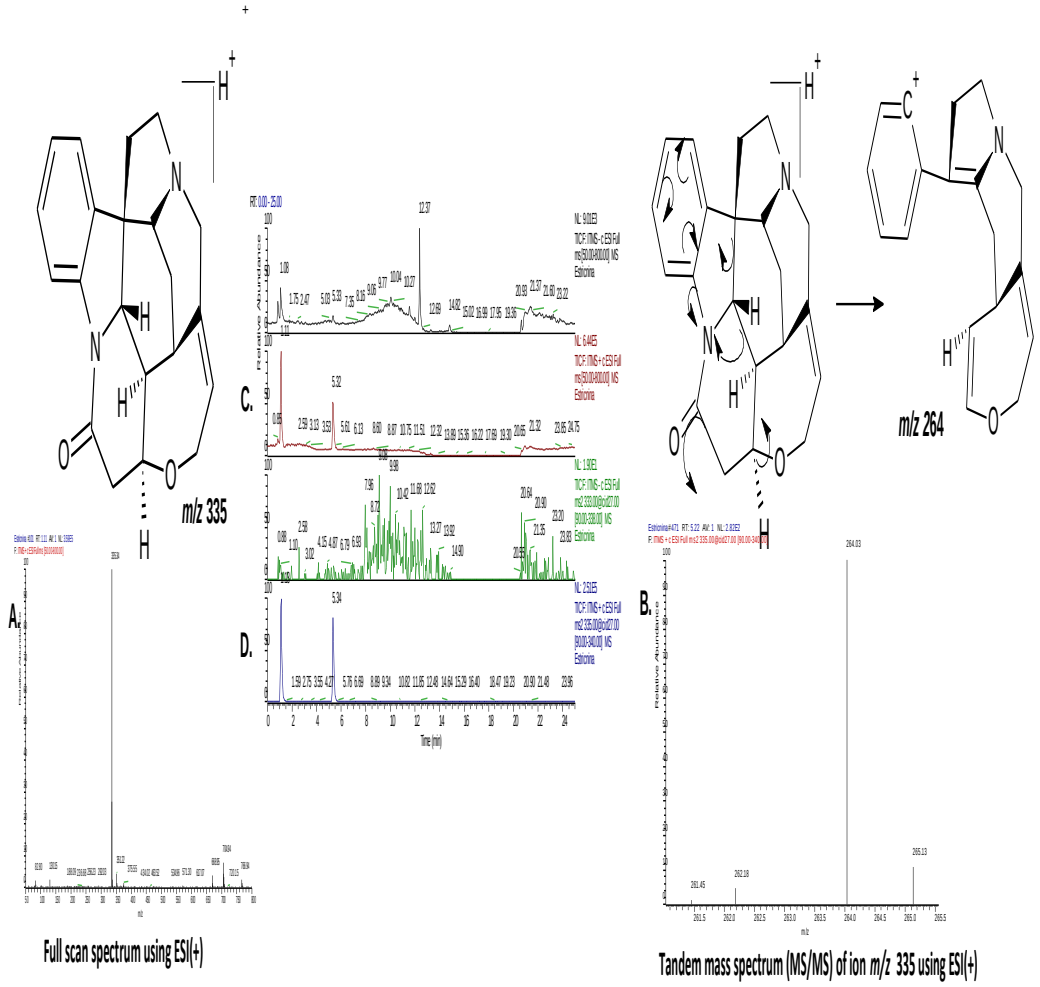


Fig 2.13. Strychnine standard: C Total ion chromatogram obtained in the full scan ESI (+) mode;
D. Extracted ion chromatogram of ion at *m/z* 335, obtained in the ESI (+) mode.
Also shown arethe full scan (A) and MS/MS (B) spectra along with the structure of the main fragment observed.

2.3 IDENTIFICATION OF PESTICIDES IN REAL SAMPLES

Several samples, obtained from LPC/PJ, were analysed using the same chromatographic conditions used for the pesticide's standards. Table 2.2 displays the origin of samples identified and thepesticides identified using this methodology. It should be stressed that samples were obtained after methanol extraction of criminal evidence samples (mostly baits) suspected of being used with the goal of killing animals. These samples have been previously analysed by LC-HRMS-QTOF analysis, which allowed the identification of the multiple pesticides depicted in table 2.2. In the current work we wanted to evaluate if low resolution-based MS analysis could be used as an alternative to the expensive LC- HRMS-QTOF analysis to detect pesticides in forensic samples.

As expected, rodenticides were the class of pesticides mostly identified in the analyzed samplesand the SGAR bromodiolone was the most frequent pesticide found in the group of samples analyzed.,Due to the similarity in the retention time and full scan spectra obtained in the ESI (-) mode with the bromadiolone standard, this rodenticide was identified in the samples B,C,D,E,F,G . However, due to the low concentration of this pesticide in sample H, LC-MS analysis did not allow its detection. Also, weshould highlight the fact that this pesticide was methanol extracted, which is not the most suitable solventfor LLE of rodenticides. Therefore, if rodenticides are suspected, a mixture of water/acetone: dichloromethane (1: 1) was used for extraction.[28] While AR warfarin was identified in the sample I (In the ESI (+) mode), LC-MS did not allow the detection of this rodenticide in sample K, most probably dueto its low concentration. The pesticides imidacloprid and parathion where identified in the samples A and J in the ESI (+) mode, respectively. Whereas flocoumafene was identified by LC-HRMS-QTOF analysis in samples L, M and O, this AR was not identified by LC-MS/MS. This result was not surprising, since the floucomafene standard was not detected under the conditions used in current work.

Table 2.2 Origin of the forensic samples used in the current work and pesticides previouslyidentified by LC-HRMS-ESI and confirmed by LC-MS, in the current work.

Sample	Sample type	Pesticides confirmed by LC-HRMS-QTOF analysis	Pesticides identifiedby LC-MS in the current work	Rt of standard	Rt of Sample	Fig.
A	Meat bait found in a backyard	IMIDACLOR PRIDE (to be confirmed with authentic pesticide standard)	IMIDACLOR PRIDE (to be confirmed withauthentic pesticide standard)		6.5	2.14
B	Bait found in a public Place	Bromadiolone	Bromadiolone	11.1	10.95	2.15
C	Meat bait found in a backyard	Bromadiolone	Bromadiolone	11.1	10.95	2.16
D	Rodenticide bait found in a backyard	Bromadiolone	Bromadiolone	11.1	10.91	2.17
E	Imperial eagle corpse	Bromadiolone	Bromadiolone	11.1	11.14	2.18
F	Claws and Glottis	Bromadiolone	Bromadiolone	11.1	10.95	2.19
G	Canid vomiting	Bromadiolone	Bromadiolone	11.1	11.01	2.20
H	Bait	Bromadiolone	Bromadiolone	11.1	11.16	2.21
I	Royal kite corpse	Warfarin	Warfarin	9.2	9.29	2.22
J	Dog food found in a backyard	Parathion	Parathion	10.5	9.14	2.23
K	Royal kite corpse	Warfarin	----			
L	Imperial eagle corpse: claws and beak	Flocoumafene	--			
M	Imperial eagle corpse: stomach contents	Flocoumafene	--			
O	Imperial eagle corpse	Flocoumafene	---			

Confirmation of the presence of pesticides was obtained upon the analysis of samples by LC- MS/MS of ion corresponding to the deprotonated or protonated molecule of the pesticides in comparisonwith data obtained from standard pesticides, retention time (Rt), preferential ionization mode - ESI (+) or ESI (-) and *m/z* values for the most abundant fragments (obtained in the MS/MS spectra). Figure 2.15 - 2.23 shows the superimposition of LC-MS data of each forensic sample with the corresponding pesticide standard identified.

Despite we could not match any of the LC-MS data parameters of sample A with the one obtained for pesticide standards. It was possible to obtain evidence that suggests the presence of one pesticide. In fact, LC-MS/MS analysis of sample A in the full scan ESI (+) mode, allowed the identificationof ion at *m/z* 256 (with the isotopic pattern expected for a mono-chlorinated molecule). The MS/MS spectrum of this ion clearly shows two fragment ions at *m/z* 175 and *m/z* 209 (this one with the isotopicpattern expected for a monochlorinated molecule). This data is in accordance with the behaviour expected for the insecticide imidacloprid, [27] thereby suggesting the presence of this pesticide in the forensic sample A, analysed. Nonetheless, the confirmation of the presence of this insecticide should only be achieved upon comparison with authentic standard. Fig 2.14. below shows the proposed structures for the fragment ions obtained.

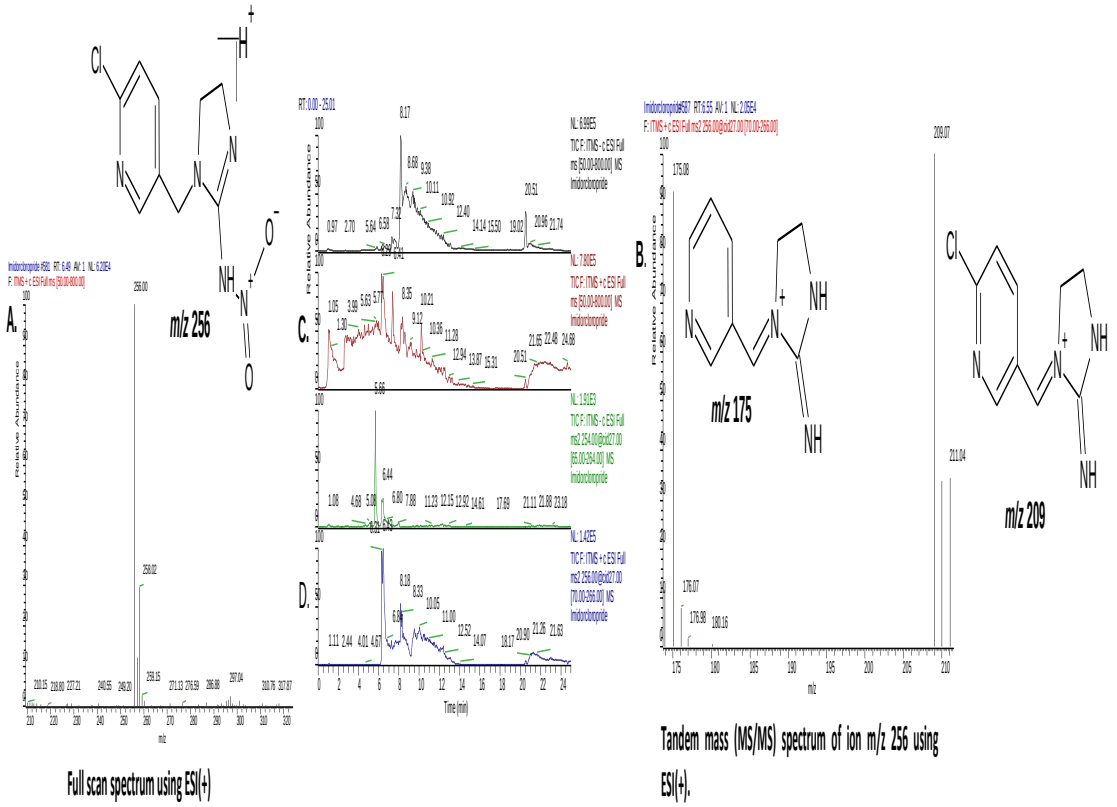
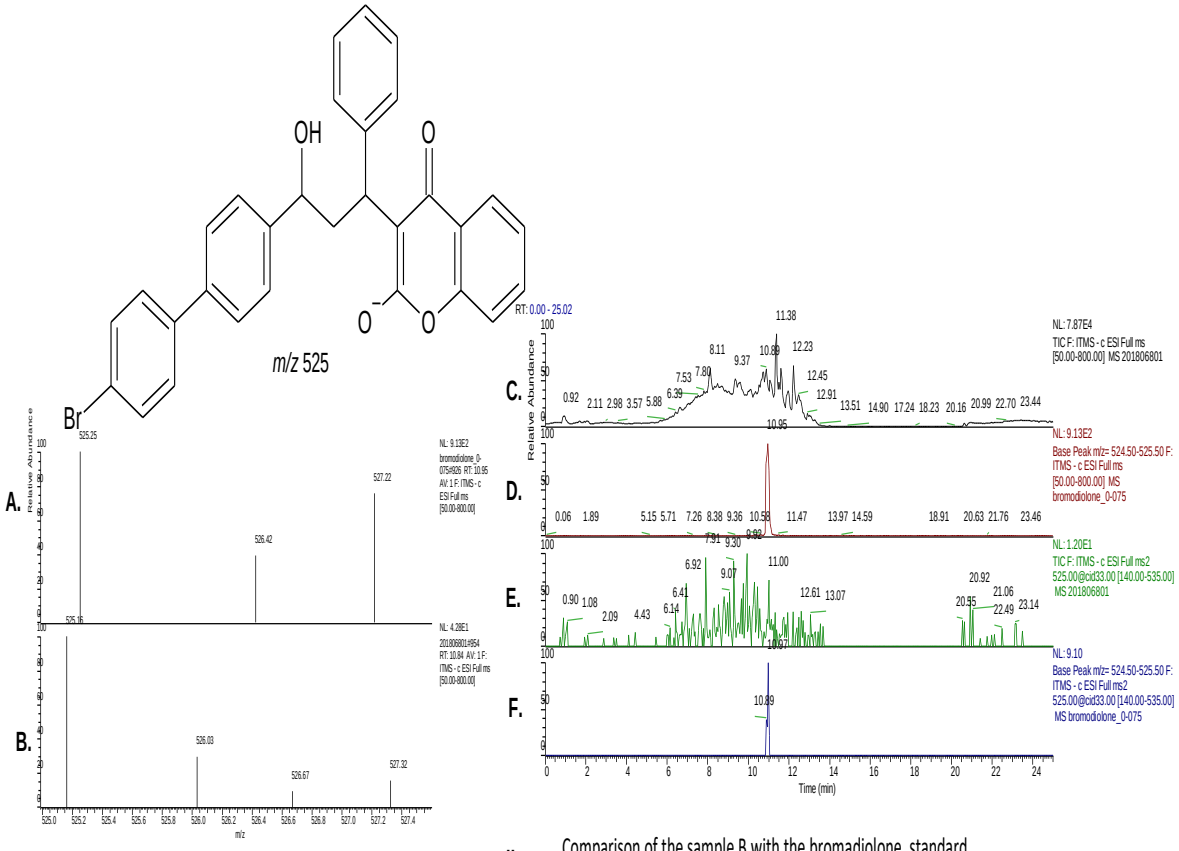


Fig 2.14. Analysis of sample A: C. Total ion chromatogram obtained in the full scan ESI (+) mode; D. Extracted ion chromatogram of ion at *m/z* 461, obtained in the ESI (+) mode. Also shown are the full scan (A) and MS/MS spectra (B) along with the structures of the main fragments observed.

2.3.1 Analysis of Real Samples

E; Total ion chromatogram of standards. **F;** Total ion chromatogram of samples. **G;** Extracted ion chromatogram of standards **H**: Extracted ion chromatogram of samples. Also shown are the superimposition of the full scan spectra for the standards and samples (**A** and **B**) respectively and MS/MS spectra of standards and samples (**C** and **D**) respectively along with the structures of the main fragments observed.

2.3.1.1 SAMPLE B

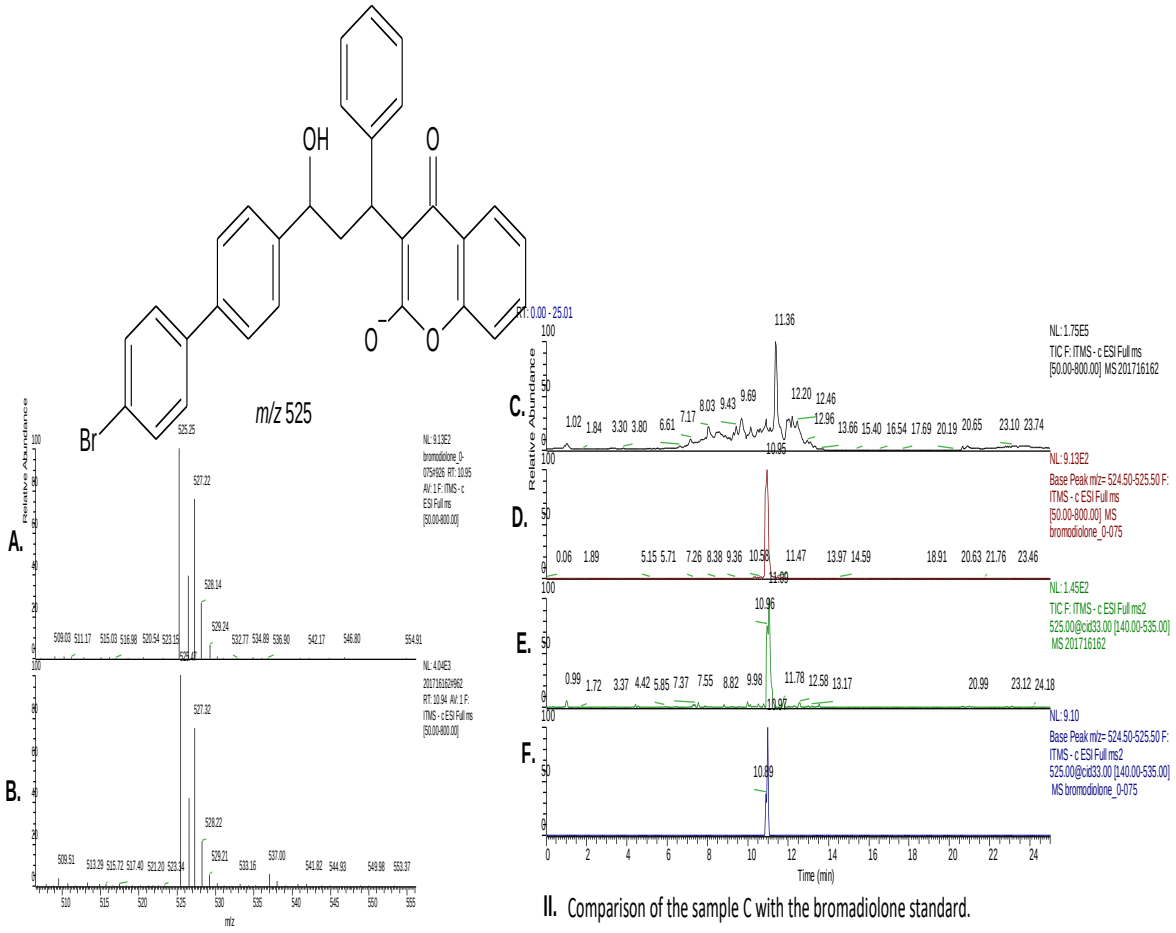


I. Comparison of the full scan spectra of Bromadilone standard (A) and sample B (B)

II. Comparison of the sample B with the bromadiolone standard.

Fig 2.15. Superposition of the full scan spectra of bromadiolone standard (A) with sample B (B).

2.3.1.2 SAMPLE C



I. Comparison of the full scan spectra of Bromadilone (A) and sample C (B)

II. Comparison of the sample C with the bromadiolone standard.

Fig 2.16 Superposition of the full scan spectra of bromadiolone standard (A) with sample C (B).

2.3.1.3 SAMPLE D

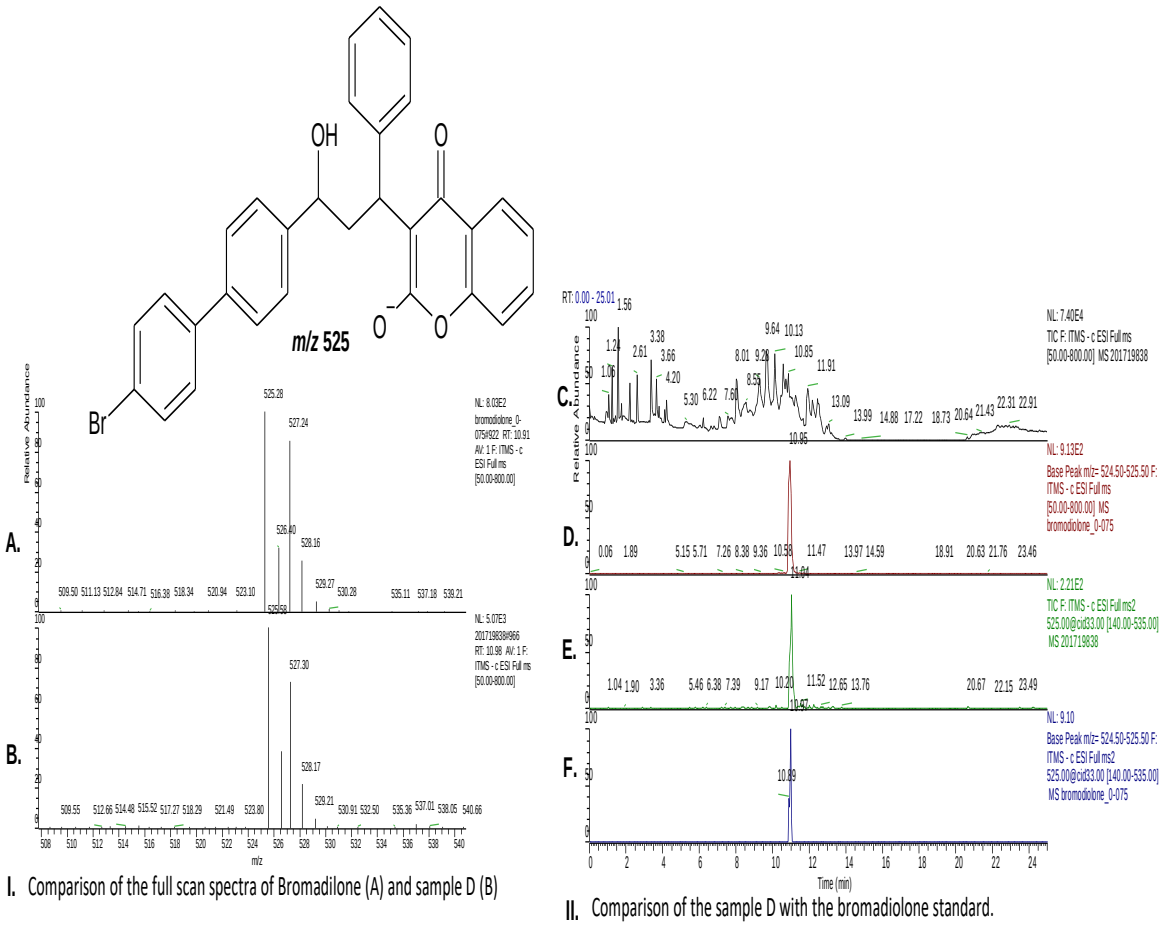


Fig 2.17 Superposition of the full scan spectra of bromadiolone standard (A) with sample D (B).

2.3.1.4 SAMPLE E

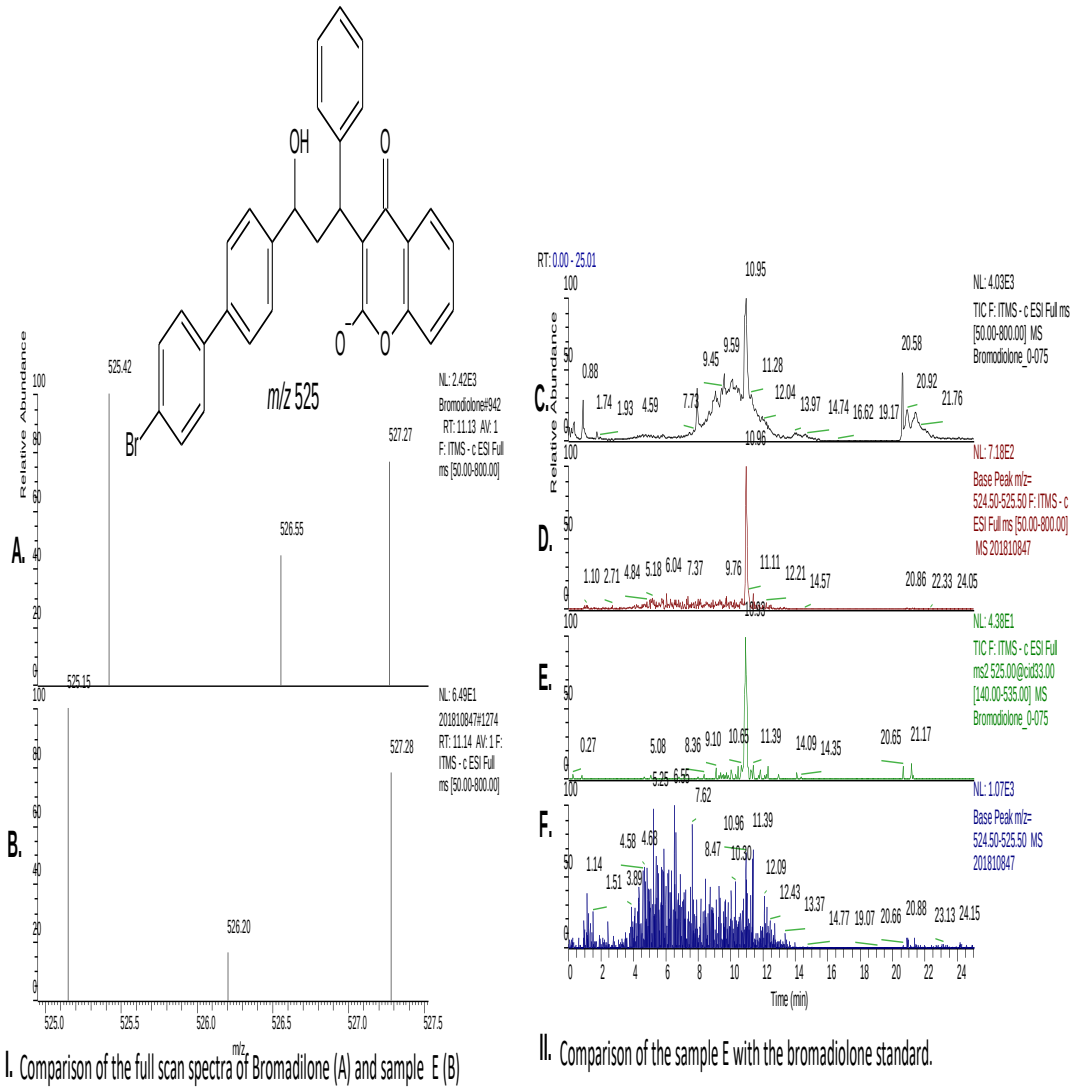
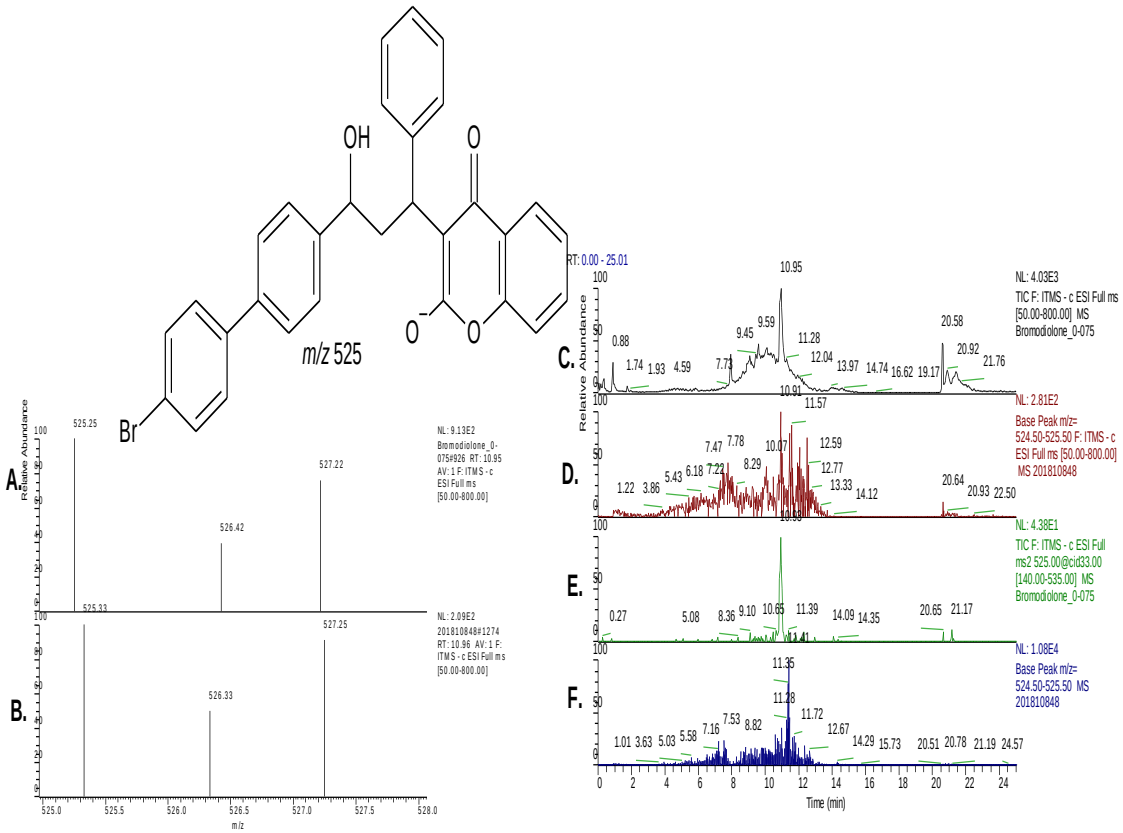


Fig 2.18 Superposition of the full scan spectra of bromadiolone standard (A) with sample E (B).

2.3.1.5 SAMPLE F



I. Comparison of the full scan spectra of Bromadilone standard (A) and sample F (B)

II. Comparison of the sample F with the bromadiolone standard.

Fig 2.19 Superposition of the full scan (A and B) and MS/MS (C and D) spectra of bromadiolone standard with sample F.

2.3.1.6 SAMPLE G

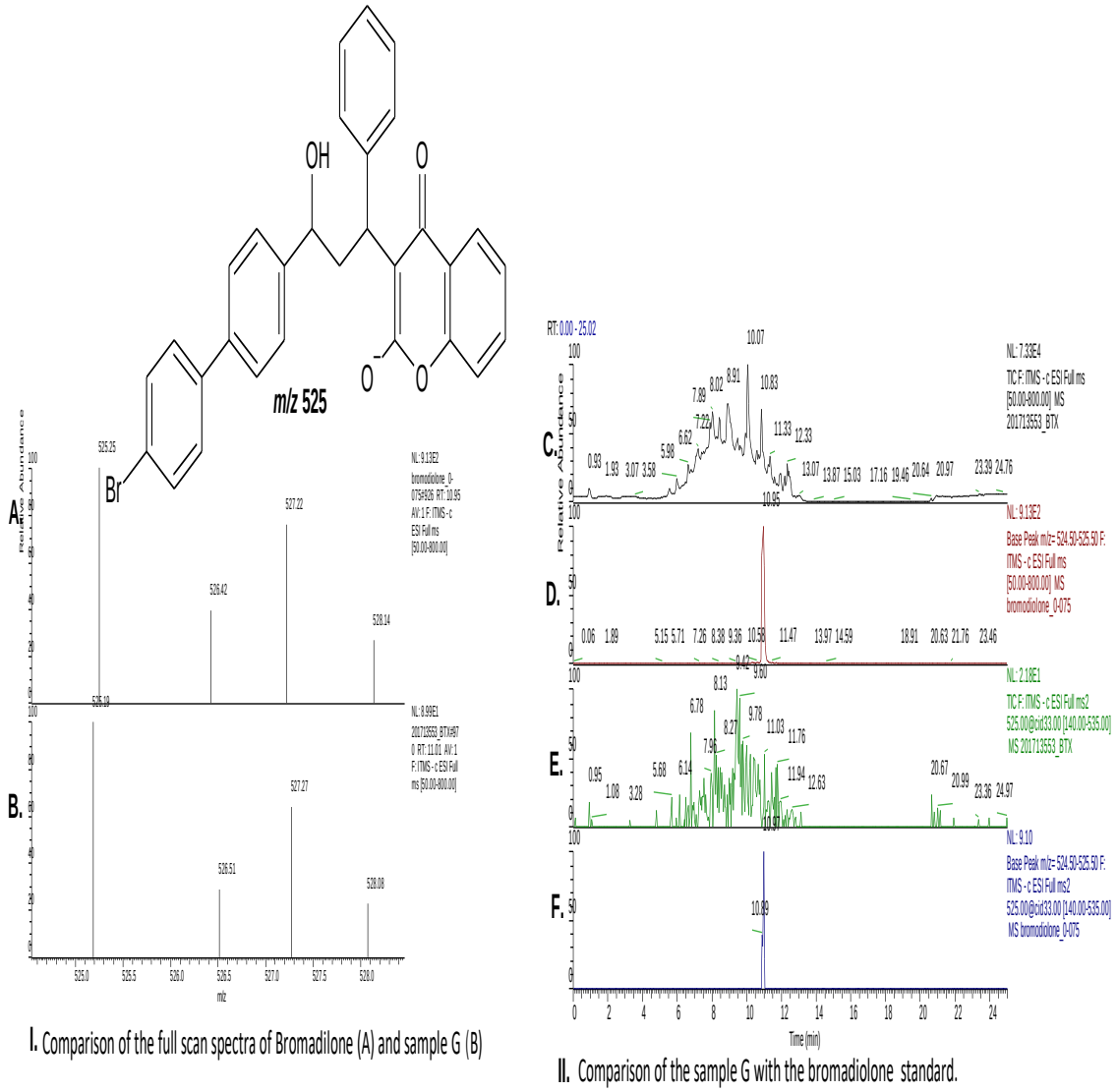


Fig 2.20 Superposition of the full scan spectra of bromadiolone standard (A) with sample G (B).

2.3.1.7 SAMPLE H

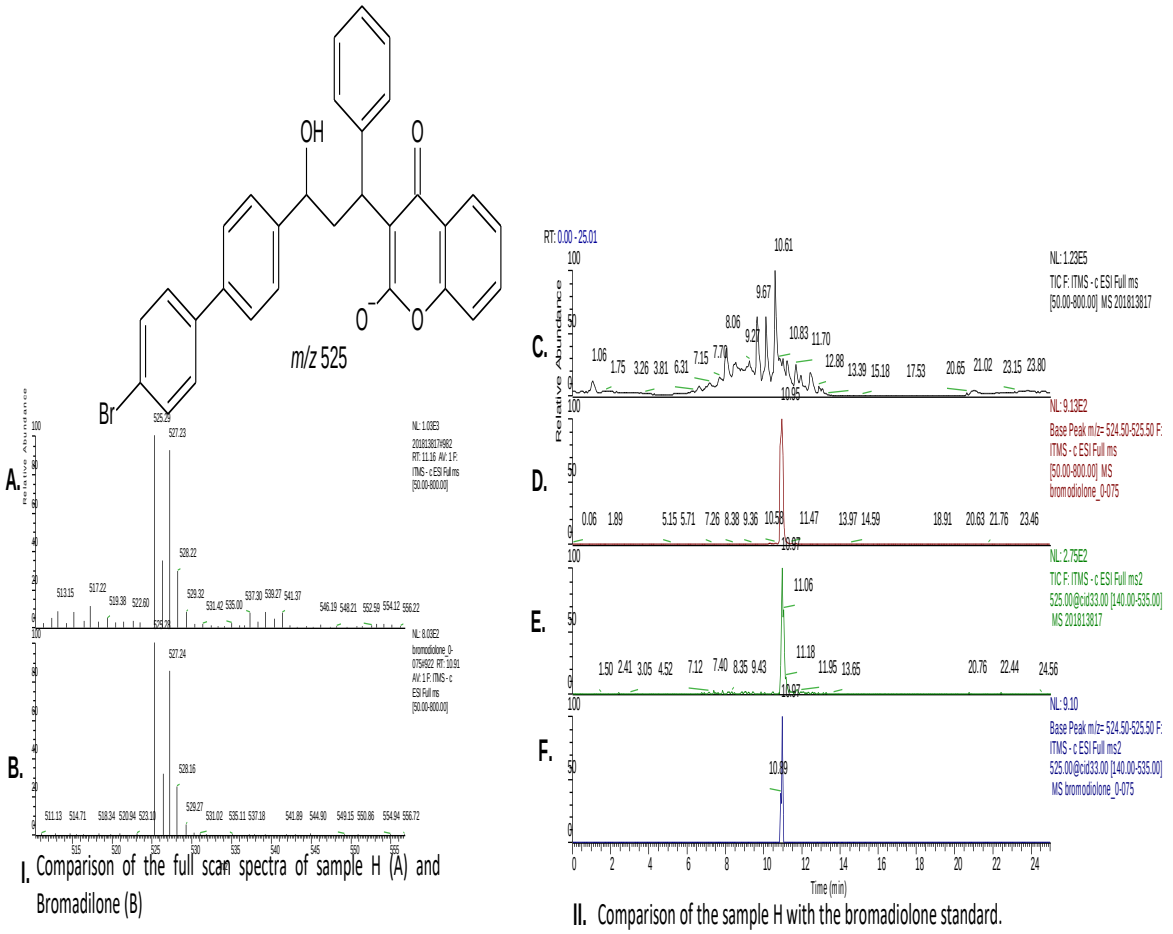


Fig 2.21 Superposition of the full scan spectra of bromadiolone standard (A) with sample H (B).

2.3.1.8 SAMPLE I

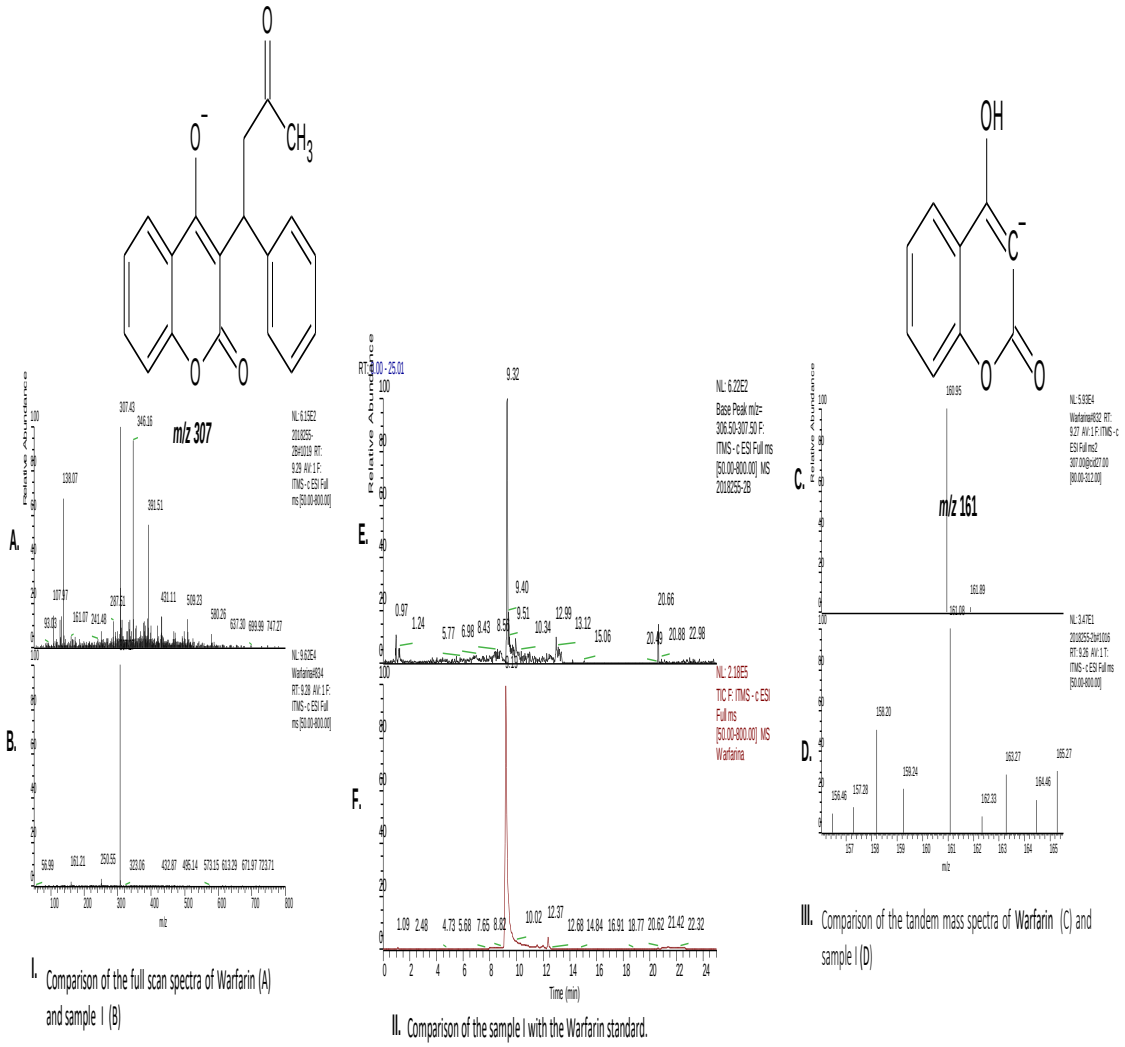


Fig 2.22 Superposition of the full scan (A and B) and MS/MS (C and D) spectra of warfarin standardwith sample I.

2.3.1.9 SAMPLE J

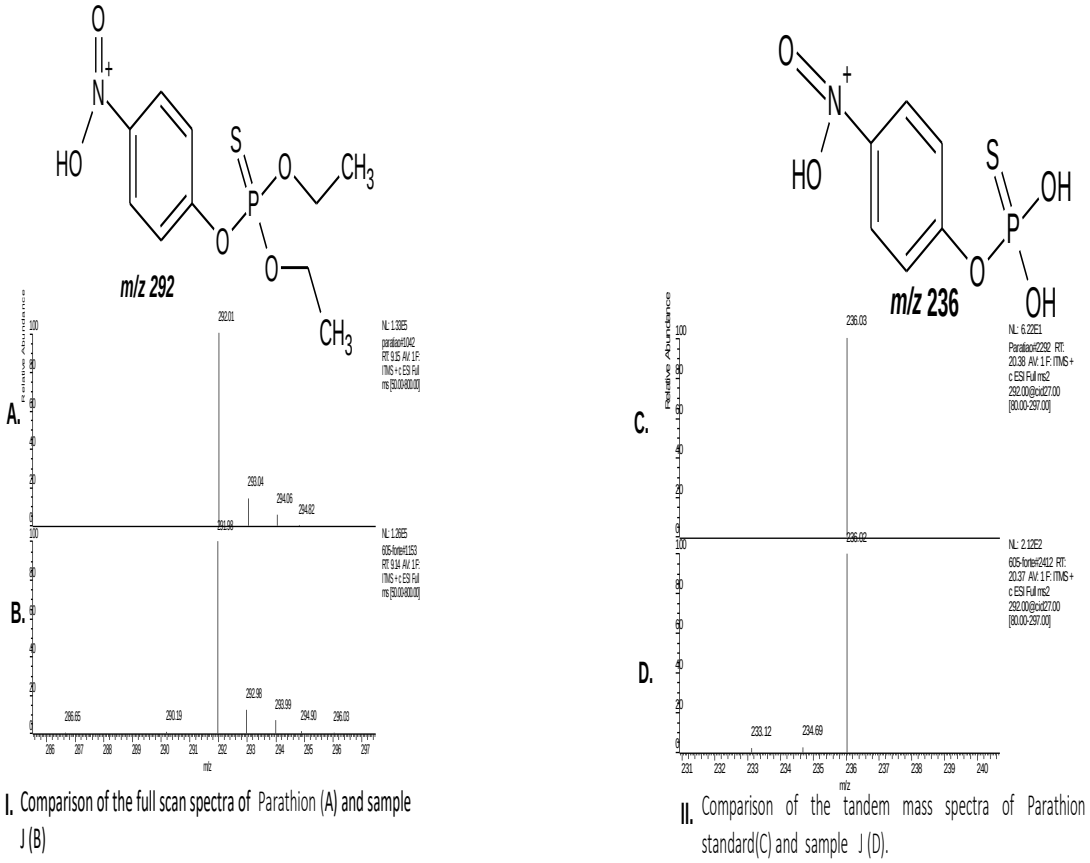


Fig 2.23 Superposition of the full scan (A and B) and MS/MS (C and D) spectra of parathionstandard with sample J.

3. EXPERIMENTAL SECTION

3.1 CHEMICALS AND REAGENTS

Standards of aldicarb, azinphos ethyl, azinphos methyl, bromadiolone, carbofuran, coumatetralyl, deltamethrin, difenacoum, flocoumafen, flocunazole, glyphosate, parathion, strychnine, terramycin, oxytetracycline, warfarin, HPLC-grade acetonitrile, were supplied by either Sigma Aldrich orby the Portuguese police department.

3.2 SAMPLE PREPARATION

We analysed ten (10) samples obtained from different poisoning cases that resulted in the death of animals and submitted by the Portuguese Scientific Police Department to our institution. Methanol was used to extract most of the pesticides from biological or food samples.

3.3 PREPARATION OF STANDARDS

Primary stock solutions of the pesticides were prepared in methanol in the concentration rangeof 0.01–10 mg/mL were obtained by further dilution with methanol.

3.4 LC-MS CONDITION

Chromatographic analysis was performed on a HPLC Dionex Ultimate 3000 system coupled in-line to an LCQ Fleet ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher Scientific, Waltham, MA). Chromatographic separation was performed on a Kinetex polar column (Phenomenex)100A, 100mmx2.1mm,x2.6um at a constant temperature of 30 °C, using an elution gradient of 0.1% formic acid in water or 50mM ammonium acetate in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 200 µL/min, The gradient elution consisted on: 100%of A for 1 min, followed by a linear gradient of 4 min to 80% B, followed by a gradient of 5 min to 100% B, these conditions were maintained for 2 min, followed by 3 min isocratic elution under these conditions.The mass spectrometer was operated in the ESI positive and negative ion modes, with the following optimized parameters: ion spray voltage, ± 4.5 kV; capillary voltage, 16/ 18 V; tube lens offset,70/58 V,sheath gas (N_2), 80 arbitrary units; auxiliary gas (N_2), 5 arbitrary units; capillary temperature, 270°C. Spectra typically corresponded to the average of 20–35 scans, and were recorded in the 100–1000 Da range. Tandem mass spectra MS/MS were obtained with an isolation window of 1 or 6 m/z units; 28– 35% relative collision energy; and with an excitation time of 30 min. Data acquisition and processing were performed using the Xcalibur 2.2 software.

The injection of each sample was followed by the injection of water.

4. CONCLUSION

Pesticides have been employed worldwide for the control of different pests that poses hazard or interference to human activity. Due to the increased potency and subsequently wide range application, some of them have been reported over the years to be employed in the illegal killing of animals, which is considered a criminal act in the European union. Thus, bans and restrictions have been placed on the use or application of these toxic compounds by unauthorized persons. Despite the ban and restrictions on the use of these toxic compounds, there are still current reports on the illegal killing of animals using these chemicals.

In this work, a database of a group of distinct pesticides was constructed based on the retentiontime for each pesticide, data from full scan mode *m/z* (Quasi molecule) of the various compounds and fragment ion tandem mass (MS/MS) spectra. Thus, from the data base, the standards of the compounds analysed were used as reference for the comparison of the samples analysed.

The methodology developed revealed to be suitable for the identification of pesticides (mostly rodenticides) in forensic samples. In fact, we were able to identify pesticides in ten samples provided by LPC/PJ. The rodenticide bromadiolone was identified in seven of the samples. Additionally, the methodology developed enabled the confirmation of the following pesticides; imidacloprid, warfarin, parathion (each in one sample). It is interesting to note that pesticides such as parathion have been banned in Portugal and according to: Decreto-lei nº 48/95 artigo 278[10] any harm caused intentionally or unintentionally to animals is punishable by the law. Regardless of these measures in place the heinous act of illegal killing of animals persist. Thus, stricter regulations should be put in place to controll the use of these toxic compounds even by professional users.

This work shows that the low resolution-based LC-MS, using an ion trap can provide the sensitivity for the identification of some pesticides. The choice of the methodology of extraction can playa key role here. Nonetheless the use of LC-HRMS-QTOF proved to be a more general methodology allowing the identification of more pesticides.

5. REFERENCES

1. European Convention for the Protection of Pet Animals, (https://www.animallaw.info/treaty/european- convention-protection-pet-animals) (accessed jun 29 2020)
2. Chiari, M., et al. (2017) "Pesticide incidence in poisoned baits: A 10-year report" *Science of the Total Environment Journal.* Vol 601-602: pp. 285–292.
3. Luzardo, O.P., et al. (2014) "Methodology for the identification of 117 pesticides commonly involved in the poisoning of wildlife using GC–MS–MS and LC–MS–MS". *Analytical Journal of Toxicology.* Vol 38:pp. 155 –163.
4. Caloni, F., et al. (2016) *"*Suspected poisoning of domestic animals by pesticides*". Science of the Total Environment Journal.* Vol 539: pp.331-336
5. UTZ (2015) " List of banned pesticides and pesticides watchlist" https://utz.org/wp-content/uploads/2015/12/EN_UTZ_List-of-Banned-PesticidesWatchlist_v1.0_2015.pdf (acessed jun 292020).
6. Teixeira H., et al. (2004) "Pesticide intoxications in the Centre of Portugal: three years analysis".
   Forensic Science International. Vol 143*:* pp 199–204.
7. Belas A ., et al. (2010) "Wildlife pesticide poisoning in Portugal: Retrospective analytical results",
   Toxicology letters. Vol 196: pp.318
8. The Portugal News (2019) "Banned pesticides found in country's groundwater".https://www.theportugalnews.com/news/banned-pesticides-found-in-countrys-groundwater/49513 (accessed jun 30 2020).
9. Gupta R.C., et al. (2013) "Agricultural Chemicals *" Haschek and Rousseaux's Handbook of ToxicologicPathology,* Ch.42, pp. 1364. Third Edition OH, USA: Vet Path Services, Inc., http://dx.doi.org/10.1016/B978-0-12-415759-0.00042-X
10. Decree-Law nº 48/95 (1995) "CRIMINAL CODE" BOOK II - Special part
    TITLE IV - Crimes against life in society Chapter III - Of crimes of common danger. Article 278 - Damage to nature
11. Koli, P., et al. (2019) "Agrochemicals: Harmful and Beneficial Effects of Climate Changing Scenarios" Climate change and Agricultural ecosystem. Ch. 4, pp 65-96. https://doi.org/10.1016/B978-0-12-816483-9.00004-9
12. Lohr M. T., et al. (2018) "Anticoagulant rodenticide use, non-target impacts and regulation: A case studyfrom Australia" *journal of Science of the Total Environment*. Vol 634: pp. 1372–1384.
13. Kostakis C., et al. (2013) "Liquid Chromatography-Mass Spectrometry in Forensic Toxicology" *ForensicToxicology*. Ch. 10, pp.255-266. Bedford Park, Australia.
14. Seljetun, K.O., et al. (2018) *"Quantitative method for analysis of six anticoagulant rodenticides in faeces,applied in a case with repeated samples from a dog"* Vol 60: pp 3-11. Department of Companion Animal Clinical Sciences, Faculty of Veterinary

Medicine, Norwegian University of Life Sciences (NMBU, Norway.

15. Kotthoff, M., et al. (2019) “First evidence of anticoagulant rodenticides in fish and suspended particulate matter: spatial and temporal distribution in German freshwater aquatic systems”. *Journal of Environmental Science and Pollution Research*. Vol 113: pp.7315–7325 https://doi.org/10.1007/s11356-018-1385-8
16. Wittrig, B., et al. (2011) *“Comprehensive Pesticide Residue Analysis by LC/MS/MS”*. Journal of Foods, Flavors & Fragrances. https://www.restek.com/pdfs/GNAD1535-UNV.pdf (accessed jun 30 2020)
17. Barbieri M.V., et al. (2019) “Analysis of 52 pesticides in fresh fish muscle by QuEChERS extraction followed by LC-MS/MS determination” journal of Science of the Total Environment. Vol 653 . 958–967 https://doi.org/10.1016/j.scitotenv.2018.10.289
18. Imran, M., et al. (2015) “Analytical methods for determination of anticoagulant rodenticides in biologicalsamples”. *Forensic Science International journal.* Vol 253: pp. 94-102.
19. Berton T., et al., (2014) “Development of an analytical strategy based on LC–MS/MS for the measurement of different classes of pesticides and their metabolites in meconium: Application and characterisation of foetal exposure in France” *Environmental Research journal.* Vol 132: pp. 311–320.
20. Meiser, H., (2005) “Detection of Anticoagulant Residues by a New HPLC Method in Specimens of Poisoned Animals and a Poison Control Case Study” *Journal of analytical toxicology.* Vol. 29: pp 556- 562
21. Sniegocki,T., *et al.,* (2019). “The Usefulness of MS^3 to Confirm Poisoning on the Example of Dog Poisoning with Strychnine” M*olecules.* Vol 24: pp. 1-8.
22. John H., et al., (2008). “LC-MS-based procedures for monitoring of toxic organophosphorus compounds and verification of pesticide and nerve agent poisoning”. *Analytical and Bioanalytical Chemistry*. Vol 391:pp. 97–116.
23. El-Aneed, A., et al. (2009) “Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers” *Applied Spectroscopy Reviews*. Vol 44: Pp. 210-230
24. Premier Biosoft Accelerating Research in life science. http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html (acessed Nov. 12. 2020)
25. Magnetic field laboratory. https://nationalmaglab.org/user-facilities/icr/techniques/tandem-ms(accessed jul 1 2020)
26. Globenewswire,https://www.globenewswire.com/news-release/2017/05/12/1113311/0/en/Kinetex-R- Polar-C18-Core-Shell-Columns-Deliver-High-Retention-and-Aqueous-Stability.html (accessed jul 12020)
27. Spectrometry, M., *et al.* (2010) “Determination of Imidacloprid in Paddy Water and Soil by LiquidChromatography Electrospray Ionization Tandem,” vol. 65, no. 8, pp. 843–847.

28. Adamowicz, P., et al. (2005) “Simple hplc method for the identification of the most commonly usedrodenticides in Poland,” *Problems of Forensic Sciences.* Vol 64: pp. 373–381.

29. Gómez-Canela C., *et al.* (2014) “Comprehensive characterization of rodenticides in wastewater by liquid chromatography-tandem mass spectrometry” *Analytical and Bioanalytical Chemistry*. Vol 406: pp.345-358.