INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO
MESTRADO EM ENGENHARIA QUÍMICA




Avaliação da Segurança de Produtos Cosméticos: Pesquisa de Contaminantes em Extractos Usados como Ingredientes Ativos

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POLITÉCNICO
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Abstract

The increasing use of cosmetics by the population around the world, particularly in developed countries, has led to a huge demand for new ingredients obtained from natural sources due to their richness in bioactive compounds associated with skin benefits as well as new concerns related with the environment. This is particularly the case of extracts obtained from the shell of chestnut (*Castanea sativa*) shells that have been investigated as a new potential cosmetic ingredient. This by-product results from chestnut production and presents a bioactive composition rich in antioxidant compounds (such as catechin or gallic acid), which are of great interest for the cosmetic field. However, it is fundamental to guarantee that the extracts coming from shells, or from any other food by-product, are safe for consumers. Therefore, the principal goal of this work was to monitor the presence of some compounds present in the Regulation (EC) nº 1223/2009, concerning cosmetic products, in chestnut shells and chestnut shells extracts obtained by green extraction techniques (ultrasound-assisted extraction (UAE) and subcritical water extraction (SWE)).

Considering this, the presence of organochlorines (OCPs), pyrethroids (PYRs) and organophosphorus (OPPs) pesticides families was evaluated in the chestnut shells as well as in the SWE and UAE extracts. For the analysis in the chestnut shells, the QuEChERS analytical method for contaminant extraction was developed and optimized. The optimization of this method was done in the cleaning step, where different sorbent combinations were tested, namely Cleanup 1 (CL1), Cleanup 2 (CL2) and Cleanup 3 (CL3). After analysing the recovery values and the matrix effect obtained when used the different combinations, it was concluded that CL2 offers better results for the OCPs and PYRs studies, while CL3 lids to better results for the OPPs. After validation, this method was applied to real samples and the obtained extracts were injected in the pesticide detection equipment, namely gas chromatograph with electron capture detector and gas chromatograph with the photometric flame detector. Although some compounds were detected, part of them were in concentrations below the limit of detection and, therefore, were considered as not detectable. The others, despite being present in concentrations between the limits of detection and quantification or even higher, are below the maximum residue limits defined by the European Union for chestnuts. Finally, the analysis of the extracts from the chestnut shells was performed. To prepare samples of these extracts, for subsequent analysis in gas chromatography, the solid-phase extraction method was used. Contrary to what was done regarding QuEChERS method, this methodology did not undergo any optimisation and consequent validation, since the analysis of these liquid extracts served only as a confirmation of the results obtained for the chestnut shells. Nevertheless, it should have

been done in order to ensure that the method applied was the most appropriate for this purpose. This work allows to conclude that, partly similar to what was obtained for the chestnut shells, the UAE and SWE chestnuts extracts are free of pesticides, namely OCPs, PYRs and OPPs, thus being safe for a possible application in cosmetic products.

Keywords: Organochlorines; Pyrethroids; Organophosphates; QuEChERS; Solid-phase extraction; Gas Chromatography; Chestnut shell; Cosmetic Industry

Resumo

Associado ao crescente consumo de produtos cosméticos e de higiene, tem-se verificado um aumento da procura de produtos de origem natural, comumente associados a diversos benefícios, quer para a saúde do consumidor, quer para o meio ambiente. Com efeito, a indústria cosmética tem vindo a expandir este campo de pesquisa e inovação, adotando o uso de extratos com origem natural como ingredientes ativos. Um dos extratos com potencial para vir a ser incorporado em produtos cosméticos é proveniente da casca da castanha. Este subproduto, resultante da produção da castanha, apresenta uma composição bioativa rica em compostos antioxidantes, os quais se revestem de interesse para a indústria cosmética devido aos seus efeitos ao nível da pele (a título exemplificado, a ação antienvelhecimento). No entanto, é fundamental garantir que os extratos provenientes de subprodutos como a casca da castanha, são seguros e, nesse sentido, desenvolveu-se o presente trabalho que assentou na monitorização de alguns compostos presentes no Regulamento (CE) nº 1223/2009, relativo aos produtos cosméticos, na casca da castanha e em extratos da casca de castanha obtidos através de técnicas verdes (extração assistida por ultra-sons (UAE, do inglês *ultrasound-assisted extraction*) e extracção subcrítica de água (SWE, do inglês *subcritical water extraction*))

Nesta dissertação, avaliou-se a presença de pesticidas da família dos organoclorados (OCPs, do inglês *organochlorines pesticides*), piretróides (PYRs, do inglês *pyrethroids*) e organofosforados (OPPs, do inglês *organophosphorus pesticides*) na casca da castanha e, posteriormente, nos extratos obtidos através de SWE e UAE a partir da mesma. Para a análise na casca da castanha, desenvolveu-se e otimizou-se o método analítico QuEChERS para a extração de pesticidas. A otimização deste método foi feita na etapa de limpeza, onde foram testadas diferentes combinações de sorbentes: *Cleanup* 1 (CL1), *Cleanup* 2 (CL2) e *Cleanup* 3 (CL3). Após analisados os valores de recuperação e o efeito matriz obtido quando utilizadas as diferentes combinações, concluiu-se que, para o estudo dos OCPs e PYRs, o CL2 oferece os melhores resultados, enquanto para os OPPs é o CL3. Após validação, este método foi aplicado em amostras reais e os extratos obtidos injetados nos equipamentos de deteção de pesticidas: cromatógrafo gasoso com um detetor de captura de eletrões e cromatógrafo gasoso com um detetor fotométrico de chama. Apesar de terem sido detetados alguns compostos, parte destes apresentava-se em concentrações inferiores ao limite de deteção e, por isso, foram considerados como não detetáveis. Os restantes, apesar de estarem presentes em concentrações entre os limites de deteção e de quantificação ou até superiores, estão abaixo dos limites máximos de resíduos definidos pela União Europeia para as castanhas. Por fim, foi feita a análise dos extratos provenientes da casca de castanha. Para a preparação de amostras destes extratos, para

posterior análise em cromatografia gasosa, utilizou-se o método de extração em fase sólida. Contrariamente ao que foi feito com o método de QuEChERS, este método não sofreu qualquer otimização e consequente validação, uma vez que a análise a estes extratos líquidos serviu apenas como confirmação dos resultados obtidos para a casca de castanha. Deste modo, concluiu-se que, em parte semelhante ao que foi obtido para a casca de castanha, os extratos provenientes desta estão isentos de pesticidas (OCPs, PYRs e OPPs), sendo assim seguros para uma possível utilização em produtos cosméticos.

Palavras-chave: Organoclorados; Piretróides; Organofosforados; QuEChERS; Extração em fase sólida; Cromatografia Gasosa; Casca da castanha; Indústria da Cosmética

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List of Abbreviations

CE	Catechin Equivalents
CL1	Cleanup 1
CL2	Cleanup 2
CL3	Cleanup 3
d-SPE	Dispersive Solid-Phase Extraction
d.w	Dry weight
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
End	Endossulfan
EU	European Union
fw	Fresh weight
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
GC-ECD	Gas Chromatography with Electron Capture Detector
GC-FPD	Gas Chromatography with Flame Photometric Detector
GC-MSD	Gas Chromatography with Mass Selective Detector
GLC	Gas-Liquid Chromatography
GSC	Gas-Solid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAE	Microwave Assisted Extraction
MRL	Maximum Residue Limit
MS	Mass Spectrometry
OCP	Organochlorine Pesticide
OPP	Organophosphorus Pesticide
PLE	Pressurized Liquid Extraction
POPs	Persistent Organic Pollutants
PYR	Pyrethroid
QuEChERS	Quick, Easy, Cheap, Effective, Robust and Safe
SFE	Supercritical Fluid Extraction
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
SWE	Subcritical Water Extraction

UAE	Ultrasound-Assisted Extraction

List of Compounds Abbreviations

ACN	Acetonitrile
C18	Octadecyl silica
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
IS	Internal Standard
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
$MgSO_4$	Magnesium sulfate
PSA	Primary Secondary Amine
S_2	Disulfur
TPP	Triphenyllphosphate

List of Symbols

$\overline{[Analyte]}$	Analyte concentration
m	Slope of the linear regression
R^2	Correlation coefficient
Rt	Retention time
RSD	Relative standard deviation
S	Absolute standard deviation
$S_{y/x}$	Standard deviation of linear regression
$\bar{X}$	Sample mean

1 Introduction

1.1 Framing and description of the project

The consumption of cosmetic and body hygiene products has grown exponentially in recent times, being crucial to ensure that the substances used in their production are safe and do not compromise or endanger public health. To control the presence of such substances, there are regulations whose European Union (EU) marketing rules have been defined to impose restrictions on the quantities allowed or, in the limit, to forbid their presence. The main regulatory framework for newly marketed cosmetics in the EU is Regulation (EC) nº 1223/2009 [1].

In the range of active ingredients used in cosmetic products, it is important to highlight extracts obtained from natural sources which are being used more often. There are a lot of benefits, however, being these natural products and mainly produced by intensive agriculture, pesticides are used during production, which may result in the contamination of the final extracts. In this sense, it becomes very important the evaluation of the chemical safety of the extracts obtained from natural products before their incorporation into cosmetic products, since many of the used contaminants are on the list of substances banned by the EU.

In parallel, the raising of the concern with the environment has promoted the use and valorisation of industrial and agricultural wastes by industries [2]. Having this concern as one of the reasons for the application of environmentally friendly measures, this work was developed considering the concerns of valorise non-toxic wastes in the production of cosmetics.

From the production of several food products, there are a lot of by-products that are considered as waste and are often discarded, as in the case of the chestnut shell. In order to generate a lower environmental impact, it is essential to understand what benefits are associated with these wastes and, subsequently, to develop processes capable of converting them into a product with a higher added value [2].

In this sense, the present work was developed based on the above-mentioned beliefs: to associate the advantages of cosmetics production using extracts of safe and natural products to the environmental advantages of offering a new use to what would be discarded beforehand.

Thus, since the chestnut shell is an agricultural waste with a rich composition in bioactive compounds of great interest for the cosmetic field, a natural extract from this by-product was used.

Consequently, the main objectives associated with this project are: (i) monitor some compounds present in the EU regulation (Regulation (EC) nº 1223/2009, concerning cosmetic products) in order to ensure that the extracts obtained from the chestnut shell are safe for future applications in the cosmetic area; (ii) develop and optimize robust analytical methods for extraction and analysis of contaminants in extracts obtained from the chestnut shell and (iii) applicate the techniques developed in the extracts under study in order to control the presence of different contaminants.

1.2 Hosting Institution

Grupo de Reação e Análises Químicas (GRAQ) is a research group founded in January 1999. In its initial phase, it was a research group of the Instituto Superior de Engenharia do Porto (ISEP) which was integrated in CEQUP (Centro de Química da Universidade do Porto) and had financial and scientific autonomy [3]. Since November 2001, this research group, whose mission is to research in the field of Chemical Engineering and Green Chemistry, belongs to REQUIMTE (Rede química e tecnologia), the largest Chemistry and Chemical Engineering organization in Portugal, recognized as an Associated Laboratory for Green Chemistry by the Portuguese Ministry of Science and Higher Education [3].

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rede de química e tecnologia

1.3 Dissertation structure

The dissertation is divided into 5 chapters, which are briefly described as follows:

- **Introduction:** framing and presentation of the project, as well as the presentation of the institution where it was developed.
- **State of the Art:** an approach to subjects that allow understanding and framing of the theme developed.
- **Materials and Methods:** detailed description of the techniques used for the evaluation of the presence of pesticides in the chestnut shell and in the extracts obtained from it. A description of all reagents and solutions used is also done.

- **Results and discussion:** description of the results obtained from the processes described in the previous chapter and discussion of them.
- **Conclusions and suggestions for future work:** overall appreciation of the work carried out and possible suggestions for future developments

2 State of the art

This section will address some concepts and theoretical foundations necessary for the framework and better understanding of this project. Firstly, a short presentation of the cosmetics industry is made, where a brief reference of the global cosmetic ingredients market and an analysis of the cosmetic market at European level regarding natural cosmetic products are made. Next, an approach to chestnut production is described, where the data of its production at a worldwide level are displayed. Within this section, the intensive and organic cultivation systems are discussed and the by-products resulting from the chestnut production are addressed, focusing on their composition, particularly the antioxidant compounds (phenolics) reported for the *Castanea sativa* Mill shell. The methods of extraction of bioactive compounds from the chestnut and its by-products are also explored and, finally, an approach is made to the pesticide residue contamination of the chestnut and its by-products, where topics such as pesticide classification and extraction and detection methods of these compounds are included.

2.1 Cosmetic industry

The cosmetics industry is an area that has been growing over the last few years and has shown signs that this trend is set to continue. If one analyses the global cosmetic ingredients market, it was valued in 2016 at $21.85 billion and is expected, according to a report published by Zion Market Research, to reach approximately $31.80 billion by 2023. The ingredients highlighted in that report have had extensive application in skin care, hair care, oral care and makeup products. If an analysis is done by region, it is observed that Europe in 2019 held the largest share of this market, amounting to 30%, followed soon by North America and Asia [4][5].

It is well known that, nowadays, industries are increasingly sensitive to sustainability issues, seeking for solutions to minimize their environmental impact. The cosmetics industry is one of these industries and has been working in this direction, increasing the demand for new ingredients from natural sources, associating environmental issues with innovation [6]. Moreover, consumer demand for products based on natural compounds is growing, as they consider them safer for human health [7]. Besides that, the level of environmental awareness is increasing [7]. All this means that the area of cosmetics where organic/natural compounds are used is growing, and when we analyse the European market for natural cosmetics this is exactly what is seen. The cosmetic market has been growing over the last few years, faster even than

the global cosmetics market itself, being valued in 2018 at €3.6 billion. In Figure 2.1 the growth rates of the European natural cosmetics market are represented [7].

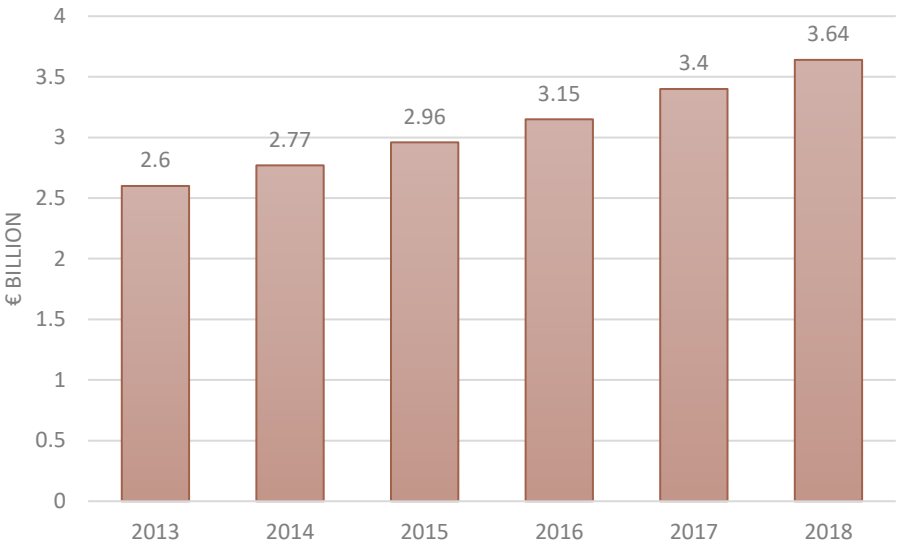


Figure 2.1 Growth Rates for the European Market for Natural Cosmetics [8].

Within the European market, Germany, France, Italy and the United Kingdom, in 2018, were the countries with the largest market shares, having 35%, 20%, 12% and 10%, respectively, as shown in Figure 2.2. Countries such as Switzerland and Greece had a lower percentage in comparison with the previously mentioned countries, with a value around 3% in both cases. The remaining European countries have about 17% of the market [7].

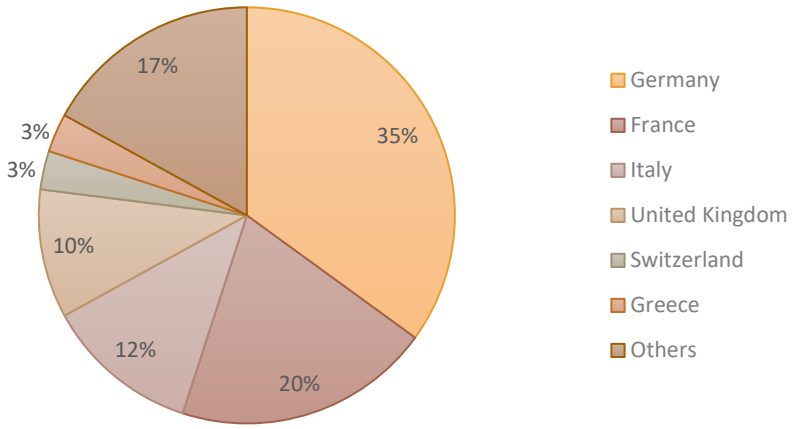


Figure 2.2 European natural cosmetics market by country (2018) [9].

Germany and France are not only the largest producers of cosmetic products based on organic compounds, but also the countries with the highest consumption, which makes them the markets that offer the best opportunities in this area [7].

Due to all the reasons mentioned above, this industry has been investing natural extracts as antioxidant sources, contributing to the advancement of this area as well as bringing several environmental and economic benefits. The employment of extracts from, for example, *Castanea sativa* Mill by-products, which are rich in bioactive compounds, is an added value for this field [10]. However, the use of products from natural sources requires deep attention to ensure the consumer safety, guaranteeing that there are no contaminants from agriculture and other sources.

2.2 Chestnut production

Castanea sativa Mill, commonly known as chestnut, is a species that belongs to the Fagaceae family. This kind of species are widely present in the south part of Europe (in countries like Portugal and Greece) and Asia. Chestnut cultivation has a long tradition in Europe, since it is a tree species suitable for timber and fruit productions. As a result of its many applications, sweet chestnut has a huge economic and environmental importance [11].

Chestnut can be divided into three distinct parts, namely fruit, pericarp, and integument (outer and inner shell). Its production process has three general steps: (1) calibration, (2) peeling of the outer shell in a high-temperature process and (3) removal of the inner shell using steam and mechanical processes [11]. From these production processes, several by-products are generated, which have the advantage of being a valuable source of bioactive molecules [12]. Considering this, and the fact that waste valorisation is associated with economic and environmental benefits, numerous studies have been developed for this purpose [13].

2.2.1 Worldwide chestnut production

According to the Food and Agriculture Organization of the United Nations Statistics (FAOSTAT), from 2000 to 2018, the chestnut farming and production increased from 940,911k tonnes to 2,353,825k tonnes and, consequently, the chestnut by-products generated [14]. Analysing the data provided by FAO, shown in Figure 2.3, it is possible to conclude that Asia is the world's major chestnut producer (88.6%), followed by Europe (7.7%) and America (3.7%) [14].

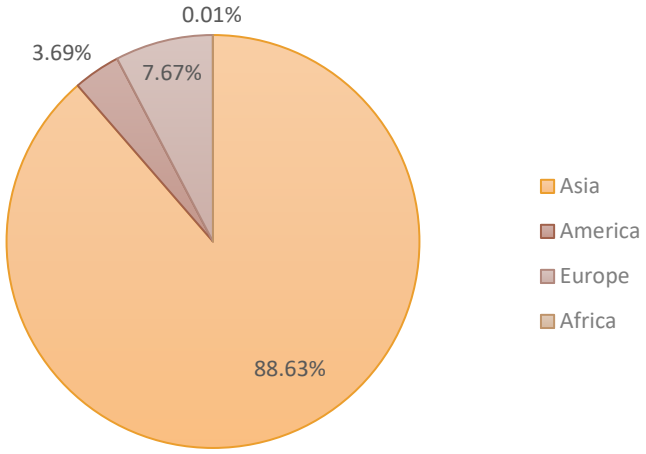


Figure 2.3 Production share of Chestnut by region [14].

In 2018, China produced over 1965,000 tonnes of chestnuts, being the highest producer country worldwide, followed by Bolivia, Turkey, Italy, Republic of Korea, Italy, Greece, Portugal, Japan, Spain and Democratic People's Republic of Korea [14]. Figure 2.4 shows the top 10 chestnut world producers.

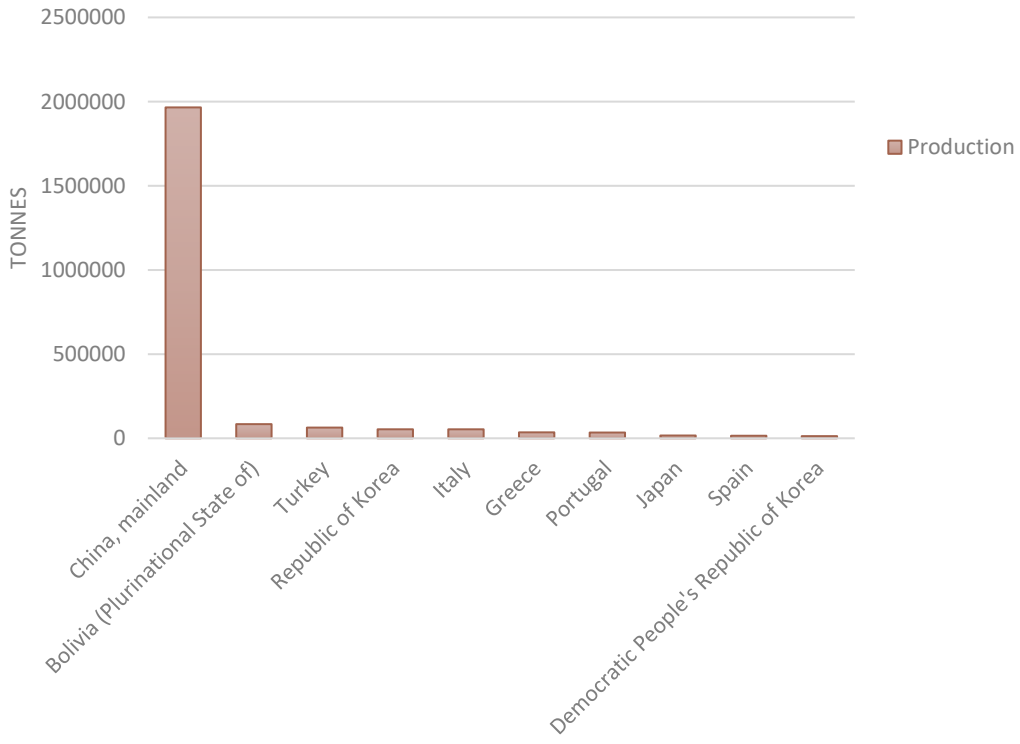


Figure 2.4 Production of Chestnut, top 10 producers [14].

2.2.2 Chestnut cultivation: Intensive and Organic agriculture

With the evolution of society, agriculture has increased its production in all sectors, including the chestnut one. This fact has been achieved due to the intensive agriculture, a system characterised by the intensive use of inputs, labour and technology and also by several positive points, of which can be highlighted the high crop yields, greater food variety, higher efficiency and affordable food prices [15]. However, this practice also has some allied downsides, including intensive use of natural resources and an exponential increase in pollution. Intensive agriculture is the main contributor to greenhouse gases, biodiversity loss, soil degradation (continuous cultivation) and agrochemical pollution [16]. Regarding agrochemicals, pesticides are the most widely used chemicals in the world, being particularly dangerous to the human health and the environment. These compounds have an extremely negative effect on the human body when consumed in large quantities, leading to different diseases, including cancer and, in the worst case, death [17].

The challenge of feeding a growing population, which is expected to reach 9-10 billion by 2050, and simultaneously protecting the environment, is a task that will have to be overcome sooner or later. The adoption of large-scale sustainable organic farming systems is a good alternative to ensure food and ecosystem security [18]. This type of agriculture combines traditional conservation farming methods with modern technologies, promoting soil quality, crop rotations, animal and plant diversity, biological processes, and animal welfare. Simultaneously, irradiation, sewage sludge, genetic engineering and the use of chemicals, as synthetic pesticides, and fertilizers are generally prohibited [18].

2.2.3 Chestnut by-products

As already mentioned at the beginning of section 2.2, from the chestnut production several by-products are generated, such as wood, flowers, leaves, shells, barks, and burs [19]. These products are often considered as waste and are discarded, but there are, in most cases, ways to take advantage and convert them into products with a higher added value [2].

2.2.3.1 Chestnut by-products composition

The chestnut, as well as its by-products, have a rich bioactive composition, particularly in antioxidant compounds, which make them interesting to be explored. These bioactive compounds are associated with health benefits, such as antioxidant, anticarcinogenic and

cardioprotective properties [11]. Therefore, several studies have been developed to valorise the chestnut by-products, mainly leaves, but also the remaining ones [12][13].

Shells represent approximately 10% of the chestnut production and can be divided into two parts: the pericarp (outer shell) and the integument (inner shell). The antioxidant compounds present in shells, as well as in the remaining by-products, are mainly phenolic compounds, some of which are exemplified in Figure 2.5. Within these compounds, the most predominant ones are:

- **Phenolic acids**: divided into benzoic and cinnamic acids. Two examples of benzoic acid are present in chestnuts shells, namely ellagic acid and gallic acid [19]. Phenolic acids, due to their antioxidant, anticarcinogenic and anti-inflammatory character, have many benefits for health [11].
- **Flavonoids:** examples of these compounds are rutin, quercetin and apigenin. Flavonoids may contribute significantly to human health at the level of the circulatory system, for example [11].
- **Tannins:** there are two types of tannins, condensed and hydrolysable, including vescalagin, castalagin, acutissimin A and B [19].

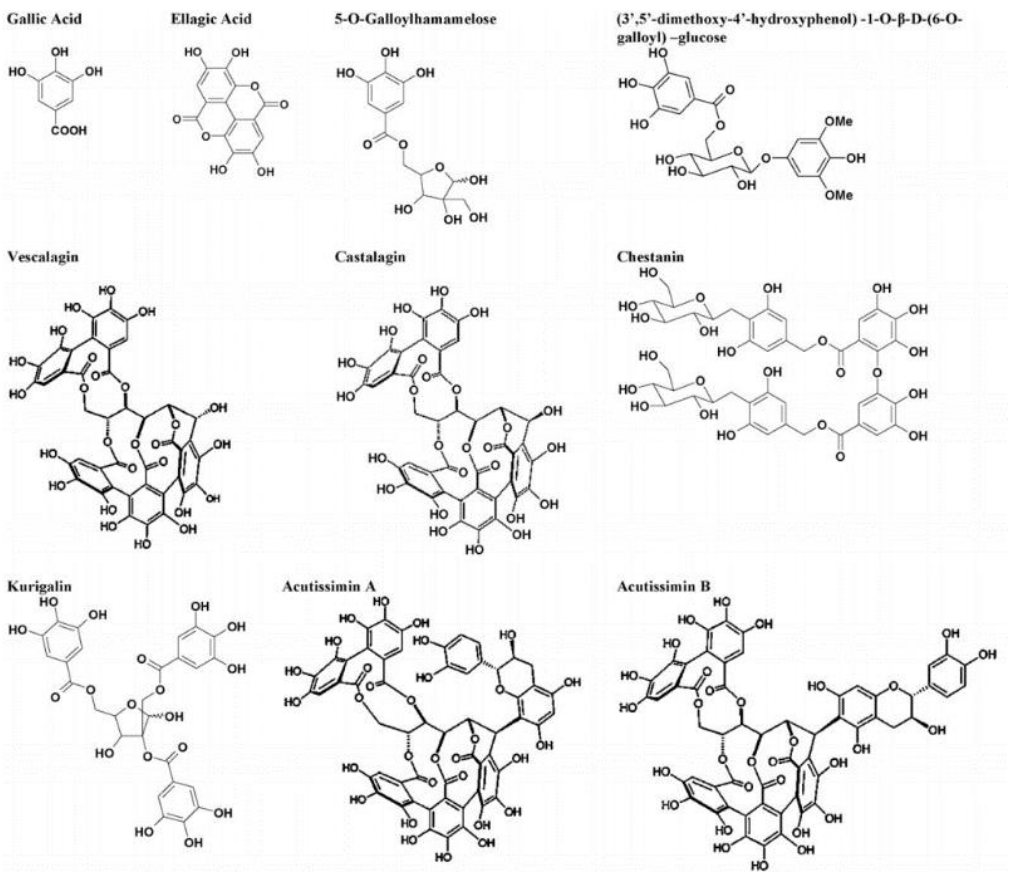


Figure 2.5 Chemical structure of the principal phenolic compounds present in chestnut shells [20].

Table 2.1 summarizes the phenolic compounds reported for *C. sativa* shells (outer and inner), according to different authors.

To extract phenolic compounds from the outer and inner shell of chestnuts, Barreira *et al.* used water as solvent at a temperature of 100°C for 30 minutes [21], while Živković *et al.* employed a binary mixture of ethanol and water (50:50 v/v) during 30 minutes using ultrasound-assisted extraction (UAE) [22]. Barreira *et al.* obtained for the outer shell 510 mg GAE/g phenolic acids and 503 mg CE/g extract of flavonoids and for the inner shell 475 mg GAE/g extract of phenolic acids and 330 mg CE/g extract of flavonoids. On the other hand, Živković *et al.* [22] for the outer shell obtained 12 mg GAE/g d.w. of phenolic acids and 6.5 mg CE/g d.w. of flavonoids and for the inner shell 5.9 mg GAE/g d.w. of phenolic acids and 0.70 mg CE/g d.w. of flavonoids.

More recently, De Vasconcelos *et al.* used as solvent water at a temperature of 20°C during 1 h and at 70°C during 48h as well as 70% acetone at 20°C and methylethylketone at 20°C, both for 48 h [20]. For the outer shell, the authors reported a phenolic acid content for each method of (10.90-18.40) mg GAE/g f.w., 13.75-22.13 mg GAE/g f.w., 68.51-105.66 mg GAE/g f.w. and 2.22-3.25 mg GAE/g f.w., respectively. For the inner shell, the values obtained were 23.49-65.82 mg GAE/g f.w., 15.06-44.42 mg GAE/g f.w., 91.02-120.85 mg GAE/g f.w. and 3.37-6.62 mg GAE/g f.w., respectively.

For the extraction of phenolic acid and flavonoids from chestnut shells the authors, Barreira *et al.* [23] and Vella *et al.* [24], used as solvent water. , Barreira *et al.* [23] obtained a phenolic acid content of 533.81-805.74 mg GAE/g extract and a flavonoid amount of 42.92-146.08 EC/g extract. Lastly, Vella *et al*. [24] reached a phenolic acid and flavonoid content of 17.68 mg GAE/g d.w and 7.36 mg EC/g d.w, respectively.

Table 2.1 Studies of the composition of phenolic compounds in the chestnut shell.

	Extraction method	Phenolic acids[1]	Flavonoids[2]	Reference
Outer shell	Water 100°C 30 min	510 mg GAE/g extract	503 mg CE/g extract	Barreira *et al.* [21]

(continued on next page)

[1] GAE: Gallic acid equivalents; d.w.: dry weight; f.w.: fresh weight
[2] CE: Catechin equivalents

Table 2.1 (continued)

	Extraction method	Phenolic acids[3]	Flavonoids[4]	Reference
	50% Ethanol ultrasound 30 min	12 mg GAE/g d.w.	6.5 mg CE/g d.w.	Živković *et al.* [22]
	Water 20°C 1 h	10.90–18.40 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
	Water 70°C 48 h	13.75–22.13 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
	70% Acetone 20°C 48 h	68.51–105.66 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
	Methylethylketone 20 °C 48 h	2.22–3.25 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
Inner shell	Water 100°C 30 min	475 mg GAE/g extract	330 mg CE/g extract	Barreira *et al.* [21]
	50% Ethanol ultrasound 30 min	5.9 mg GAE/g d.w.	0.70 mg CE/g d.w.	Živković *et al.* [22]
	Water 20°C 1 h	23.49–65.82 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]

(continued on next page)

[3] GAE: Gallic acid equivalents; d.w.: dry weight; f.w.: fresh weight
[4] CE: Catechin equivalents

Table 2.1 (continued)

	Extraction method	Phenolic acids[5]	Flavonoids[6]	Reference
	Water 70°C 48 h	15.06–44.42 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
	70% Acetone 20 °C 48 h	91.02–120.85 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
	Methylethylketone 20°C 48 h	3.37–6.62 mg GAE/g f.w.	–	De Vasconcelos *et al.* [20]
Shell	Water 1 h	533.81–805.74 mg GAE/g extract	42.92–146.08 CE/g extract	Barreira *et al.* [23]
	Water	17.68 mg GAE/g d.w	7.36 mg CE/g d.w	Vella *et al*. [24]

In the investigation carried out by De Vasconcelos *et al.* [20], the influence of different solvents, extraction time and temperature were evaluated. The authors concluded that the outer and inner shells have a high content in phenolic acids, and the highest value obtained was achieved with 70% acetone at 20°C for 48 h (Outer shell: 68.51-105.66 mg GAE/g f.w.; Inner shell: 91.02-120.85 mg GAE/g f.w.).

It is possible to state that the best solvent for the extraction of phenolic compounds is water at a temperature of 100°C, obtaining a total of 510 mg GAE/g of phenolic acids extract and 503 mg

[5] GAE: Gallic acid equivalents; d.w.: dry weight; f.w.: fresh weight
[6] CE: Catechin equivalents

CE/g of flavonoids in the outer shell; in the inner shell, a value of 475 mg GAE/g of phenolic acids extract and 330 mg CE/g of flavonoids was obtained.

Shortly, with the analysis of the results reported by the different studies, in addition to understanding, which is the best way to extract these compounds, it is also confirmed that the chestnut shell has a composition rich in phenolic acid and flavonoids. This shows the potential that this by-product presents for a possible application in the cosmetic field.

2.2.3.2 Circular economy

The concept of circular economy has become increasingly popular in recent years. The circular economy is an economic model based on the reduction, reuse, recovery and recycling of materials and energy. The chestnut industry can indeed be an example of this challenge, as the production of chestnuts and the processing of by-products fit almost entirely into the Sustainable Development Goals of the 2030 Agenda. These by-products can integrate a form of recycling for these companies considering their chemical composition, through developing cost-efficient processing methods and decreasing the environmental impact (as the waste accumulation is reduced) and providing several economic advantages [12].

2.3 Extraction methods of bioactive compounds from chestnut and its by-products

Extraction is a fundamental step to obtain bioactive compounds from different foods or plants. Therefore, the use of a proper extraction process is crucial for the separation, identification and characterisation of these compounds [25]. There are several extraction techniques, conventional and non-conventional, that follow common objectives [25]:

- Extract bioactive compounds from complex plant samples;
- Increase the selectivity of analytical methods;
- Increase the sensitivity of biological activity by increasing the concentration of the compounds;
- Converse bioactive compounds into a more suitable form for detection and separation;
- Provide a robust and reproducible method that is independent of sample matrix variations;

The following chapters will describe some examples of conventional and non-conventional techniques suitable for obtaining bioactive compounds.

2.3.1 Conventional techniques

Conventional extraction techniques are characterized by a process where the sample is placed in contact with the solvent and the soluble components contained in the solid matrix diffuse into the solvent [13]. There are several classical extraction techniques, however, in this work only Soxhlet extraction, maceration and hydrodistillation will be considered.

2.3.1.1 Soxhlet Extraction

Soxhlet extraction is frequently used for the extraction of bioactive compounds and serves as a reference for evaluating new alternative extraction techniques [25]. The extraction process begins with the introduction of the dried sample into the thimble, being then placed in a distillation flask containing the solvent. When the overflow level is reached, the solution in the thimble is aspirated by a siphon that unloads it with the extracted components into the flask. The solvent passes back through the solid bed and the process is repeated until the extraction is completed [25].

2.3.1.2 Maceration

The first step of maceration technique is to grind the sample, reducing the particle size. By reducing the particle size, the contact area increases and, consequently, the extraction efficiency improves. The next step is to add the solvent to a closed container with the sample at room temperature. After some time, the liquid is strained, and the solid residue (pomace) is pressed to recover a large amount of occluded solution. The last step is to mix the liquids obtained and to purify the solution by filtration [15][16].

2.3.1.3 Hydrodistillation

Hydrodistillation is a technique that can be used for the extraction of bioactive compounds and essential oils from plants [25]. There are three distinct types of hydrodistillation, namely distillation into water, distillation into water and steam and direct steam distillation. This extraction method can be divided into the following steps: (1) packing the sample in a still

compartment, (2) adding water and boiling it or injecting direct steam, (3) cooling the sample indirectly by water, condensing the vapour mixture of water and oil and (4) separating the oil and bioactive compounds from the water. Hydrodistillation involves the physicochemical processes of hydrodiffusion, hydrolysis and heat decomposition. One of the limitations associated with this technique is related to the high temperature since it leads to the loss of volatile components [25].

2.3.2 Non-conventional techniques

Non-conventional extraction techniques have emerged to overcome the limitations related to conventional techniques. These limitations are mainly associated with the low extraction rate and selectivity, thermal decomposition of thermolabile compounds, high energy consumption and use of large amounts of solvent with high purity and price [1][15].

The most promising extraction methods are enzyme-assisted extraction, microwave-assisted extraction (MAE), pulsed electric field assisted extraction, pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and subcritical water extraction (SWE) [15]. These "green techniques" are economically more attractive for industrial processes and have a lower environmental impact as they use safe solvents, renewable raw materials and have inherently safer chemistry for accident prevention, etc [3][15].

In this dissertation, only UAU, SFE and SWE will be studied. These methods will be detailed in the following sections.

2.3.2.1 Ultrasound-Assisted Extraction (UAE)

The UAE consists in applying mechanical waves with a frequency above the limit of human hearing (20 kHz) [11]. The use of ultrasound aims to intensify the extraction by a phenomenon named cavitation, characterized by the formation, growth and collapse of micro-bubbles within the liquid phase, as shown in Figure 2.6 [1][17]. The bubbles collapse generates high temperatures (around 5000°C) as well as high pressures (almost 100 MPa). Thus, the phenomenon of cavitation facilitates the entry of solvent, in addition to increasing heat and mass transfer [11].

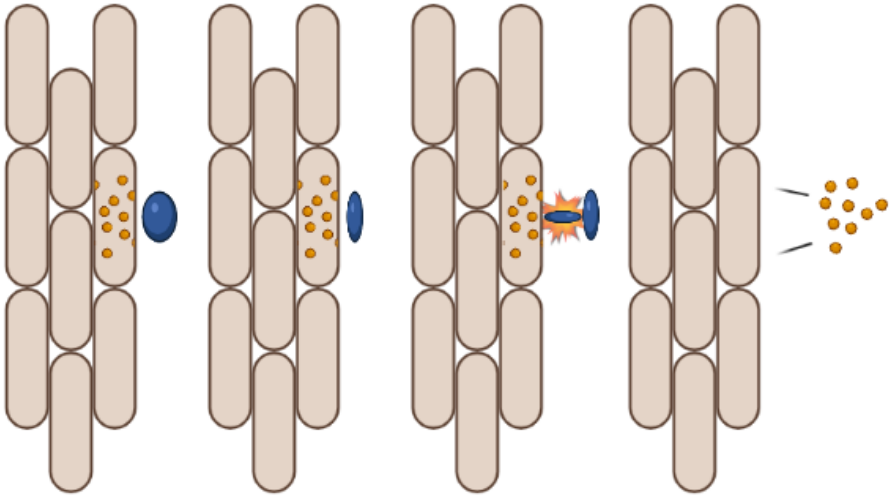


Figure 2.6 Cavitation process [27].

The UAE can be performed in a variety of equipment's; the most frequently used are the ultrasound bath and the ultrasound probe. The ultrasound bath is a relatively simple and cheap device with capacity for many samples. However, it has some disadvantages compared to the probe-type ultrasound equipment, due to the low reproducibility and low power of the ultrasound reaching the sample (resulting in a loss of intensity caused by the water in the bath and by the glass container) [11][28]. In the case of the ultrasound probe, it is immersed in the solution, resulting in a direct delivery of ultrasonic energy with minimal losses, favouring the extraction process. Nevertheless, there is a temperature rise resulting from the absorption of high-intensity ultrasonic energy, so it is convenient to have a cooling system in the reactor.[28].

In summary, the use of ultrasonic energy has several advantages [25]:

- Reduced extraction time and quick start-up;
- Reduced consumption of energy;
- Reduced use of solvent;
- More efficient mixing;
- Fast energy transfer;
- Low extraction temperature and reduced thermal gradients;
- Small equipment size;
- Quick response to extraction process control system;
- Increased production and elimination of process steps.

2.3.2.2 *Supercritical Fluid Extraction (SFE)*

A supercritical fluid is a substance whose temperature and pressure are beyond its critical point, in which there is no distinction between the liquid and gaseous phase. This fluid has, therefore, typical characteristics of liquids, such as solubility and density, and gases, like diffusivity, viscosity and surface tension [25]. One of the most used supercritical solvents is carbon dioxide (CO_2) since its critical temperature is 34°C, close to room temperature, and its critical pressure is 74 bar, allowing it to operate at moderate pressures. The only negative point of this solvent is its low polarity, being not ideal for the extraction of polar compounds. However, this limitation can be easily overcome by using small amounts of chemical modifiers or co-solvents [25]. Figure 2.7 represents the instrumentation diagram of a supercritical fluid extraction using CO_2 as solvent.

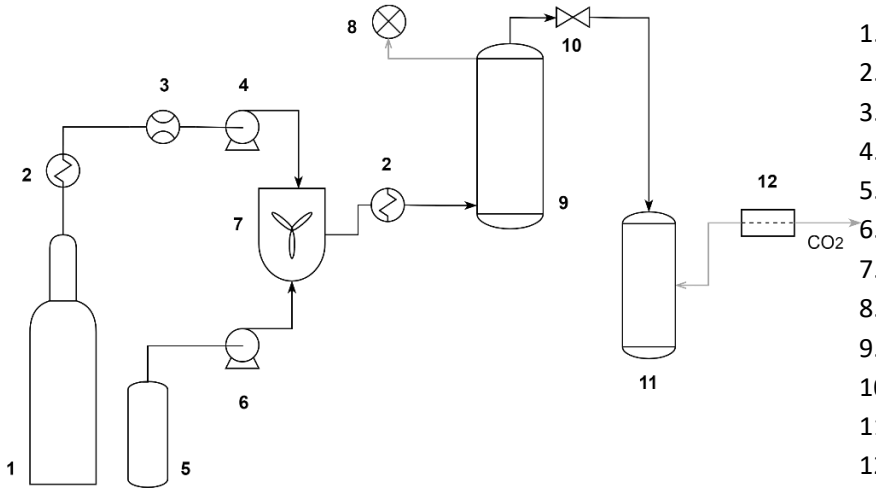


Figure 2.7 Installation of a supercritical liquid extraction with CO2 [25].

This extraction process has significant advantages [25]:

- Supercritical fluid has a low viscosity, low surface tension and high diffusion coefficient compared to a liquid solvent, which results in a greater penetration into the sample, favouring the mass transfer;
- Multiple passages of the supercritical fluid in the sample allow a complete extraction;
- High selectivity of the supercritical fluid, since its properties can be modified by pressure and temperature variations;
- Depressurisation of the supercritical fluid prevents the separation of solvent from solute at the end of the process;

- Operation at room temperature makes possible the extraction of thermolabile compounds;
- Minimal waste production due to the recycling and re-use of the supercritical fluid;
- Feasible on both laboratory and industrial scale.

2.3.2.3 Subcritical Water Extraction (SWE)

SWE, also known as Pressurized Hot Water Extraction, uses water as solvent, whose temperature is between boiling (100°C) and critical (374°C) points, under pressures below critical conditions (between 10 and 100 bar, approximately) [3][16]. Under these conditions, the dielectric constant of water decreases from about 80 to 30ε, a value characteristic of organic solvents, such as methanol, ethanol and acetone. This condition improves the extraction of bioactive compounds, making SWE a potential alternative. However, it should be noted that under these conditions some phenolic compounds may be degraded, requiring a prior study of the applicability of this method to the sample to be extracted [13].

Therefore, SWE is a very promising method, because [13]:

- It has a short extraction time;
- It has a low energy consumption;
- It uses a green solvent (water);
- It has the potential to be scaled up and implemented at an industrial level.

2.4 Pesticide residue contamination of chestnut and its by-products

As already mentioned in chapter 2.2.2, obtaining high yield productions has become increasingly emergent. One of the reasons is the rapid urbanisation that the world is experiencing, causing less lands availability for agricultural activity. Added to this fact, the growth of the human population and the consequent increase in the demand for food are key factors in the search for solutions with effective answers. As such, based on its effectiveness, the use of pesticides has suffered a growth in recent times, since these compounds control the spread and proliferation of pests in crops, generating food with excellent quality-price [29][30]. However, some pesticides are part of a group of toxic substances, called persistent organic chemical pollutants (POPs) and their excessive application can leave harmful residues either in soil, water, or food, thus posing a risk to both environment and humans. As for humans, they can be susceptible to pesticides through ingestion, dermal contact and inhalation of contaminated air [30].

It is, therefore, of great importance to analyse the presence of contaminants before incorporating any type of extract whose origin is natural, whether in cosmetic products (as is the case of the extracts studied) or into other types of products. In chestnut cultivation, as well as in dried fruit cultivation in general, pesticides are widely used, so it is necessary to perform this analysis in order to guarantee that the extracts obtained are safe [31]. Still, if one looks at the 2018 report of EFSA (the institution through which the EU reports data on pesticides in food), no exceedances of maximum residue levels (MRLs) were detected for chestnuts after analysing several samples [32].

Having said that, the determination of pesticide residues using analytical methods involves the steps present in Figure 2.8 [29]:

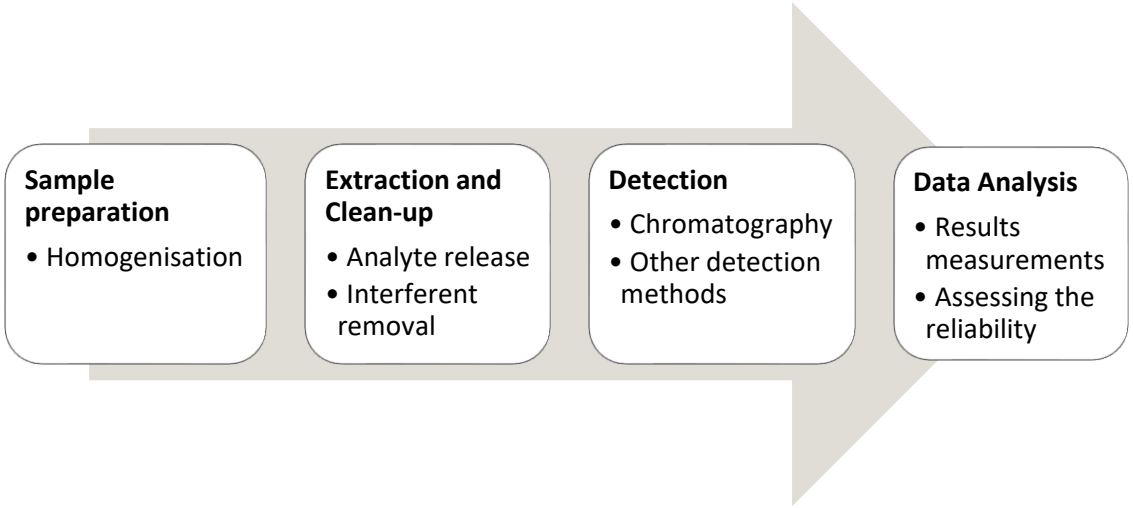


Figure 2.8 Steps involved in the determination of pesticides residues [29].

Over the years, several techniques have been developed for the determination of pesticides. In the case of detection techniques, these compounds can be determined by conventional techniques or by advanced techniques. In the scheme of Figure 2.9 there are some examples of these techniques. Within the conventional analytical methods are gas chromatography (GC), liquid chromatography (LC), high performance liquid chromatography (HPLC), enzyme-linked immunosorbent essays and capillary electrophoresis. Advanced techniques, on the other hand, are essentially based on the sensor principle [29].

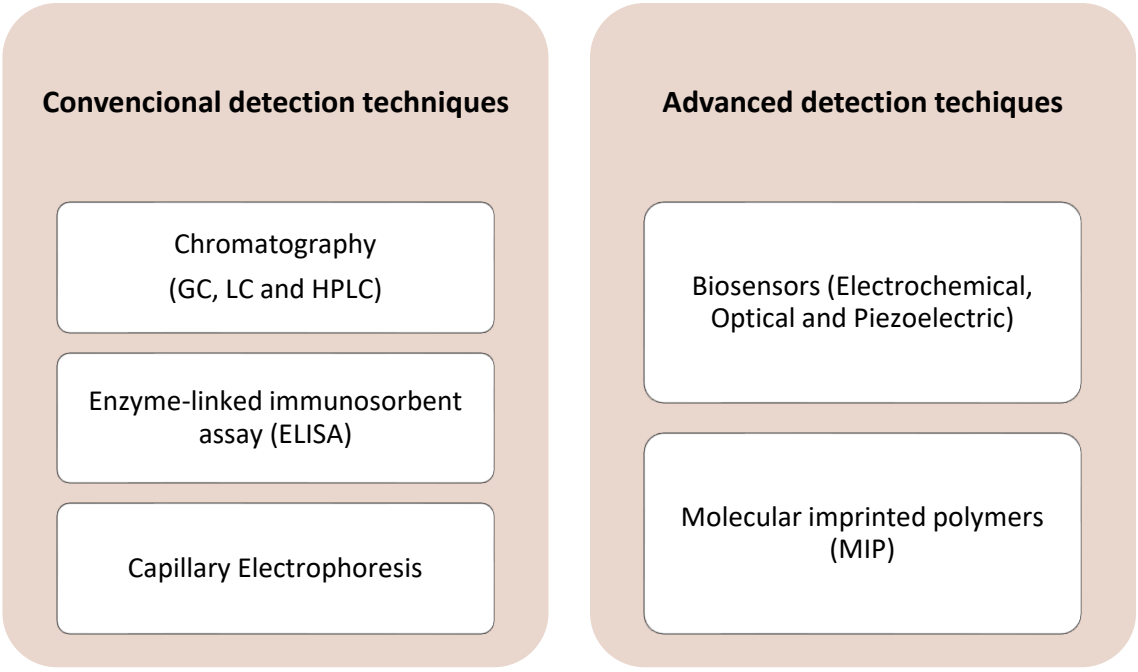


Figure 2.9 Example of detection techniques for pesticides residues in food of plant origin [29].

These techniques are quite different from each other. Advanced biosensor-based methods have emerged with the potential to complement or even replace conventional chromatographic techniques, simplifying sample preparation and making analysis consequently more simple, faster and less costly [33]. Conventional methods are based on expensive and complex instruments, requiring skilled labour. Both methods have high sensitivity and selectivity at low detection limits, although conventional methods can reach a level of detection that advanced methods cannot, highlighting also the fact that the first ones analyse several types of compounds simultaneously, while the second ones analyse only one compound or compounds of the same family [29].

This section will include the most common classification of pesticides, some methods used in the step of extraction and clean-up and also some methods of detection of pesticides, noting that chromatographic techniques are widely used when it comes to the determination of these contaminants and for that reason were the ones addressed in this work [34].

2.4.1 Pesticide's classification

Classification of pesticides is performed according to various criteria like toxicity or hazardous effects, use or purpose, chemical composition, mode of action, how or when it works, its

formulations, and its source of origin. In the Figure 2.10 is shown a scheme with the most commonly used classification [17].

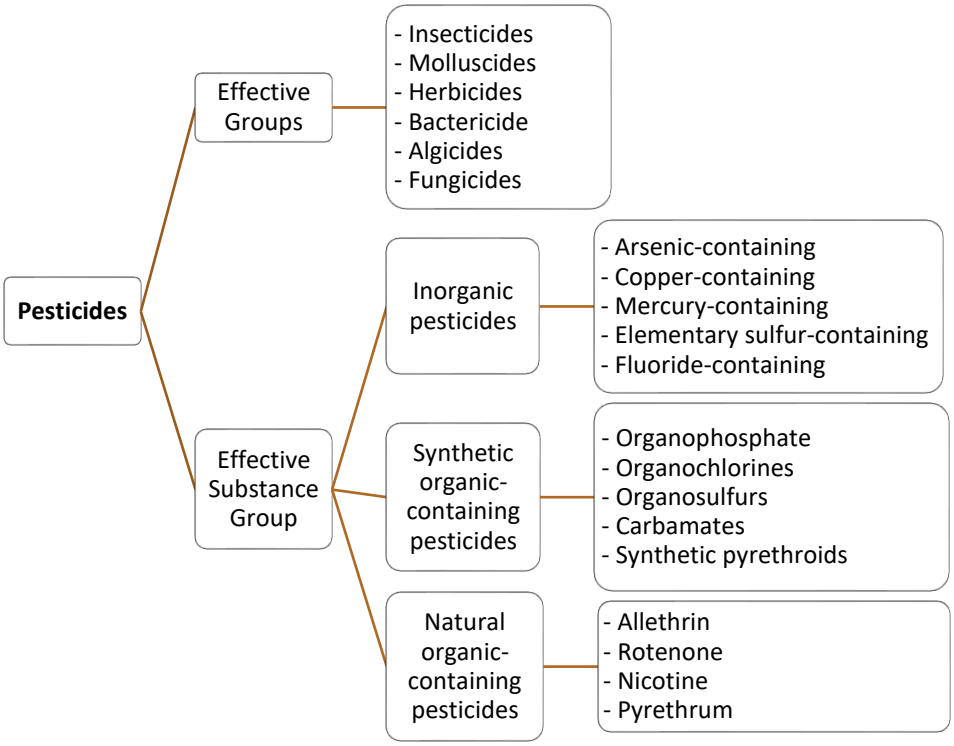


Figure 2.10 Pesticide's classification.

Pesticides can then be divided into four major groups: organochlorines, organophosphates, carbamates and pyrethroids.

Organochlorine pesticides (OCPs) are persistent organic pollutants with high toxicity and slow environmental degradation. They are lipophilic compounds and can be absorbed by organisms leading to their bioaccumulation. OCPs have already been banned in North America, Europe and partially in South America, however, they can still be found due to their long-life cycles in the environment and because they are easily carried over very long distances through sea and air [29]. Some examples of OCPs are α-hexachlorocyclohexane (α-HCH), hexachlorobenzene (HCB), lindane, β-hexachlorocyclohexane (β-HCH), δ-hexachlorocyclohexane (δ-HCH), aldrin, α-endossulfan (α-end), p,p'- dichlorodiphenyldichloroethylene (p,p'-DDE), dieldrin, endrin, β-endossulfan (β-end), p,p'- dichlorodiphenyldichloroethane (p,p'-DDD), dichlorodiphenyltrichloroethane (DDT), and methoxychlor.

Synthetic pyrethroids (PYRs) pesticides are derived from esters present in chrysanthemum flower extracts, called pyrethrins. These substances possess pesticidal properties and are,

therefore, used for the industrial production of PYRs. Synthetic PYRs show selective insecticidal activity and low toxicity to mammals and, compared to their natural form, have greater stability and a longer half-life of environment [29]. There are several types of PYR, including bifenthrin, cyhalothrin, permethrin, cyfluthrin, cypermethrin, α-fenvalerate, β-fenvalerate and deltamethrin.

Organophosphorus pesticides (OPPs) generally exist as esters. They are among the most widely used pesticides, along with carbamates, because of their broad spectrum of use, their very attractive price, their easy availability, shorter life cycles than OCPs. They are, therefore, biodegradable and fat-soluble compounds. The mechanism of action of OPPs is mainly through enzyme inhibition [29]. Dimethoate, chlorpyrifos-methyl, parathion-methyl, malathion, chlorpyrifos and, chlorfenvinphos are some examples of organophosphorus compounds that we will address in this work.

To analyse pesticide residues (OCPs, OPPs and PYRs) in chestnut shells and other by-products, the first step is to prepare the samples properly. This step involves the elimination of the interfering matrix and the extraction and concentration of the substances of interest. Among the conventional extraction techniques, Soxhlet and solid-phase extraction (SPE) are the ones that stand out, while the QuEChERS technique (Quick, Easy, Cheap, Effective, Rugged, Safe) has become more prevalent in recent years [34].

2.4.2 Extraction techniques of pesticides

2.4.2.1 Soxhlet Extraction

The Soxhlet extraction of pesticides is based on the same principles as presented in chapter 2.3.1.1, using the processes of temperature increase and distillation, but in this case to extract pesticides [34]. This method begins with the introduction of a small dry solid sample into a thimble, which is then placed in a distillation flask and subjected to successive extractions by evaporation and condensation of the solvent [25]. Although it seems simple, this method is very time consuming and environmentally unfriendly, and for these reasons it has been replaced by more efficient methods, such as the QuEChERS method [35].

2.4.2.2 Solid-phase extraction (SPE)

Solid-phase extraction is commonly used to analyse pesticides in liquid samples namely, water, tea and wine, but it is also used in other agricultural samples such as fruits, vegetables and other

plants [36]–[38]. This is a simple, reproducible technique and is easily coupled with detection instruments [39].

Solid-phase extraction occurs in four steps: 1) conditioning the cartridge with a suitable solvent to adjust the forces between the elution solvent and the sample solvent; 2) introduction of the sample into the cartridge; 3) cleaning of the cartridge with solvent (clean-up) and 4) elution of the analyte. These steps result in the extraction of the analytes from the sample together with the interfering compounds [40].

There are many types of SPE cartridges, and these can be divided into three classes on the markets: silica-based cartridges, polymer-based cartridges and adsorption-based cartridges. An organic solvent is generally used to remove the interfering compounds and another solvent is used to wash out the analytes of interest [34]. The right adsorbent choice is critical for a good recovery. Considering the properties of the adsorbents, they can be classified as normal phase (e.g. diatomite, silica gel, etc.), reversed phase (e.g. C8 and C18), polymeric sorbent and mode-mixed sorbent [39].

2.4.2.3 QuEChERS

QuEChERS, as the name suggests, is a fast, easy, cheap, effective, robust and safe sample preparation technique. Developed by Anastassiades *et al*.[41], in 2003, and validated by Lehotay *et al*. [42], it is a method used for the analysis of multi-class or multi-residue pesticides in complex matrices [39]. The QuEChERS extraction technique is low susceptible to errors as it involves a series of simple analytical steps. Thanks to the above aspects and to its versatility, this method has become very popular and, in order to expand its application to other products (food, biological fluids and environmental samples, for example) and to other contaminants (antidepressants, polyphenols, amines and environmental pollutants, for example), it has been modified over time. QUEChERS is based on the dispersion of salts to extract (salting-out effect), isolate a wide range of analytes (first step) from very complex matrices in addition with a second step called clean-up of the extract obtained in the first step. [35]. The first step of this approach is extraction with an organic solvent and is followed by an extraction/partition step with addition of a salt mixture. The second step is solid-phase dispersive extraction (d-SPE), where extract cleaning is performed using various combinations of porous sorbents and salts to remove interfering substances from the matrix [35][43]. Some examples of such sorbents are primary secondary amine (PSA), octadecyl silica (C18), magnesium sulphate ($MgSO_4$) and Supel QuE Z-Sep. PSA is used to remove various polar organic acids, fatty acids, sugars, and polar pigments.

C18 is mainly used to remove interfering compounds that are non-polar (e.g. lipids) and $MgSO_4$ to remove water from the organic solvent and sample [44]. Finally, Supel QuE Z-Sep, which consists of C18 and zirconia bonded to the same silica particles, is used to remove fats and pigments that PSA and C18 do not remove with the same efficiency [45]. The final organic extract is analysed using traditional analytical methods with different types of detectors, including GC [35][43].

Despite its simplicity, the QuEChERS method depends on several factors to be effective, among them the properties of the target analytes, the matrix composition, the analytical techniques and the equipment used. It is in this sense that it becomes important to take into account all the parameters that may affect its performance and optimise them if necessary [35]. The most used versions of the QuEChERS method are the Oirginal version, the AOAC Official Method 2007.01 and the CEN Standard Method EN 15662. The last two procedures emerged thanks to an attempt to overcome an inconvenience associated with the non-buffered conditions under which the original method is performed: the degradation of compounds sensitive to high pH and low pH. To this end, citrate buffer, which has a relatively low buffer capacity, and/or acetate buffer, with a strong buffer capacity, were added, thereby increasing the efficiency of the extraction. Nevertheless, these three approaches continued to undergo modifications over the last years, mainly in the partitioning salts and the d-SPE sorbents. However, besides these parameters, there are others that must be considered by researchers using this method, among them the amount of sample, extraction solvent and the sample/solvent ratio [35].

To the best of our knowledge, any study was found that specifically used the QuEChERS method for the extraction of pesticide residues in chestnut or its by-products. However, some were found where it was applied in similar samples, as is the case of the study developed by Cebi *et al*. [46] where the presence of 77 pesticides in hazelnut samples was evaluated applying this method together with a liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology. Another example was the work developed by Barci *et al*. [47] where a method based on the QuEChERS sample preparation procedure and liquid chromatography-tandem mass spectrometry analysis was developed and validated to monitor the presence of 47 pesticides in pecans. There are examples of other studies where this method has been applied to other types of foods, such as the work prepared by Varela-Martínez *et al*. [48] where the three versions of QuEChERS (Original version, AOAC 2007.01 and CEN 15662) were evaluated for the extraction of 38 multiclass pesticides from a mixture of five nuts, including strawberry, blackberry, passion fruit, pineapple and grapes.

2.4.3 Analytical methods for detection of pesticides

Chromatographic techniques are, as already stated, widely employed in the identification and quantification of pesticides [34]. Gas chromatography is a process where the sample is vaporized and injected at the top of the chromatographic column. The eluent is an inert gas that acts as the mobile phase. Its function is to transport the analyte along the column without interacting with it. There are two types of gas chromatography: gas-solid chromatography (GSC) and gas-liquid chromatography (GLC), the second being the most widely used. GSC is based on the partition of the analyte between the mobile phase and the liquid stationary phase (inside the column) [49]. The analytes with greater affinity for the mobile phase will have shorter retention times, and those with greater affinity for the stationary phase will spend more time retained on the column. Retention time is defined as the time it takes for the analyte to reach the detector after the sample has been injected [50].

The basic components of gas chromatography equipment are shown in Figure 2.11.

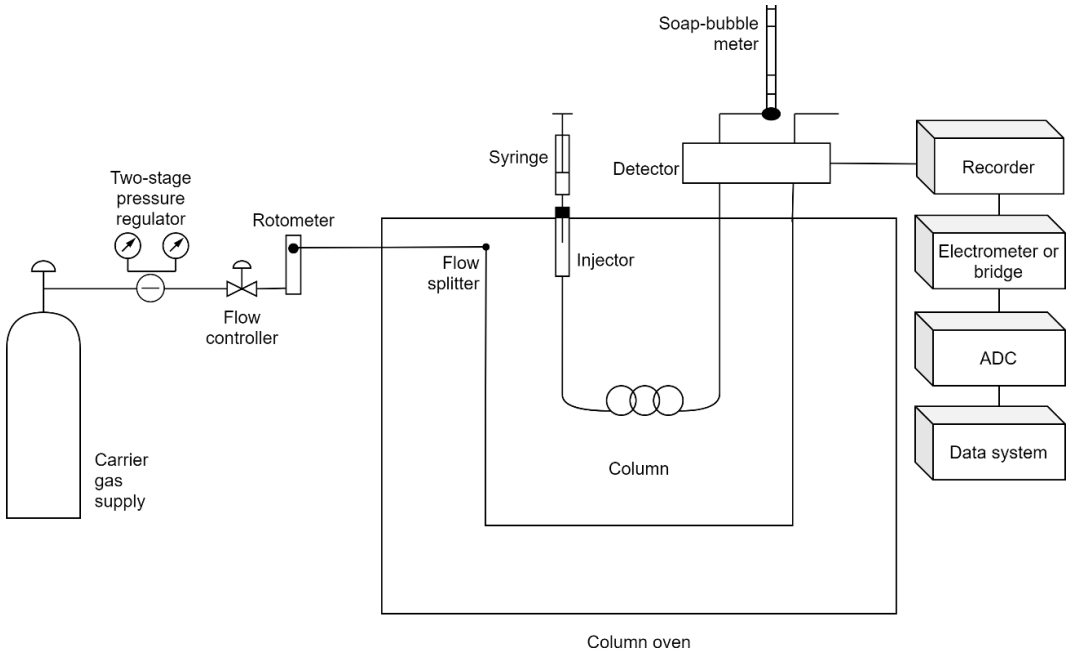


Figure 2.11 Scheme of a gas chromatograph [49].

For gas chromatography, an ideal detector should have the following characteristics [49]:

- Adequate sensitivity;

- Good stability and reproducibility;
- Linear response for solutes extending over several orders of magnitude;
- High reliability and ease of use;
- A wide working temperature range;
- Fast response, independent of flow rate;
- Similarity of response for all solutes or a highly predictable and selective response for one or more solute classes;
- No sample decomposition.

For the separation and determination of OCPs, PYRs and OPPs compounds are normally employed detectors such as electron capture detector (ECD), flame photometric detector (FPD) or mass selective detector [51].

ECDs selectively detect halogens contained in substances such as pesticides. The effluent from the column passes over an emitter (usually nickel-63), ionising the inert gas and producing a burst of electrons. In the absence of organic species, the ionisation process results in a constant current, whereas in the presence of these species, the current decreases significantly because the organic molecules tend to capture the electrons. This detector is selective, highly sensitive to molecules containing halogen groups, peroxides, quinones and nitro groups and has the advantage of not significantly altering the sample. However, its linear response is limited to a small range [49].

The FPD has been widely applied in pesticide analysis because it selectively detects sulfonated and phosphorus compounds. In this detector, the eluent passes through a low temperature hydrogen/air flame, which converts phosphorus to an HPO species emitting radiation around 510 and 526 nm. On the other hand, the sulphur is converted into S_2, which emits radiation in a band centred at 394 nm. Appropriate filters are used to isolate these radiation bands, and the intensities are subsequently registered photometrically [49].

Finally, by coupling a mass spectrometer (MS) to the gas chromatograph, in addition to detecting the analytes, it also identifies them [49]. Gas chromatography with mass selective detector (GC-MSD) instruments are widely used in the identification of a large number of components present in natural and biological species. An MS may be composed of a sample introduction system, an ion source, an ion mass analyser and an electron multiplier, besides the detector and the data acquisition system, as shown in Figure 2.12. Regarding mass analysers, the most common are the sector, quadrupole, ion trap, time of flight and Fourier transform ion cyclotron resonance [52].

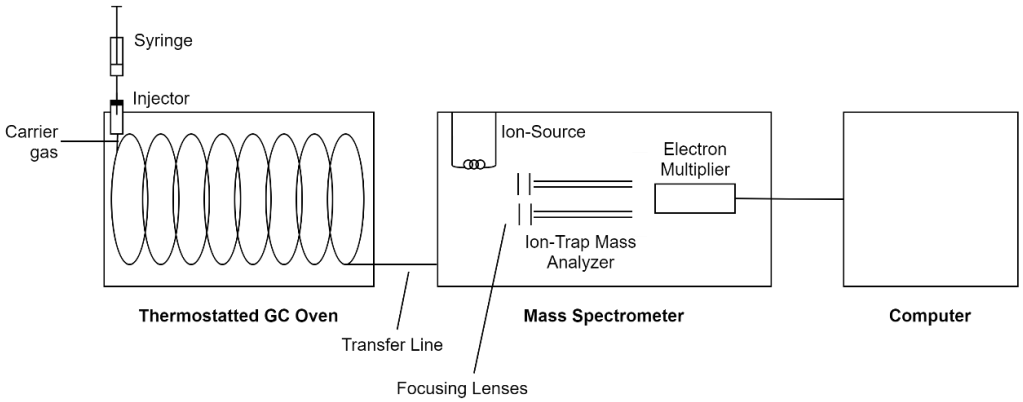


Figure 2.12 Scheme of GC/MS system [53].

The flow rate from capillary columns is usually low enough to allow direct connection of the column to the ionisation chamber of the spectrometer. If the columns are packed or megadiameter capillaries, a jet separator should be used to remove most of the carrier gas from the analyte [49]. The simplest mass detector for use in gas chromatography is the ion trap detector, where ions are generated from samples eluted by ion impact or chemical ionisation. The ions that have been trapped are subsequently injected into an electron multiplying detector in a controlled manner to ensure scanning based on the mass-to-charge ratio. MS detectors have two modes of displaying results: real-time and computer based [49].

3 Materials and Methods

As already mentioned, the chestnut shells, thanks to its composition rich in bioactive compounds, in particular antioxidant compounds, has a great potential to be applied in cosmetic products. However, it is necessary to ensure that they are safe for this purpose, and it is essentially in this sense that this work was developed. In order to use the extracts obtained from the chestnut shells, the study developed had the following main objectives:

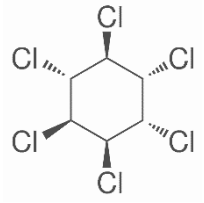
- Monitoring some compounds present in Regulation (EC) No 1223/2009 regarding cosmetic products;
- Development and optimization of robust analytical methods for the extraction and analysis of contaminants in the extracts obtained from the chestnut shell;
- Application of the developed techniques in the extracts under study, in order to control the presence of different contaminants.

In this chapter, the techniques and procedures that have been followed to reach the proposed objectives will be detailed.

3.1 Reagents

The analytical standards of the pesticides studied during this work have a purity of more than 95% and are represented in Table 3.1, along with their chemical group, chemical structure, brand, molecular mass, and boiling temperature.

Table 3.1 Physiochemical properties of pesticides compounds.

Chemical group	Compounds	Chemical Structure	Brand	Molecular mass (g/mol)	Boiling temperature (°C)	Toxicity
Organochlorine	**α-HCH**		Sigma-Aldrich	290.83	288.0 °C	Possibly carcinogenic to humans (Group 2B)

(continued on next page)

Table 3.1 (continued)

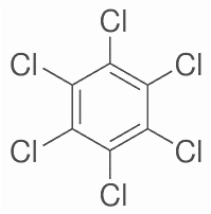
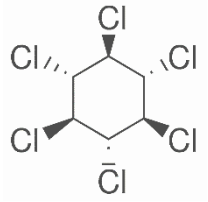
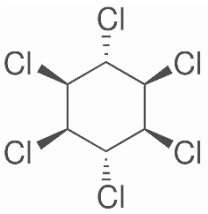
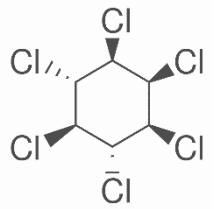
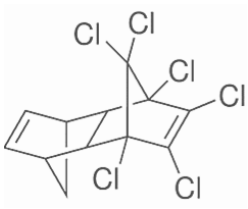
Chemical group	Compounds	Chemical Structure	Brand	Molecular mass (g/mol)	Boiling temperature (°C)	Toxicity
Organochlorine	**HCB**		Sigma-Aldrich	284.78	325.0 °C	Possibly carcinogenic to humans (Group 2B))
	β-HCH		Sigma-Aldrich	290.83	60.0 °C at 0.50 mmHg	Possibly carcinogenic to humans (Group 2B)
	Lindane (γ-HCH)		Sigma-Aldrich	290.83	323.4 °C at 760.00 mmHg	Possibly carcinogenic to humans (Group 2B)
	δ-HCH		Sigma-Aldrich	290.83	323.4 °C at 760.00 mmHg	Possibly carcinogenic to humans (Group 2B)
	Aldrin		Sigma-Aldrich	364.91	145.0 °C at 2.00 mmHg	Possibly carcinogenic to humans (Group 2B)

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Table 3.1 (continued)

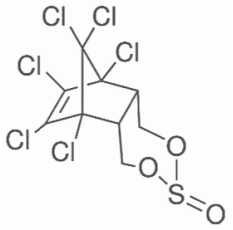
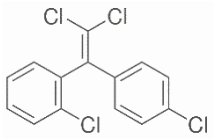
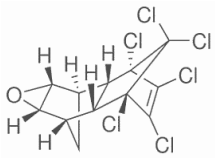
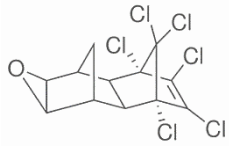
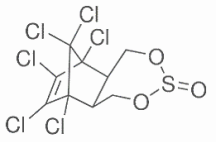
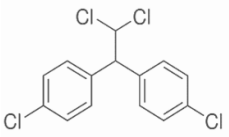
Chemical group	Compounds	Chemical Structure	Brand	Molecular mass (g/mol)	Boiling temperature (°C)	Toxicity
Organochlorine	**α- Endosulfan**		Sigma-Aldrich	406.93	No data	No data
	p.p´-DDE		Sigma-Aldrich	318.03	316.5 °C at 760.00 mmHg	Possibly carcinogenic to humans (Group 2B)
	Dieldrin		Sigma-Aldrich	380.91	Decomposes	Possibly carcinogenic to humans (Group 2B)
	Endrin		Sigma-Aldrich	380.91	Decomposes	Not carcinogenic to humans (Group 3)
	β- Endosulfan		Sigma-Aldrich	406.93	No data	No data
	p.p´-DDD		Sigma-Aldrich	320.04	316.5 °C at 760.00 mmHg	Possibly carcinogenic to humans (Group 2B)

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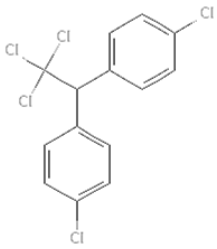
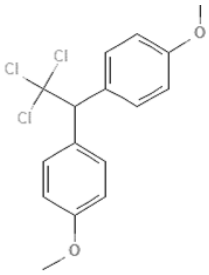
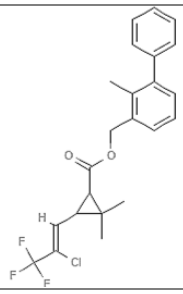
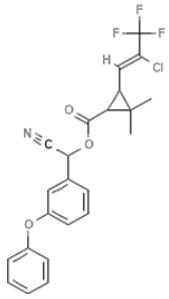
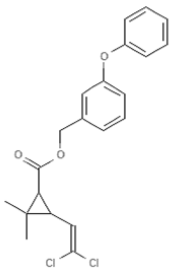
Table 3.1 (continued)

Chemical group	Compounds	Chemical Structure	Brand	Molecular mass (g/mol)	Boiling temperature (°C)	Toxicity
Organochlorine	**DDT**		Sigma-Aldrich	354.49	260.0 °C	Possibly carcinogenic to humans (Group 2B)
	Methoxychlor		Sigma-Aldrich	345.65	Decomposes	Not carcinogenic to humans (Group 3)
Pyrethroid	**Bifenthrin**		Sigma-Aldrich	422.87	No data	No data
	Cyhalothrin		Sigma-Aldrich	449.85	No data	No data
	Permethrin		Sigma-Aldrich	391.29	> 290.0 °C at 0.76 mmHg	Not carcinogenic to humans (Group 3)

(continued on next page)

Table 3.1 (continued)

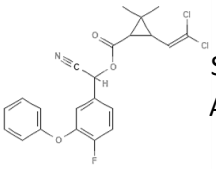
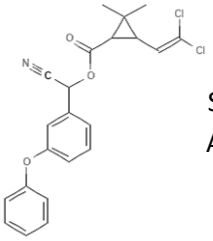
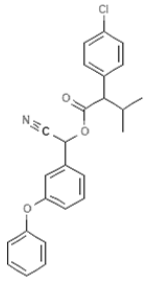
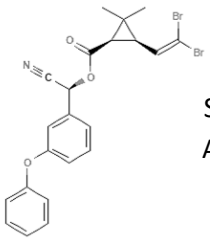
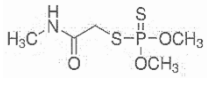
Chemical group	Compounds	Chemical Structure	Brand	Molecular mass (g/mol)	Boiling temperature (°C)	Toxicity
Pyrethroid	**Cyfluthrin**		Sigma-Aldrich	434.29	No data	No data
	Cypermethrin		Sigma-Aldrich	416.30	No data	No data
	Fenvalerate		Sigma-Aldrich	419.90	Decomposes	Not carcinogenic to humans (Group 3)
	Deltamethrin		Sigma-Aldrich	505.20	No data	Not carcinogenic to humans (Group 3)
Organophosphorus	**Dimethoate**		Sigma-Aldrich	229.26	117.2 °C at 0.10 mmHg	No data

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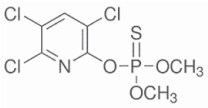
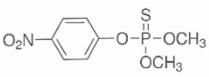
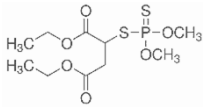
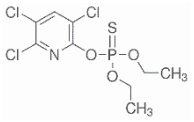
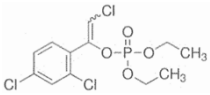
Table 3.1 (continued)

Chemical group	Compounds	Chemical Structure	Brand	Molecular mass (g/mol)	Boiling temperature (°C)	Toxicity
Organophosphorus	**Chlorpyrifos--methyl**		Sigma-Aldrich	322.53	No data	No data
	Parathion--methyl		Sigma-Aldrich	263.21	118.9 °C at 0.13 mmHg	Not carcinogenic to humans (Group 3)
	Malathion		Sigma-Aldrich	330.31	156.1 to 157.2 °C at 0.70 mmHg	Probably carcinogenic to humans (Group 2A)
	Chlorpyrifos		Sigma-Aldrich	350.59	Decomposes	No data
	Chlorfenvinphos		Sigma-Aldrich	359.57	390.0 °C at 0.76 mmHg	No data

Source: Merck. 2021; PubChem. 2021; National Toxicology Program. 2021 [54]–[56]

In addition to the analytical standards pesticides, triphenyllphosphate (TPP, purity 99%), and the 4,4'-Dichlorobenzophenone (purity 99%) were used as internal standard (IS), also provided by Sigma-Aldrich. The analytical grade solvents used as acetonitrile (ACN), *n*-hexane and methanol were obtained from Carlo Erba Reagents, Supelco and VWR Chemicals, respectively. The QuEChERS (AOAC) used in the extraction process of pesticide compounds were obtained from Agilent Technologies. The salts used in the dispersive solid-phase extraction (d-SPE) clean-up step, such as magnesium sulphate ($MgSO_4$), primary secondary amine (PSA), octadecylsilane (C18) and Supel QuE Z-Sep were supplied by Phenomenex.

3.2 Preparation of standards solutions

Individual solutions of each pesticide (PYRs, OCPs and OPPs) were previously prepared in *n*-hexane at the concentrations of 2000.00, 500.00 and 150.00 µg L^{-1}, respectively, and stored at 4°C.

In order to analyse the recovery and matrix effect, working standard mixture solutions were prepared in *n*-hexane at a concentration of 50.00 µg L^{-1}. Also, for validation studies, six calibration standard solutions were prepared in a blank matrix of the chestnut shell (with concentrations of OCPs and PYRs between 6.00-49.97 µg $kg^{-1}$and of OPPs of 0.90-8.99 µg kg^{-1}) and in solvent, *n*-hexane, (with concentrations of OCPs, PYRs and OPPs between 15.00-150.00 µg L^{-1}). In all experiments, the internal standard (IS) was added with a concentration of 50.00 µg L^{-1}.

3.3 Evaluation of pesticides in chestnut shell samples and extracts

In this section, the procedures that were followed to evaluate the presence of pesticides in the samples under study will be detailed. Initially, the sample preparation procedures will be described, that is, the QuEChERS method, which was used to prepare solid samples of chestnut shell, and the SPE method, which was used to prepare the samples of chestnut shell extracts coming from the UAE and SWE extraction methods. It should be noted that since the QuEChERS method has undergone some modifications in order to improve its performance, it has been validated and, in this section, this will also be addressed. Finally, a description of the gas chromatography with electron capture detector (GC-ECD) and gas chromatography with flame photometric detector (GC-FPD) equipment and their respective analysis conditions will be given.

3.3.1 Chestnut shell sample preparation using QuEChERS method

In this study the AOAC version of the QuEChERS method was used since, according to the literature, it provides better results than the original and CEN versions for dried fruits [48].

The general process is divided into two main steps, the extraction process and the sample cleaning process, and is done as follows: (1) weight 2.5 g of the ground chestnut shell sample in a 50 mL tube; (2) add 10 mL of acetonitrile and vortex (VWR) the tube for 1 minute; (3) add the buffer salts (6.0 g magnesium sulphate and 1.5 g sodium acetate), shake the tube vigorously for 5 minutes on vortex and centrifuged at 4500 rpm for 5 minutes; (4) transfer 1 mL of the supernatant to a 2 mL tube already containing the dispersive SPE, shake on the vortex for 1

minute and centrifuged at 4500 rpm for 5 minutes in a centrifuge (Thermo Scientific™, Megafuge™ 16); (5) take 1 mL of the extract and dry in a nitrogen flow; (6) subsequently, redissolve the extract in the same volume taken from *n*-hexane; (7) transfer 140 µL to labelled vials, add 10 µL of internal standard (IS) namely, TPP for the OPPs analysis and 4,4´ - Dichlorobenzophenone for OCPs and PYRs analysis. Finally, the extracts were shaked on vortex and (8), inject 1 µL into the gas chromatograph.

For optimization of this method, three different d-SPE sets were tested in the clean-up step. The first set tested (Cleanup 1) was composed of 50 mg PSA, 50 mg C18 and 150 mg $MgSO_4$. The second (Cleanup 2) had in its composition 25 mg PSA, 25 mg C18, 100 mg $MgSO_4$ and 5 mg Supel QuE Z-Sep. Finally, Cleanup 3 was composed of 25 mg PSA, 25 mg of C18, 50 mg of $MgSO_4$ and 5 mg of Supel QuE Z-Sep.

Finally, it should be noted during the application of this method, the extracts obtained were concentrated (2:1) and (3:1) in order to improve the detection of trace amounts of the pesticides under study.

A schematic representation of the process described is shown in Figure 3.1.

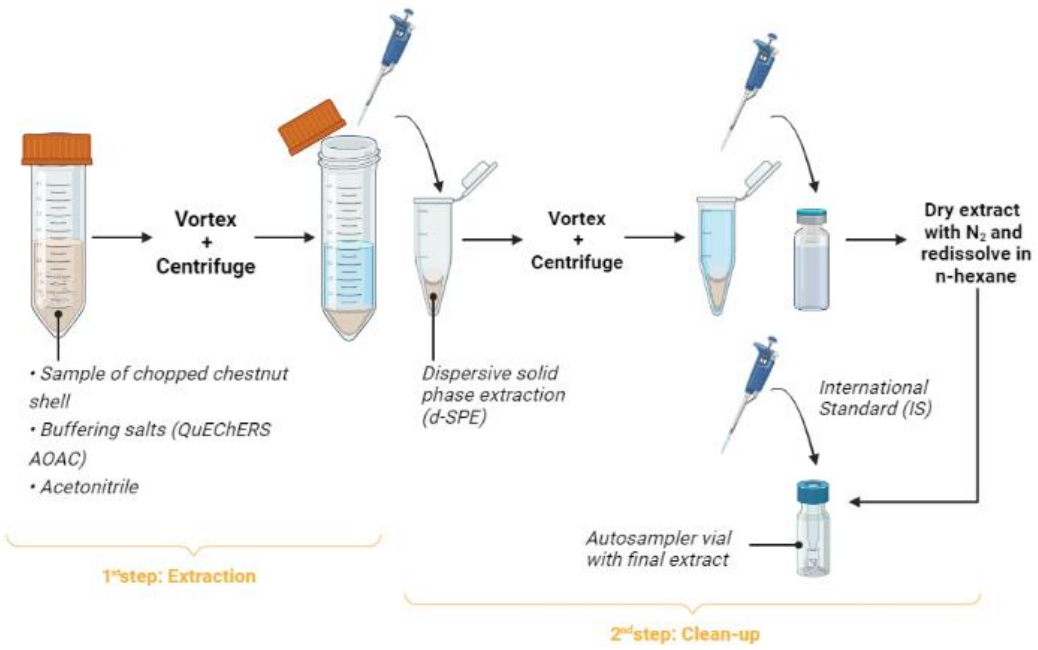


Figure 3.1 Sample preparation using the QuEChERS technique [57].

3.3.1.1 Recovery and matrix studies

The recovery of each analyte was used to evaluate the efficiency of the extraction step of the QuEChERS method. The procedure to obtain the recovery of the pesticides was performed as described in chapter 3.3.1, with the samples being spiked with working standard mixture solutions of 50.00 µg L^{-1} concentration before the extraction step (pre-spiking) in one assay and after the extraction step (post-spiking) in another assay. Thus, the process recovery was calculated as described in equation 5.1 [58].

$$\%\, Recovery = \frac{Peak\ area\ _{Pre-spiking}}{Peak\ area\ _{Post-spiking}} \times 100 \qquad \textit{equation (3.1)}$$

According to the European Commission Guidance Document nº SANTE/12682/2019, the average recovery values should be between 70 and 120% [59].

As for the matrix effect, this was evaluated by comparing the response of the analyte in the presence of matrix (post-spiking) and standard solvent (*n*-hexane), with the same concentration in both situations (50.00 µg L^{-1}). This was calculated as described in equation 5.2.

$$Matrix\ effect = \frac{Peak\ area\ _{Post-spiking}}{Peak\ area\ _{Standard}} \qquad \textit{equation (3.2)}$$

According to the European Commission Guidance Document nº SANTE/12682/2019, matrix effects are considered significant if they exceed ±20% [59].

3.3.1.2 Analytical method validation

In order to ensure that the modified QuEChERS method is suitable for its intended use, it should be subjected to a validation process. The analytical parameters used to evaluate this method were: linearity, limit of detection, limit of quantification and precision [58].

- **Linearity**

For the linearity analysis, a calibration curve was plotted peak area versus analyte concentration with linear regression. The study was performed with matrix calibration standard solutions and with solvent calibration standard solutions.

- **Limit of detection and quantification**

The limit of detection (LOD) is defined as the minimum analyte concentration or mass that can be detected at a certain confidence level. The limit of quantification (LOQ) is defined as the lowest concentration at which quantitative measurements can be made/concentration of the analyte that can be measured [49].

Both limits are calculated through the standard deviation of the linear regression and its slope, being expressed as [49]:

$$LOD = 3 \times \frac{S_{y/x}}{m} \qquad \textit{equation (3.3)}$$

$$LOQ = 10 \times \frac{S_{y/x}}{m} \qquad \textit{equation (3.4)}$$

Where,

- $S_{y/x}$ = standard deviation of linear regression
- m = slope of the linear regression

- **Precision**

Accuracy is given as the degree of agreement between values that were obtained in the same way and was evaluated using the relative standard deviation (RSD) [49]:

$$\%\ RSD = \frac{S}{\bar{X}} \times 100 \qquad \textit{equation (3.5)}$$

Where,

- S = absolute standard deviation

- $\bar{X}$ = sample mean

3.3.2 Chestnut shell extract sample preparation using SPE method

The first step of the procedure is to place the SPE cartridges (Strata C18-E) in the multi-cartridge holder (Manifold) and connect this holder to the vacuum pump. Then, the cartridges are conditioned with the appropriate solvents, in this case with 2 mL of *n*-hexane, 2 mL of methanol and 2 mL of ultrapure water. It should be noted that this procedure is repeated twice with the same volume of conditioning solvent. The previously prepared samples were then added to the cartridges. The chestnut shell extracts used were obtained from two extraction techniques: UAE, whose extraction conditions were 70 °C/40min and SWE, whose extraction conditions were 220 °C/30min [9][11]. To prepare the sample solutions about 2 mg of each extract was weighed and dissolved in water (2 mL for UAE and 3 mL for SWE) with the help of ultrasonic bath. After passing the sample, it is necessary to wash the cartridge in order to remove the interfering compounds that were retained and for this 3 mL of ultrapure water was used and left to dry for about 10 minutes. At the end of this time, the cartridge is then changed into collection tubes. The compounds retained in each cartridge are eluted with 4 × 1 mL of *n*-hexane (solvent with the highest affinity for the analyte). After collection, the sample is evaporated in a nitrogen stream and 1 mL of *n*-hexane is redissolved to increase its concentration. 1 mL is transferred to the identified pathways and analysed in the GC. A schematic diagram of the described procedure is shown in Figure 3.2.

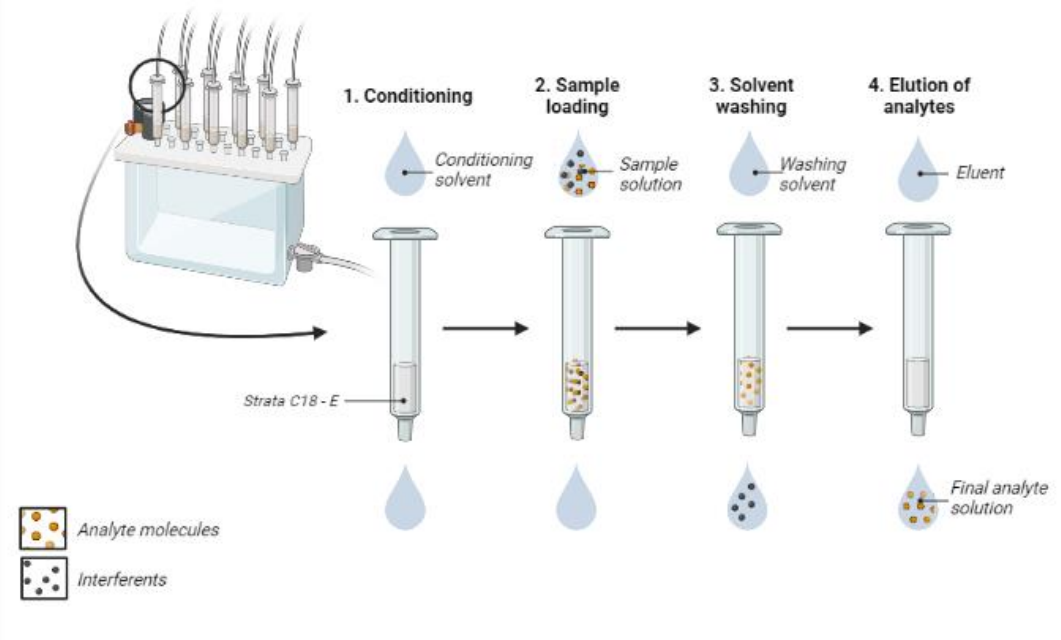


Figure 3.2 Sample preparation using the SPE technique.

3.3.3 Instrumental analysis

3.3.3.1 Gas chromatography - Electron Capture Detector (GC-ECD) analysis

OCPs and PYRs were determined by gas chromatography with an electron capture detector (Shimadzu, GC-2010). The chromatograph was equipped with an AOC-20i autosampler and injector operating at a temperature of 250°C with a splitless injection volume of 1 µL. The carrier gas used was helium with a linear velocity of 26.6 cm/sec and a flow rate of 1.09 mL/min at a pressure of 86.0 kPa. A 30 m × 0.25 mm × 0.25 µm Zebron-5MS capillary column was used. The oven temperature was programmed as follows: the initial temperature of 40 °C (1 min hold time), was increased to 120°C at a rate of 20°C min^{-1} (1 min hold time). This was followed by an increase to 150 °C at a rate of 10 °C min^{-1} (1 min hold time), then to 180 °C at a rate of 10°C min^{-1} (1 min hold time), then to 200 °C at a rate of 20 °C min^{-1} (1 min hold time) and finally an increase to 290 °C at a rate of 10 °C min^{-1} (3 min hold time). The detector temperature was operated at 300 °C.

3.3.3.2 Gas chromatography - Flame Photometric Detector (GC-FPD) analysis

OPPs were also determined by gas chromatography but with a flame photometric detector (Shimadzu, GC-2010). The chromatograph was equipped with an AOC-20i autosampler and injector operating at a temperature of 250 °C with a splitless injection volume of 1 µL. The carrier gas used was helium with a linear velocity of 25.4 cm/sec and a flow rate of 0.89 mL/min at a pressure of 92.2 kPa. A 30 m × 0.25 mm × 0.25 µm TR5 capillary column was used. The oven temperature was programmed as follows: the initial temperature of 100 °C (holding for 1 min), increased to 150 °C at a rate of 20 °C min^{-1} (1 min hold time). This was followed by an increase to 180 °C at a rate of 2 °C min^{-1} (2 min hold time) and finally an increase to 290 °C at a rate of 20 °C min^{-1} (5 min hold time). The detector temperature was operated at 290 °C.

4 Results and discussion

4.1 Identification of pesticides

Initially, the study of each pesticide of the family of OCPs, PYRs and OPPs was performed. For this, chromatographic separation of standard solutions of each compound, at a concentration of 50.00 µg L^{-1}, was performed and the chromatographic profiles present in Figures 4.1 and 4.2 were obtained. It should be noted that OCP and PYR pesticides are both separated and determined using a GC-ECD, being decided to perform the analysis of both families of compounds simultaneously, in order to facilitate the process and better manage the time. However, some adjustments had to be made in order to not have overlapping peaks.

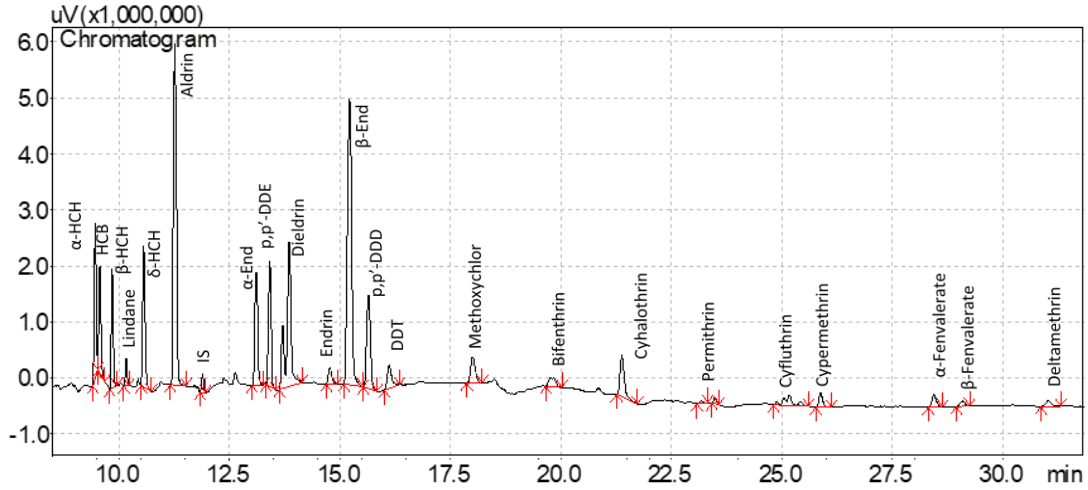


Figure 4.1 Chromatogram with the 14 OCPs and 8 PYRs studied in chestnut shell matrix at a concentration of 50.00 µg L^{-1}.

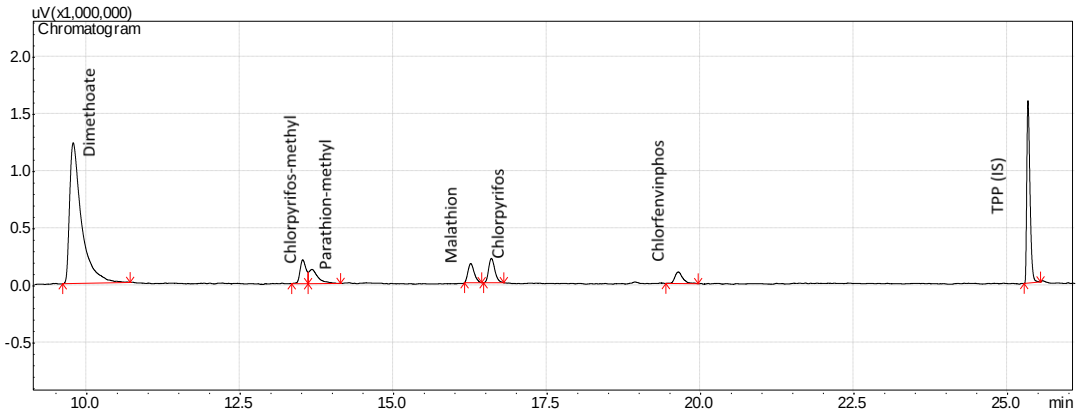


Figure 4.2 Chromatogram with the 6 OPPs pesticides in chestnut shell matrix at a concentration of 50.00 µg L^{-1}.

Thus, 14 OCPs will be studied, among them α-HCH, HCB, lindane, β-HCH, δ-HCH, aldrin, α-end, p,p'-DDE, dieldrin, endrin, β-end, p,p'-DDD, DDT and methoxychlor, 7 PYRs, of which bifenthrin, cyhalothrin, permethrin, cyfluthrin, cypermethrin, α-fenvalerate, β-fenvalerate and deltamethrin and finally 6 OPPs, these are dimethoate, chlorpyrifos-methyl, parathion-methyl, malathion, chlorpyrifos and chlorfenvinphos . These pesticides were selected taking into account the EU Regulation (EC) No 1223/2009 on cosmetic products.

4.2 Evaluation of pesticides in chestnut shell samples and extracts

4.2.1 QuEChERS sample preparation method

As previously stated, QuEChERS is a cheap and flexible method whose key parameters can be easily modified in order to optimise the process [35]. In this work, the optimization of this method consisted in improving the recovery percentage of the analytes and reducing the matrix effect that affects the sensitivity of the GC analysis. To this end, in the cleaning step, different sorbent combinations were tested in order to obtain the best results.

4.2.1.1 Selection of the d-SPE sorbents for clean-up step

The performance of the QuEChERS method was evaluated using three different combinations of d-SPE sorbents, varying their amounts. The sorbents used were PSA, C18, MgSO4 and Supel QuE Z-Sep and were combined as follows:

- **Cleanup 1 (CL1):** 50 mg PSA + 50 mg C18 + 150 mg $MgSO_4$;
- **Cleanup 2 (CL2):** 25 mg PSA + 25 mg C18 + 100 mg $MgSO_4$ + 5 mg Supel QuE Z-Sep;
- **Cleanup 3 (CL3):** 25mg PSA + 25mg C18 + 50mg $MgSO_4$ + 5 mg Supel QuE Z-Sep.

As already mentioned, in order to understand which is the best sorbent combination, studies of recovery and matrix effect were carried out. Additional results are presented in appendix A.

In Figure 4.3 is the graph that represent the recovery percentages obtained for the studied pesticides using CL1, CL2, and CL3.

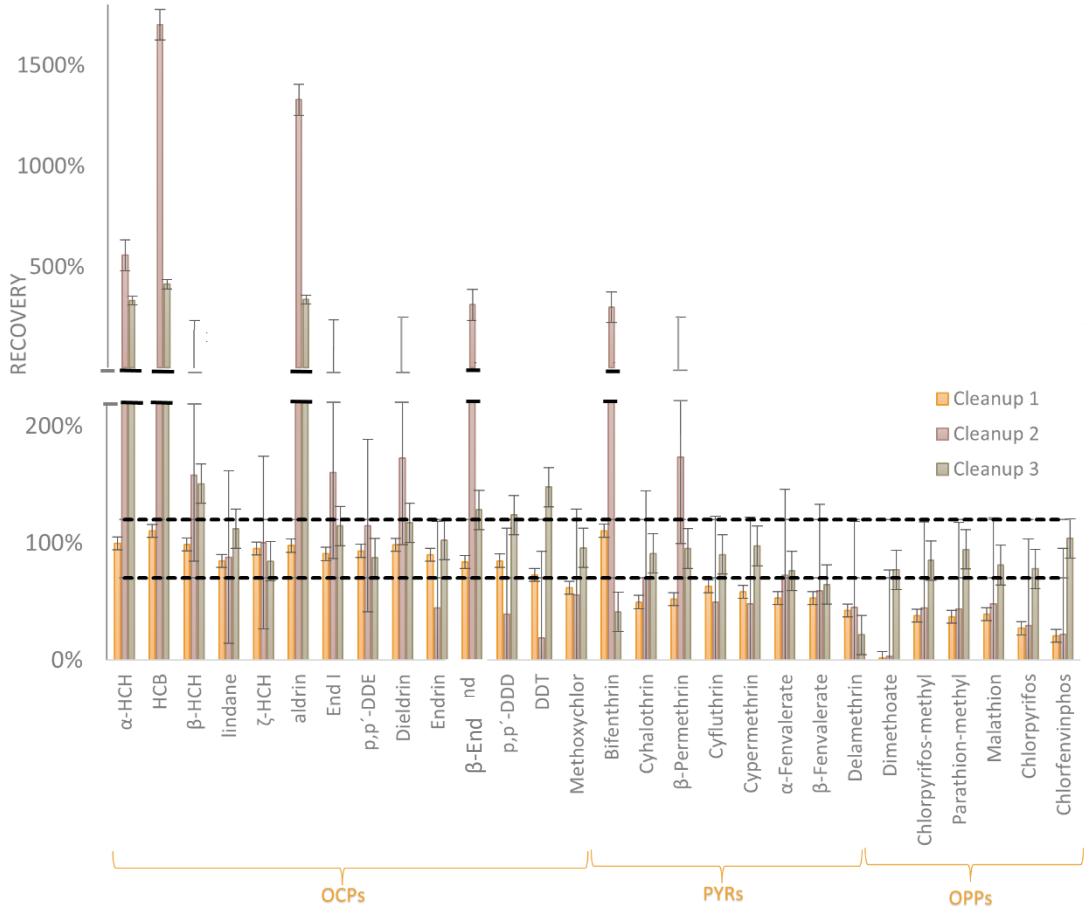


Figure 4.3 Recoveries obtained for the 28 pesticides under study, using CL1, CL2, and CL3.

When analysing the values of the recoveries obtained with CL1, it presented quite satisfactory values for the OCPs, in the range 73-110%. The only exception was methoxychlor that presented a recovery of 62%, a little below the minimum required by the EC Guidance Document nº SANTE/12682/2019 (70-120%). In the case of PYRs, it was only for bifenthrin that a value within the expected range was obtained, with a recovery of 111%. The recoveries of the remaining PYRs ranged from 42% to 63%. For the OPPs, recoveries above 70% were not obtained, reaching only values between 2 and 39%.

Therefore, based on the low recoveries achieved for some pesticides using CL1, it was decided to decrease the amounts of sorbents in the new d-SPE combination for the next test, assuming that they were in excess in the first set and therefore some analytes were being adsorbed in addition to the interfering compounds. Furthermore, it was decided to add a small amount of Supel QuE Z-Sep to ensure that all interferents were removed. Thus, regarding the recoveries

obtained using CL2, these underwent a small improvement for some pesticides compared to CL1. However, there are still some compounds where the recoveries decreased or did not change much: in OCPs, endrin showed a value of 45%, p,p'-DDD of 39%, DDT of 19% and methoxychlor of 56%; in PYRs, cyfluthrin presented a value of 49%, cypermethrin of 48%, β-fenvalerate of 59% and deltamethrin of 45% and in OPPs the recoveries remained below 70% for all compounds, ranging from 3 to 48%. On the other hand, as can be seen in Figure 4.3, excessively high recoveries were also obtained for the pesticides α-HCH, HCB, aldrin, β-end and bifenthrin, reaching the values of 557%, 1701%, 1329%, 311% and 299%, respectively. When analysing the RSD values obtained after 3 injections in the GC apparatus, none of the mentioned compounds presents very high values (with the exception of HCB, whose value is 80.24%), indicating that, in general, the data are not very dispersed in relation to the average and that the essays were well performed. It is believed that these errors may be equipment related. Moreover, according to the literature, continuous injections of sample solutions can cause the massacring of active sites or the formation of new ones, by the accumulation of little volatile matrix components along the GC system, causing matrix effect [58][59]. This may be one of the reasons for obtaining such high values, since this phenomenon can result in variations in the intensity and shape of the peaks [45]. The reason that it only occurred for some of the analytes, can be explained by the fact that the matrix effect, besides being influenced by the type of food matrix and by the analyte/matrix concentration ratio, is also influenced by the chemical structures and physical properties of analytes [44][60] Another reason may be the pre- spiking and post-spiking essays that were performed in different days, varying the essay conditions.

Finally, for CL3 it was decided to maintain the amounts of PSA, C18 and Supel QuE Z-Sep used in CL2, decreasing only the amount of $MgSO_4$. This approach was chosen based on two assumptions: (i) OPPs could be adsorbed by this sorbent and (ii) since the function of $MgSO_4$ is to remove water from the organic phase, along with it some OPPs could also be being removed, as these pesticides are somewhat soluble in water [62]. Analysing the values obtained, very high results are observed once again for α-HCH, HCB and aldrin (331%, 413% and 337%, respectively), supporting the hypothesis that this mismatch of values is due to possible equipment problems or matrix interferences. Even so and taking into account that with CL1 this signal increase was not observed, there is also the possibility that part of this problem may be related to the decrease in the amount of PSA in CL2 and CL3. However, in order to prove or disprove the assumptions made, further studies would be necessary to understand such results. The remaining OCPs presented quite satisfactory values, from 84% to 151%. For the PYRs, only for bifenthrin and deltamethrin were obtained values out of the required range, 41% and 21%,

respectively, while the recoveries of the others varied between 64% and 95%. For the OPPs, the best values were obtained compared to the other tests, with recoveries in the 77-104% range.

Figure 4.4 compares the average recoveries obtained for OCPs, PYRs and OPPs, not including the values for α-HCH, HCB, aldrin, β-end and bifenthrin with CL2 and for α-HCH, HCB and aldrin with CL3. Due to the disparity of the recoveries presented by the mentioned pesticides, these were excluded in the calculation of the mean as they would negatively influence the evaluation of the method performance.

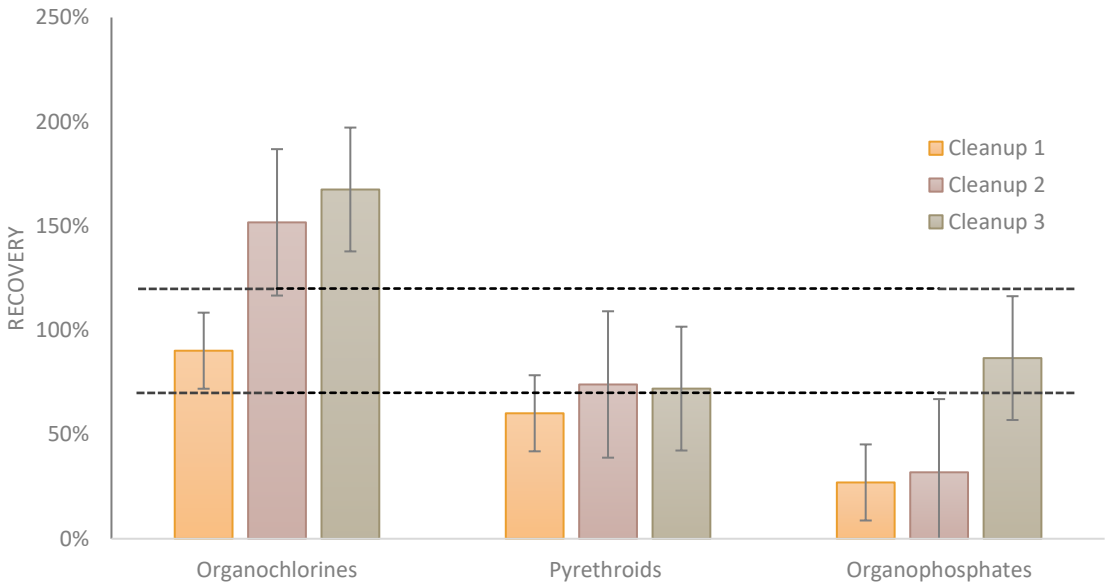


Figure 4.4 Average recoveries obtained for OCPs, PYRs and OPPs using CL1, CL2 and CL3.

By analyzing the Figure 4.4, it is clear that for the groups of pesticides studied, the method presented lower recoveries when CL1 was used. For OCPs the average result obtained was 90%, for PYRs it was 60% and for OPPs 27%, with the last being clearly the lowest value. When CL2 and CL3 were used, similar average recoveries were obtained for OCPs and PYRs, the big difference in the results obtained is in the OPPs. Thus, with CL2 the OCPs presented values of 152%, the PYRs of 74% and the OPPs of 32%. With CL3, OCPs reached 168%, PYRs 72% and OPPs 87%.

From the analysis made, the choice of the best combination of d-SPE sorbents would fall on either CL2 or CL3. However, it is important to analyse what the matrix effect of the studied compounds is.

In Figure 4.5 it is represented the results obtained in the matrix effect study using the three sets of d-SPE.

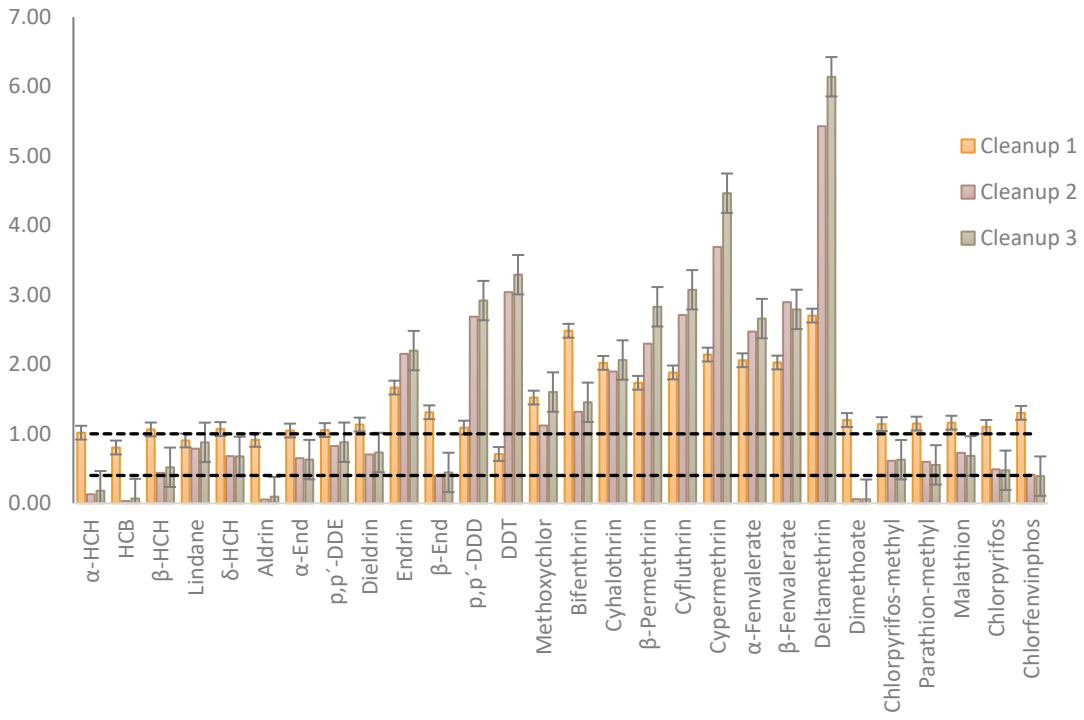


Figure 4.5 Matrix effect values obtained for the 28 pesticides under study using CL1, CL2 and CL3.

Observing the results obtained with CL1, 10 of the 14 OCPs presented matrix effect values within the range 0.80-1.20, the only exceptions being endrin, β-end, DDT and methoxychlor, with values of 1.67, 1.31, 0.71 and 1.52, respectively. For PYRs, no values within this range were obtained, while exactly the opposite was observed for OPPs, where all compounds are within the required range. With CL2, only compounds such as p,p'-DDE and methoxychlor presented acceptable values for the matrix effect, being 0.82 and 1.12, respectively. When CL3 was used, it was also observed, in most cases, values out of the stipulated, being the only compounds to escape the rule the lindane and the p,p'-DDE, both with a value of 0.88. The values obtained for the matrix effect outside the required, for the referred compounds, can be justified by equipment errors or even by the presence of interferents in the extract at the same retention time.

Therefore, based on these results, it is thought that obtaining recoveries much higher than 120% for the compounds α-HCH, HCB, aldrin, β-end and bifenthrin using CL2 and CL3 is not supported by the matrix effect studies since, for these pesticides, the matrix effect values are below the

range 0. 80-1.20 (with the exception of bifenthrin which obtained values of 1.32 with CL2 and 1.45 with CL3), which should be indication of a suppression of the signal and not an increase. In the case of PYR, the highest values for the matrix effect were obtained, especially when CL3 was used, in the range 2.06-6.14. The exceptions were β-Fenvalerate whose highest value (2.90), was obtained with CL2 and Bifenthrin (2.48), which was obtained with CL1. For OPPs, the matrix interferences were higher for all compounds with CL1, obtaining values between 1.10 and 1.30.

In Figure 4.6 the mean values of the matrix effect for the groups of pesticides studied are compared.

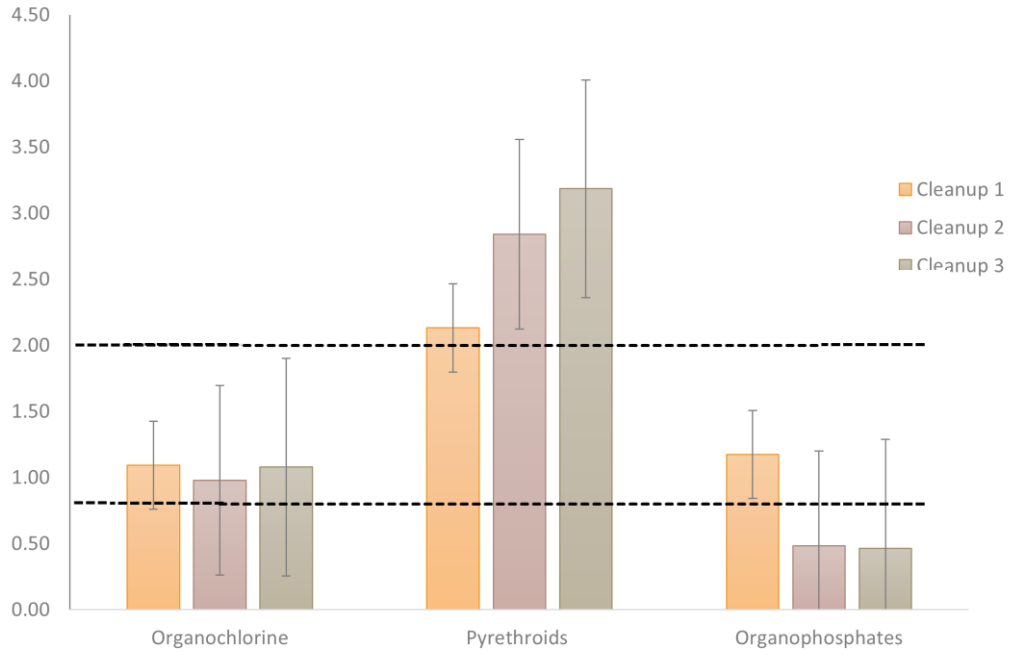


Figure 4.6 Average matrix effect values obtained for OCPs, PYRs and OPPs using CL1, CL2 and CL3.

For OCPs, the matrix effect was not felt, since the values are within the required range. PYRs are the compounds where the matrix effect is most felt, with the highest value reached when CL3 was used, at a value of 3.18. None of the studies found in the literature justifies this phenomenon, demonstrating that this topic remains a challenge for analysts and requires further studies. As for the OPPs, when CL2 and CL3 were used, the average values were slightly below the established limit, reaching values of 0.48 and 0.46, respectively.

In summary, CL2 was chosen for the study of OCPs and PYRs and CL3 for OPPs. For OPPs, clearly better recoveries were obtained when CL3 was used, possibly because as the chestnut shell has a low percentage of water and the OPPs are more polar compounds, the low percentage of

$MgSO_4$ that this d-SPE set contains causes less of these compounds to be adsorbed, increasing their percentage recovery. For OCPs and PYR, both CL2 and CL3 gave good results in terms of recoveries (above 70%). The choice ended up being CL2 because, before CL3 was tested (essentially in an attempt to optimise the method for OPPs), the method with the first set (CL2) had already been applied in the analysis of OCPs and PYRs compounds. As no significant improvements were observed for these compounds when using CL3, the need to repeat the analysis was not felt, moreover, with this set of sorbents, the PYRs reached the highest value of the matrix effect. However, it should be noted that ideally the same extract should have been optimised for all the pesticides studied.

4.2.1.2 Validation of the Analytical Method

To prove the applicability of the QuEChERS method when using either CL2 or CL3 as d-SPE sorbent combinations, in the clean-up step, it was validated in terms of matrix or solvent calibration, LOD, LOQ and precision.

For the chestnut shell extracts, matrix adjusted curves were obtained for the OCPs and PYRs at 6 concentration levels (6.00, 9.99, 19.99, 29.98, 39.98, 49.97 µg kg^{-1}), and it was not possible to obtain solvent adjusted curves (*n*-hexane) due to equipment error. As for the OPPs, both the matrix and solvent calibration curves were obtained, and in this case, the 6 concentration levels were 0.90, 1.50, 3.00, 4.50, 6.00, 7.50, and 8.99 µg kg^{-1} and 15.00, 25.00, 50.00, 100.00, 125.00 and 150.00 µg L^{-1}, respectively.

In the following tables are the equations of the calibration curves (represented in apendix B), the respective determination coefficients for the 28 compounds studied, as well as the values of $S_{y/x}$, LOD, LOQ and the average RSD (which corresponds to the average of the RSDs obtained for each compound at the different concentration levels).

Thus, in Table 4.1 are the results for OCPs and PYRs, which were obtained by duplicate analysis in GC.

Table 4.1 Method validation data for OCPs and PYRs (matrix-matched calibration).

Compound	Calibration curve in matrix	Coefficient R^2	$S_{y/x}$	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	RSD
α-HCH	y = 212209x - 1869357	0.9960	288865.7	4.08	13.61	6.2%

(continued on next page)

Table 4.1 (continued)

Compound	Calibration curve in matrix	Coefficient R^2	$S_{y/x}$	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	RSD
HCB	y = 128417x - 642844	0.9978	206820.1	4.83	16.11	4.5%
β-HCH	y = 437711x - 2288480	0.9975	418108.0	2.87	9.55	3.6%
Lindane (γ-HCH)	y = 124403x - 235993	0.9987	89744.8	2.16	7.21	5.8%
δ-HCH	y = 586057x - 408861	0.9971	640777.0	3.28	10.93	7.6%
Aldrin	y = 475315x - 15976645	1.0000	-	-	-	16.4%
α-Endosulfan	y = 410263x + 675397	0.9932	691897.2	5.06	16.86	5.0%
p.p´-DDE	y = 873663x + 1371268	0.9956	1184109.4	4.07	13.55	7.0%
Dieldrin	y = 857517x + 6987940	0.9971	972635.5	3.04	11.34	5.7%
Endrin	y = 136398x + 1284492	0.9998	46556.1	1.02	3.14	22.0%
β-Endosulfan	y = 3159837x - 7647379	0.9961	5264766.4	5.00	16.66	20.3%
p.p´-DDD	y = 4485711x + 5211871	0.9944	6242094.6	4.17	13.92	5.6%
DDT	y = 808053x + 6846205	0.9946	1210542.8	4.49	14.98	5.7%
Methoxychlor	y = 263126x + 2524778	0.9911	566224.7	6.49	21.52	10.4%
Bifenthrin	y = 287840x - 828367	0.9937	441678.3	4.60	15.34	13.1%
Cyhalothrin	y = 311318x + 2267322	0.9912	728263.9	7.02	23.39	10.5%
β-Permethrin	y = 24365x + 350176	0.9940	46086.4	5.67	18.92	6.6%
Cyfluthrin	y = 328103x + 6723530	0.9991	206734.8	1.89	6.30	5.7%
Cypermethrin	y = 148895x + 635393	0.9935	354307.3	7.14	23.80	-
α-Fenvalerate	y = 107646x + 1285895	0.9929	168731.3	4.70	15.67	13.9%
β-Fenvalerate	y = 112094x + 948378	0.9971	119905.2	3.21	10.70	11.8%
Deltamethrin	y = 77075x + 2081175	0.9941	127829.8	4.98	16.59	10.0%

As can be seen, all the OCPs and PYRs presented determination coefficients higher than 0.9911, however it should be noted that for some compounds it was necessary to remove some points of the straight line in order to obtain a good linearity. The limits of detection (LOD) and quantification (LOQ) presented values between 1.02-7.14 µg kg^{-1} and 3.14-23.80 µg kg^{-1}, respectively. These values, in general, are adequate for the analysis of pesticides in chestnuts, according to the MRL defined by the EU [63]. It stands out the aldrin, for which it was not possible to obtain LOD and LOQ values, because for lower concentrations this compound was not detected, being only possible to perform a calibration curve with two standards, which is not the most appropriate since it does not cover the whole range of concentrations desired for

this work. As for the RSD, taking into account values obtained in another study for these pesticides, its value should be less than 10.0%, however, compounds such as aldrin, endrin, β-end, methoxychlor, bifenthrin, cyhalothrin, α-fenvalerate and β-fenvalerate showed values above the defined limit [64]. For cypermethrin, it was not possible to calculate the RSD because in the second analysis, this compound presented peak area values very different from those expected and it was opted to use the values only from the first analysis.

In Tables 4.2 and 4.3 are the results obtained for the OPPs by triplicate analysis in GC.

Table 4.2 Method validation data for OPPS (matrix-matched calibration).

Compound	Calibration curve in matrix	Coefficient, R^2	$S_{y/x}$	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	RSD
Dimethoate	y = 7617984x - 753670	0.9979	1009553.2	0.40	1.33	2.8%
Chlorpyrifos-methyl	y = 736263x - 71673	0.9979	97083.8	0.40	1.32	2.9%
Parathion-methyl	y = 632847x - 29125	0.9963	110822.8	0.53	1.75	6.5%
Malathion	y = 676998x + 42914	0.9981	85050.3	0.38	1.26	3.3%
Chlorpyrifos	y = 796592x + 35048	0.9987	82346.8	0.31	1.03	3.7%
Chlorfenvinphos	y = 440172x + 49206	0.9967	73296.8	0.50	1.67	3.9%

Table 4.3 Method validation data for OPPS (solvent calibration)

Compound	Calibration curve in solvent	Coefficient, R^2	$S_{y/x}$	LOD (µg L^{-1})	LOQ (µg L^{-1})	RSD
Dimethoate	y = 19491x -358	0.9962	74451.3	11.46	38.20	7.3%
Chlorpyrifos-methyl	y =19622 - 24185	0.9985	47661.1	7.29	24.29	3.9%
Parathion-methyl	y = 12896x + 80365	0.9973	41349.0	9.62	32.06	5.4%
Malathion	y = 15966x + 35812	0.9986	37050.6	6.96	23.21	3.8%
Chlorpyrifos	y = 14832x + 7945	0.9961	57847.2	11.70	39.00	3.7%
Chlorfenvinphos	y = 6864x + 674	0.9977	15951.3	6.97	23.24	5.6%

The analytical matrix-matching curves for the OPPs showed excellent R^2, with values greater than 0.9963. Furthermore, the RSD values were all below the 10.0% set as limit. The limits of detection and quantification showed the values of 0.31-0.53 and 1.03-1.75 µg kg^{-1}, in that order, which, as what had already occurred for OCPs and PYRs, is appropriate for the analysis of these contaminants taking into account the MRLs set by the EU [63].

For these pesticides (OPPs) it was also possible to obtain the analytical curves developed in *n*-hexane, where the correlation coefficients reached values higher than 0.9961. The LOD and LOQ ranged between 6.96 and 11.70 µg L^{-1} and 23.21 and 39.00 µg L^{-1}, respectively. As for the RSD, values below 10.0% were obtained for all compounds.

4.2.1.3 Application of the QuEChERS method for chestnut shell analysis

The developed and validated methodology was applied in order to perform the monitoring of pesticides in *Castanea sativa* shell samples.

The first step was to inject two samples of the extract (Essay 1 and Essay 2) under study and plotting their chromatogram on GC-ECD. Figure 4.7 shows the graphs obtained side by side with a matrix standard.

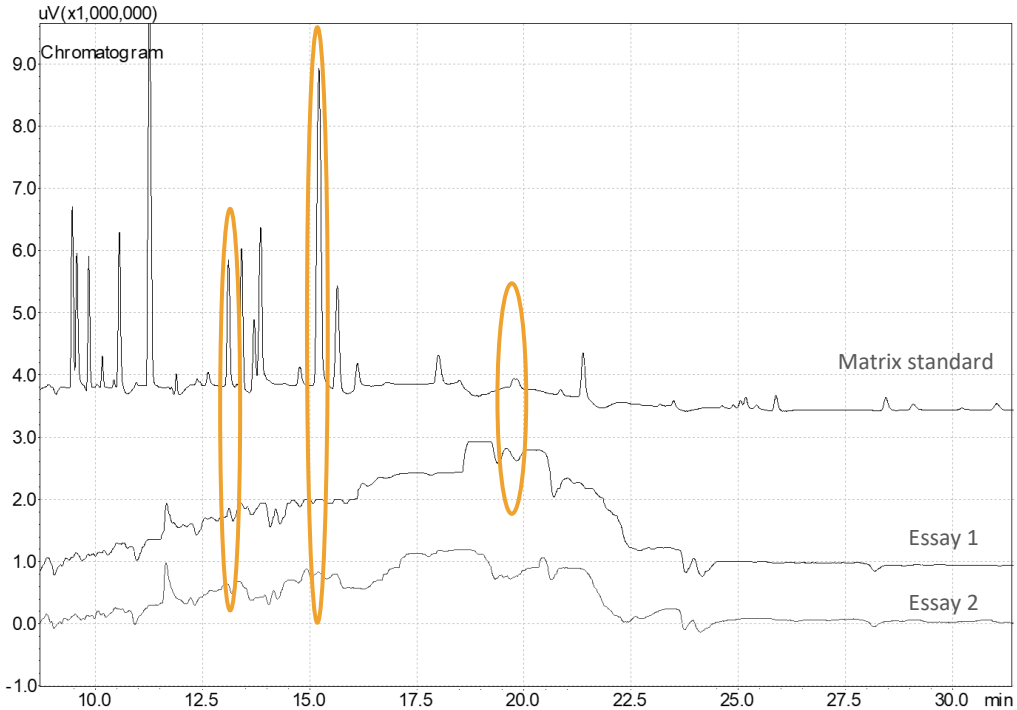


Figure 4.7 Chromatograms of a chestnut shell extract (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-ECD).

When analysing the graphs for both Essay 1 and Essay 2, some peaks were detected that could correspond to contaminants. In Figures 4.8 and 4.9 these peaks can be seen more clearly.

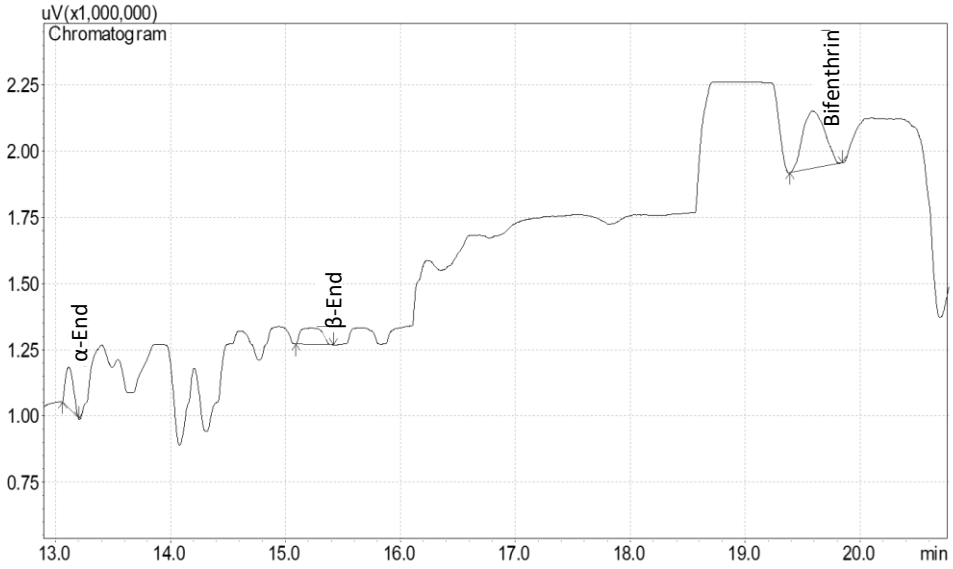


Figure 4.8 Chromatogram of a chestnut shell extract (Essay 1).

After a more detailed analysis of the graph obtained for Essay 1, it was realized that the peaks detected could correspond to compounds such as α-end, β-end and bifenthrin. Table 4.4 shows the retention time and area values of the detected peaks and the analyte concentrations calculated using the calibration curves present in Table 4.1.

Table 4.4 Obtained values for OCPs and PYRs chestnut extract - Essay 1.

Compound	Rt (min)	Peak area, A	[Analyte] (μg kg^{-1})	
α- Endosulfan	13.12	740939	0.16	< LOD
β- Endosulfan	15.24	787283	2.67	< LOD
Bifenthrin	19.58	2878484	12.89	Between LOD and LOQ

Thus, the levels found of α-end and β-end were lower than the LOD, the value of which for these compounds is 5.06 and 5.00 µg kg^{-1}, respectively. As for bifenthrin, the concentration detected is between the LOD (4.60 µg kg^{-1}) and the LOQ (15.34 µg kg^{-1}) values.

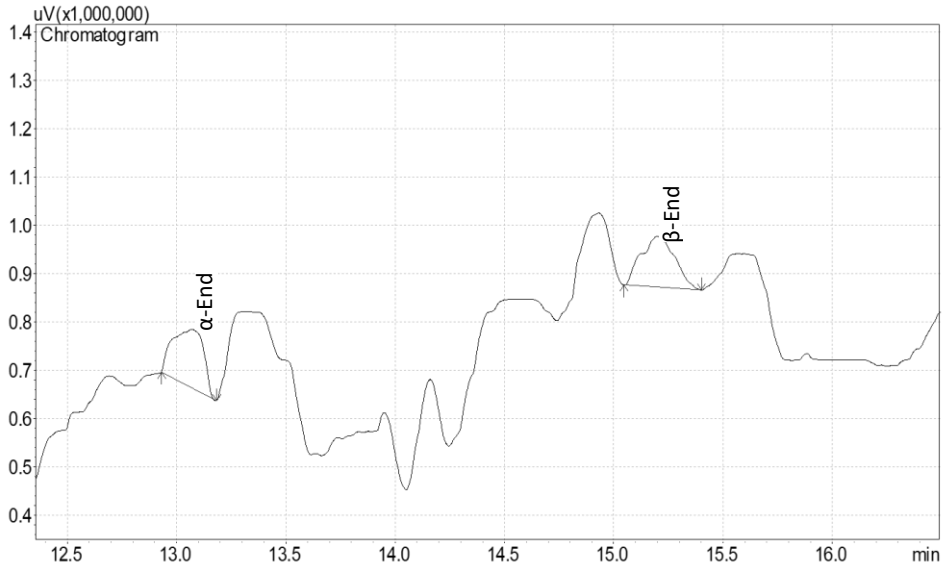


Figure 4.9 Chromatogram of a chestnut shell extract (Essay 2).

After analysis of the graph corresponding to Essay 2, the compounds α-end and β-end were also detected, as in Essay 1. However, in this essay no evidence of the presence of bifenthrin was detected, which does not support the results obtained in Essay 1 for this analyte. Thus, Table 4.5 shows the values obtained for each analyte detected in this assay.

Table 4.5 Obtained values for OCPs and PYRs chestnut extract - Essay 2.

Compound	Rt (min)	Peak area, A	[Analyte] (µg kg^{-1})	
α-Endosulfan	13.07	1163143	1.19	< LOD
β- Endosulfan	15.20	1114858	2.77	< LOD

For both α-end and β-end, the concentrations obtained were below the detection limit, which does not verify the possibility of the presence of these compounds in the sample under study.

In summary, based on the results obtained, it could be concluded that the chestnut shell sample under study is free of the target 14 OCP and 8 PYR analysed. However, it was decided to make new injections (Essay 1 and 2), this time with concentrated extracts (3:1), in order to detect trace concentrations of the contaminants studied. Figure 4.10 shows, then, the chromatograms obtained and the chromatogram of the standard with similar concentration.

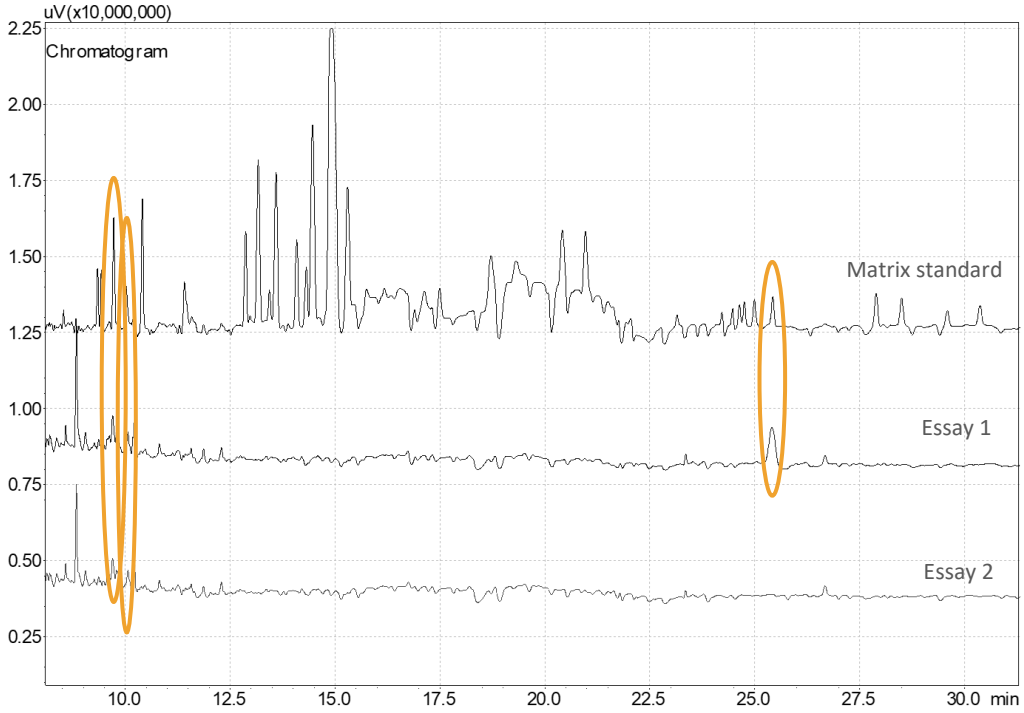


Figure 4.10 Chromatograms of a concentrated (3:1) chestnut shell extract (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-ECD).

After analysing the graphs, three pesticides were found to be possible in Essay 1, two of which also seem to be present in Essay 2. In the graphs of Figures 4.11 and 4.12 the peaks of these compounds are more evident.

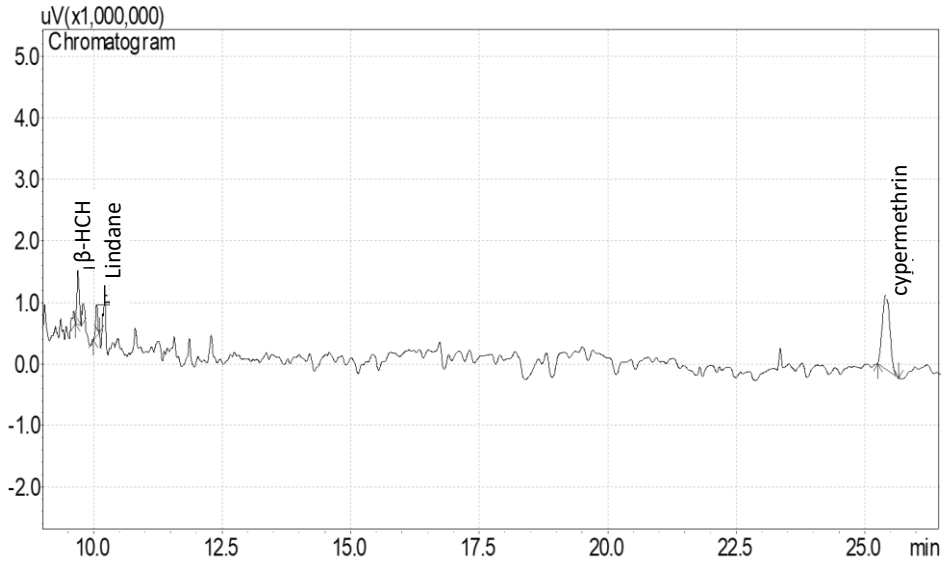


Figure 4.11 Chromatogram of a concentrated (3:1) chestnut shell extract (Essay 1).

As can be seen in Figure 4.11, taking into account the retention times at which the peaks came out, the compounds detected in Essay 1 are β-HCH, lindane and cypermethrin. Table 4.6 shows the values obtained for this essay, noting that the concentrations of the analytes will be divided by three since the LOD and LOQ values, previously calculated, were obtained with a not concentrated extract

Table 4.6 Obtained values for OCPs and PYRs in the concentrated extract (3:1) - Essay 1.

Compound	Rt (min)	Peak area, A	$[Analyte]/_3$ (µg/kg)	
β-HCH	9.69	2810621	3.88	Between LOD and LOQ
Lindane	10.06	1407410	4.40	Between LOD and LOQ
Cypermethrin	25.41	12410303	26.36	> LOQ

By the analysis of the results, it is concluded that the concentrations obtained for β-HCH and lindane are between the respective LOD and LOQ, that is, the concentration of β-HCH is between 2.87 and 9.55 µg kg^{-1} and lindane between 2.16 and 7.21 µg kg^{-1}. The amount of cypermethrin detected is slightly higher than the LOQ, which has a value of 23.80 µg kg^{-1}.

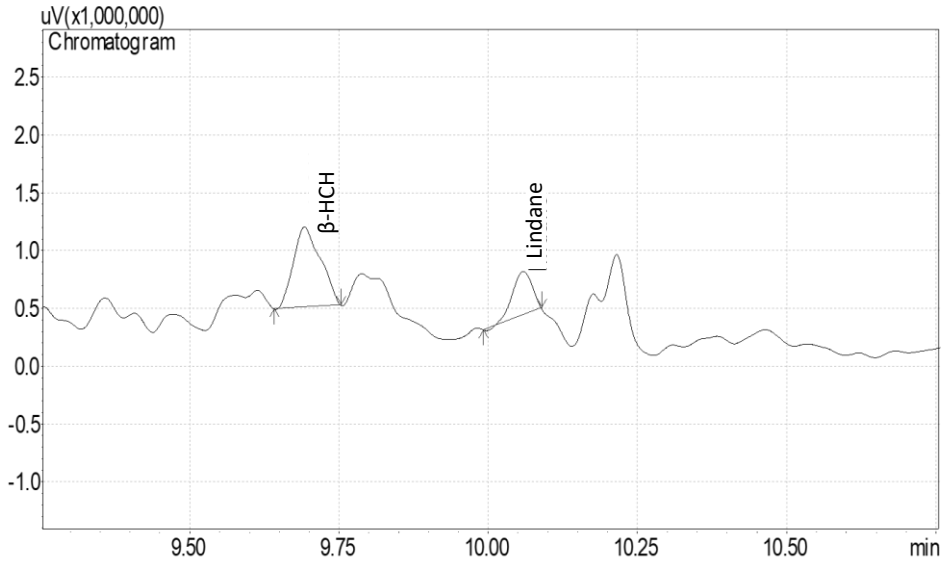


Figure 4.12 Chromatogram of a concentrated (3:1) chestnut shell extract (Essay 2).

Regarding the peaks detected in Essay 2, as shown in Figure 4.12, these correspond to the compounds β-HCH and lindane, repeating partially what happened in the first essay, since in this one the presence of cypermethrin was not detected. Table 4.7 shows the results obtained for these two analytes.

Table 4.7 Obtained values for OCPs and PYRs in the concentrated extract (3:1) - Essay 2.

Compound	Rt (min)	Peak area, A	$[Analyte]/_3$ (μg kg^{-1})	
β-HCH	9.69	2166520	3.39	Between LOD and LOQ
Lindane	10.06	825047	2.84	Between LOD and LOQ

In agreement with what was obtained in the first essay, the concentrations of β-HCH and lindane are between the respective LOD and LOQ. As for cypermethrin, as already mentioned, it was not detected in this essay, not supporting the result obtained in Essay 1.

Table 4.8 shows the mean values of the concentrations of the analytes detected in the previous essays, as well as the respective maximum residue limits (MRLs) defined by the EU.

Table 4.8 Average of the concentrations obtained for the analytes detected and their maximum required limits (MRLs) for the EU, in µg kg^{-1}.

Compound	$\overline{[Analyte]}$ (µg kg^{-1})	MRL (µg kg^{-1})
α- Endosulfan	0.67	100.00
β- Endosulfan	2.72	100.00
Bifenthrin	12.88	50.00
β-HCH	3.64	10.00
Lindane	3.62	10.00
Cypermethrin	26.36	50.00

Source of MRL values: EU Pesticides Database [63]

In summary, of the 6 pesticides detected in the samples under study, most were OCPs (4). Of these 6 compounds, 2 were found at concentrations below the LOD, which indicates that they are present in very low amounts and can be considered as non-detectable. These pesticides are α-end, whose average concentration was 0.67 µg kg^{-1} and β-end with an average concentration of 2.72 µg kg^{-1}. The others, bifenthrin (12.88 µg kg^{-1}), β-HCH (3.64 µg kg^{-1}) and lindane (3.62 µg kg^{-1}) presented concentrations between the respective LOD and LOQ and cypermethrin was the only compound with concentration higher than its LOQ, being also the contaminant present in greater amount in the sample (26.36 µg kg^{-1}). In any case, despite presenting values higher than the LOD, these 4 compounds are below the MRLs, as can be seen by the values present in Table 4.8.

In a study conducted by Liu *et al.* [31], using a different pesticide analysis method than the one used in this work, α-end was detected in chestnut just like what occurred in this study, but in a higher concentration range. This was not the only OCP to be detected in chestnut by the author, dieldrin was also detected, as well as quintozene (which was not the target of study in this dissertation). As for the PYRs, fenvalerate traces were found, contrary to what occurred in this work where the only PYRs detected were bifenthrin and cypermethrin. Analysing in general, most of the contaminants detected in the research carried out by Liu *et al.* [31], are in relatively low concentrations for chestnut similarly to what was obtained in the work developed in this dissertation.

In addition to the aforementioned study, two other studies were found on two different nuts, this time both using the QuEChERS preparation method. In the first, prepared by Cebi *et al.*[46],

the presence of 77 pesticides in hazelnuts was analysed. It was verified in that work that, similarly to what was obtained in this dissertation, all the pesticides detected were within the levels imposed by the EU (MRL), adding the fact that high recoveries and low LOD and LOQ values were obtained. In the second study, 47 compounds, were monitored in pecans. According to the author of this work, Barci *et al*.[47], the pesticides under study were not found in concentrations above the MRLs, however, he suggests monitoring a higher number of samples to ensure consumer safety.

Finally, there is a study developed by Varela-Martinez *et al*.[48], where the analysis of 38 multiclass pesticides is performed in a mixture of five dried fruits: strawberry, blackberry, passion fruit, pineapple and grapes. The author evaluated the presence of some OCP and PYR compounds that were also studied throughout this dissertation (lindane, endrin, etc.), yet no pesticides of this family were detected similarly to what occurred in this work.

Therefore, it can be concluded that the chestnut shell sample under study is not contaminated with OCPs or PYRs since most of the analytes were not present in the sample and those that were, were at levels below the MRLs defined by the EU for chestnuts.

Next, the extracts were injected into GC-FPD in order to analyse the presence of OPPs in the chestnut shell samples under study.

Figure 4.13 shows the chromatograms obtained from three injections of a chestnut shell extract (Essay 1, 2 and 3) and its standard.

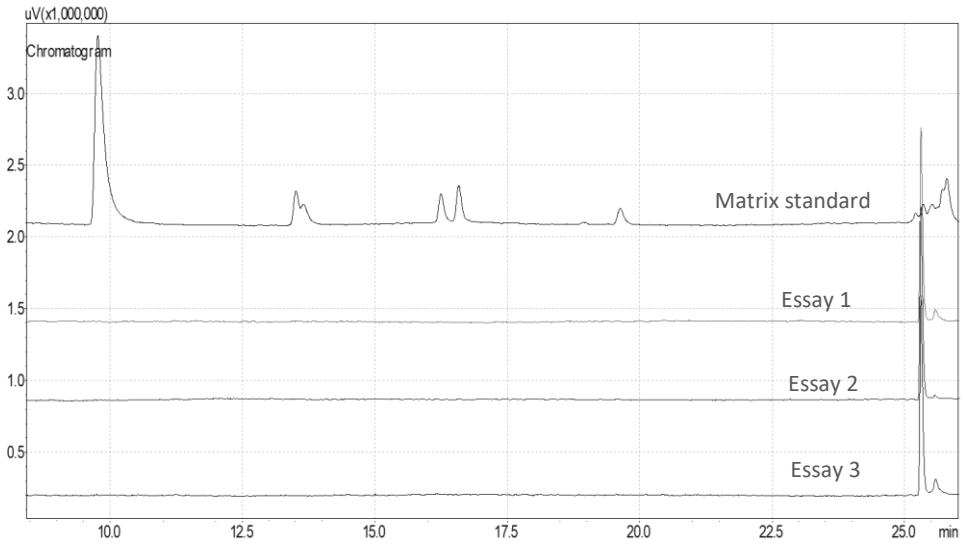


Figure 4.13 Chromatograms of a chestnut shell extract (Essay 1, 2 and 3) and chromatogram of a matrix standard (all four obtained in a GC-FPD).

As it is easy to see, no peaks were detected in the three essays performed. To ensure that the above results are correct and that there are no pesticides in trace concentrations, two more injections were performed, one of the concentrated extract (2:1) performing two essays (Essay 1 and Essay 2) and another of the concentrated extract (3:1) performing three essays (Essay 1, 2 and 3). In Figures 4.14 and 4.15 are the chromatograms obtained for the essays where the chestnut shell extract was concentrated (2:1) and (3:1), respectively.

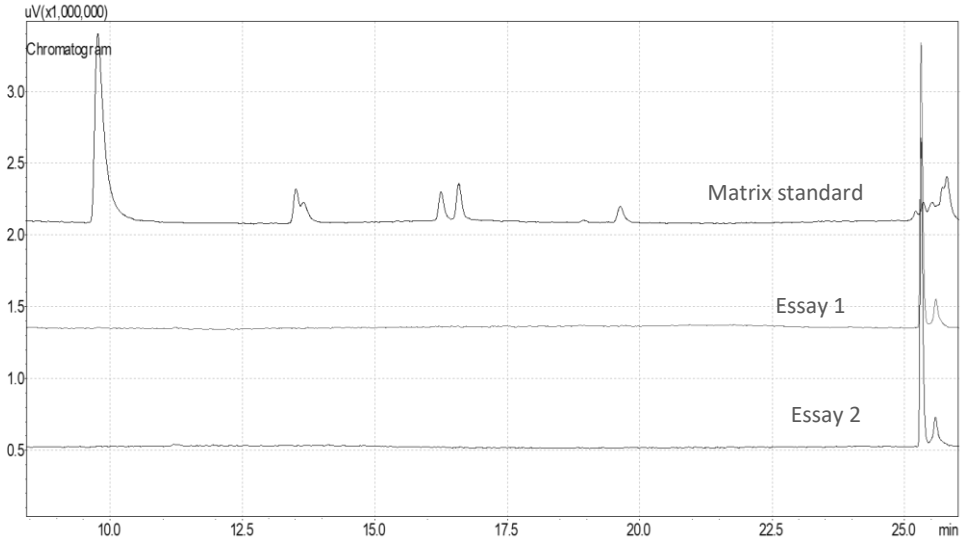


Figure 4.14 Chromatograms of a concentrated (2:1) chestnut shell extract (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-FPD).

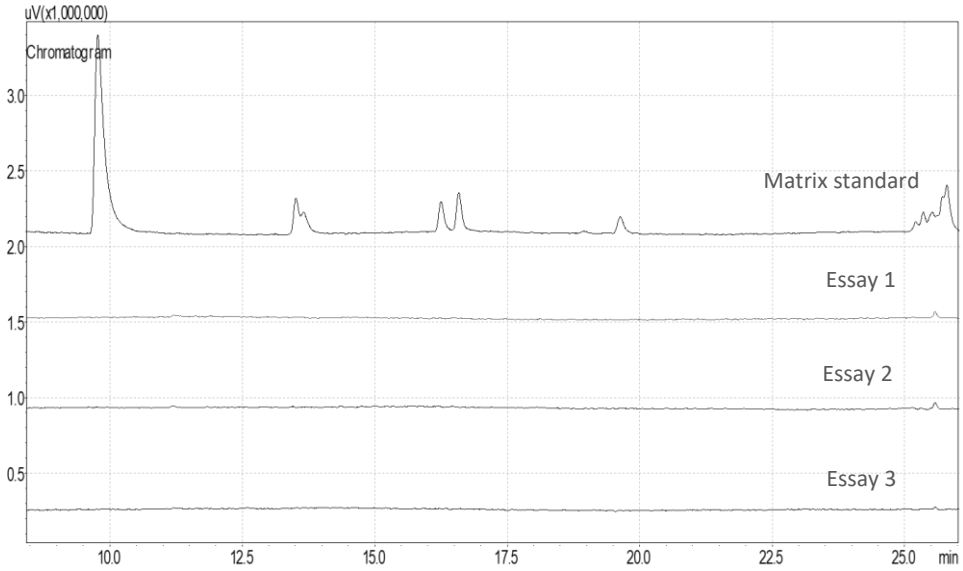


Figure 4.15 Chromatograms of a concentrated (3:1) chestnut shell extract (Essay 1, 2 and 3) and chromatogram of a matrix standard (all four obtained in a GC-FPD).

In the chromatogram of Figure 4.13, as well as in Figures 4.14 and 4.15, the only peaks that seem to stand out are those of the standard, so there are no signs of contamination of OPPs in any of the tests performed at different concentrations.

If we compare the results obtained by Liu *et al.* [31] for OPPs with those obtained in these tests, it is possible to notice that they are quite concordant, since the author of this research, for chestnut, also did not detect any organophosphorus compounds in the analyses performed.

In the study developed by Cebi *et al*. [44], within the several pesticides analysed, malathion was one of them. However, this was not detected in any of the hazelnut samples. In the study carried out by Barci *et al*. [45], the presence of dimethoate, in this case in pecan, was also evaluated. However, similarly to what was obtained in previous studies and in the study developed in this dissertation, its presence was not detected.

Finally, in the study developed by Varela-Martinez *et al.* [46], the author evaluated the presence of compounds such as chlorpyrifos-methyl, parathion-methyl, malathion, chlorpyrifos, but only chlorpyrifos was detected in two of the samples under study (blackberry and grape). In addition to this pesticide, iprodione and metalaxyl were also detected, but their presence was not evaluated in the sample studied throughout this work. According to the author, there are no defined MRLs for chlorpyrifos in the samples in question, however, in one of them it was found in an apparently slightly high concentration, even so, it cannot be confirmed whether this is an amount that can endanger the health of the consumer. The remaining compounds were either below the lowest calibrated level (metalaxyl) or below the MRL (iprodione).

Thus, taking into account the results obtained throughout these tests, it is concluded that the sample of chestnut shell under study is not contaminated by OPPs.

4.2.2 Application of SPE sample preparation method for UAE and SWE chestnut shells extracts

For the reasons already mentioned throughout this work, cosmetics is the field of interest for the application of chestnut shell extracts and, therefore, the analyse of contaminants present in the extracts have some relevance. Considering that the extracts are in liquid state, the pesticide extraction method to be applied could not be the QuEChERS (since this is commonly applicable to solid samples) but a method appropriate to the physical characteristics of the sample, namely the solid-phase extraction (SPE). The SPE method was applied to extracts resulting from the application of two extraction methods for bioactive compounds - UAE and SWE - which were

described in chapter 2.3.2. The results obtained will be discussed in the following sections. It should be noted that the SPE method applied was not validated.

Figure 4.16 shows the three chromatograms obtained on a GC-ECD, two of which result from the injection of the UAE extract after solid-phase extraction and one standard to facilitate the analysis of the results. Two injections of the extract under study were performed and are represented on the chromatograms by Essay 1 and Essay 2.

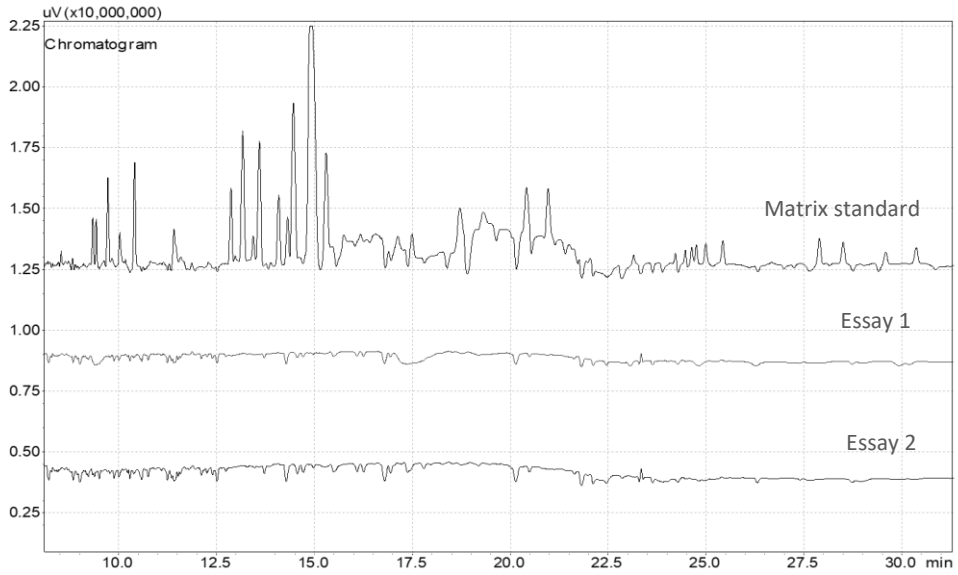


Figure 4.16 Chromatograms of an extract obtained from the UAE extraction method (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-ECD).

As it can be observed, in none of the two injections of this extract was evidence of peaks that could correspond to any of the analytes under study. However, another extract from chestnut shell was analysed, this time obtained from the SWE method. The results of this analysis are shown in Figure 4.17. Two injections of a sample of this extract were performed on the GC-ECD and chromatograms were plotted (Essay 1 and Essay 2).

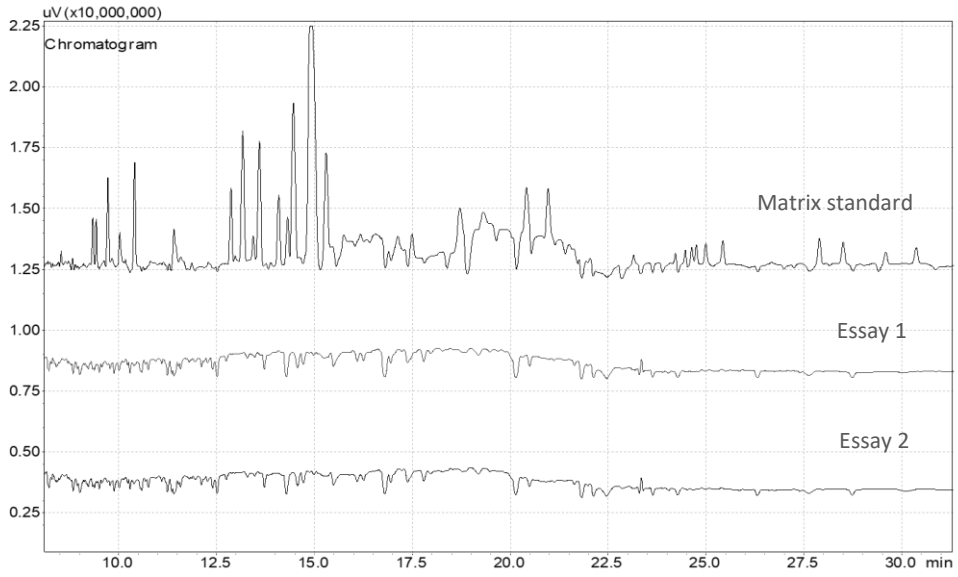


Figure 4.17 Chromatograms of an extract obtained from the SWE extraction method (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-ECD).

As already observed with the UAE extract, also in the SWE extract no contaminants from the family of OCPs and PYRs were detected. Thus, the presence of OPPs in these samples was analysed and for this purpose, two injections (Essay 1 and 2) of each extract (from UAE and from SWE) were performed in the GC-FPD resulting in the chromatograms shown in Figures 4.18 and 4.19.

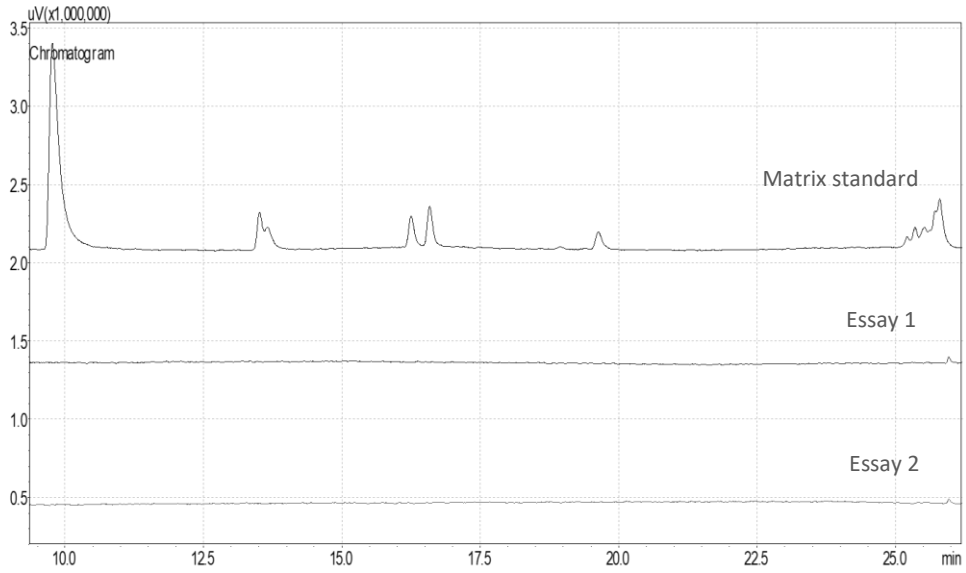


Figure 4.18 Chromatograms of an extract obtained from the UAE extraction method (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-FPD).

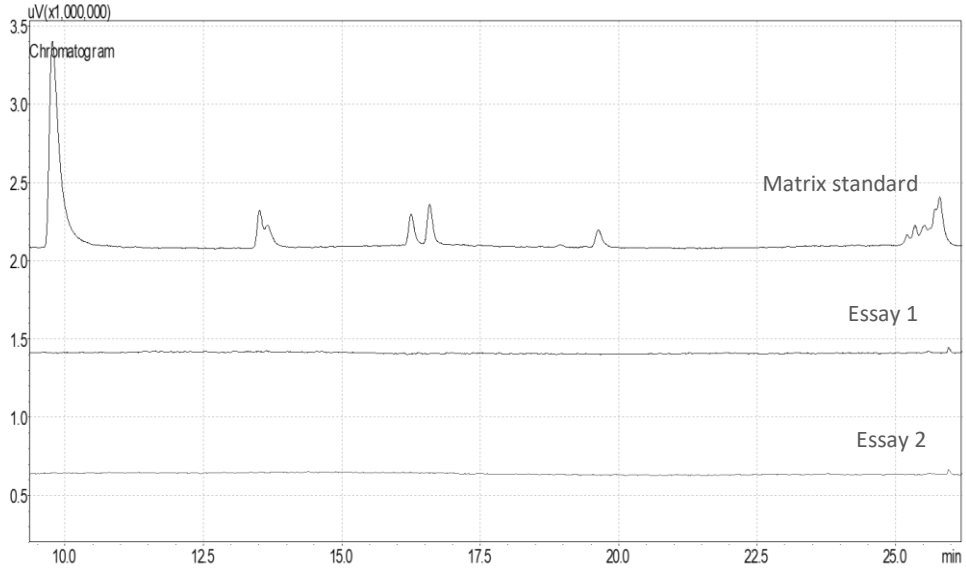


Figure 4.19 Chromatograms of an extract obtained from the UAE extraction method (Essay 1 and 2) and chromatogram of a matrix standard (all three obtained in a GC-FPD).

As already occurred in the analysis of OCPs and PYRs, no OPPs were detected in the UAE and SWE extracts. Therefore, it could be concluded that these extracts are safe for application in cosmetic products regarding the 28 pesticides evaluated. However, as previously mentioned, the SPE method did not undergo any kind of validation in this work but considering that by using the validated QuEChERS method there were also no results demonstrating that the chestnut shell had contaminants, it is believed that the UAE and SWE extracts do not have them either. In any case, the QuEChERS method and the SPE method are quite different when it comes to extraction of contaminants, and so, the most appropriate procedure would be to also validate the solid-phase extraction method to ensure that it was suitable for the intended use and if not, to modify it to meet the specified requirements.

4.3 Safety Data Sheet of chestnut shell extract

The main objective of this work was to evaluate the safety of chestnut shell extracts for possible application in cosmetic products since these compounds are an excellent source of antioxidants. After evaluating the extracts, no pesticides were detected, as it can be observed in section 4.2. Having this as a basis, its safety data sheet was developed.

***Castanea sativa* shells aqueous extract**

1. **Product and supplier identification**

   Trade Name: *Castanea sativa* shells aqueous extract

   Supplier Name: Sortegel

2. **Product details**

   General description: Aqueous extract of chestnut shells

   Composition: Rich in antioxidants, particularly ellagic acid, catechin and epigallocatechin.

3. **Registration Status**

   Suitability for cosmetics: tests on skin cell lines demonstrated the absence of irritation. The extract presented elastase and hyaluronidase inhibition properties.

4. **Data on manufacture**

   Origin:

   - Origin: Portugal (Trás-os-Montes)
   - Species: *Castanea sativa*
   - Plant parts used: shells
   - Animal origin involved? No

   Contaminants:

   - 14 tested organochlorine pesticides: not detected.
   - 8 tested pyrethroids pesticides: not detected.
   - 6 tested organophosphorus pesticides: not detected.

Conclusions and suggestions for future work

In this work, the presence of contaminants in the chestnut shell and in the UAE and SWE extracts obtained from it were evaluated. These extracts are intended to be used as active ingredients in cosmetic products and, due to this reason, it is of utmost importance their safety.

For the analysis in the chestnut shell, the first step was to apply an appropriate preparation method, in this case, the QuEChERS one. This method was optimised in the cleaning step by testing three different combinations of d-SPE sorbents (CL1, CL2 and CL3) and and analysing with which one the best recovery and matrix effect values were obtained. It was possible to conclude that CL2 offered good results for the sorbents study, while the best values of OPPs were obtained when CL3 was used. In a more detailed approach, for OCPs and PYRs the choice fell on CL2 because, when CL3 was tested, the QuEChERS method had already been validated for these pesticides and applied on real samples using CL2. Considering that no significant improvements were obtained for these compounds with CL3, the need to repeat the analysis was not felt. For the OPPs, clearly better recoveries were obtained when CL3 was used, probably due to the chestnut shell low percentage of water as well as the OPPs polarity. In fact, the low percentage of $MgSO_4$ that this sorbent set contains, leads to an inferior absorption of these compounds. After improving the method performance, it was validated in terms of matrix or solvent calibration, LOD, LOQ and precision and then applied to monitoring the pesticides in *C. sativa* Mill shells samples. After sample preparation using this method, the extracts were injected in the GC-ECD for the OCPs and PYRs analysis and in GC-FPD for the OPPs analysis. In the case of OCPs and PYRs, a total of about 6 pesticide compounds were detected during the analyses, 2 of these (α-end and β-end) were found at concentrations below the LOD, which indicates that they are present in very low amounts and can be considered as non-detectable. The remaining ones were either presented in concentrations between their respective LOD and LOQ, namely bifenthrin, β-HCH and lindane, or presented in concentrations higher than their LOQ, respectively cypermethrin. These 4 compounds, despite having been detected at values higher than the LOD, are below the MRLs set by the EU in chestnuts. In the case of OPPs, none of these compounds was detected in the analysed chestnut shell samples. Finally, since the aim of this work is to search for contaminants in extracts potentially used as active ingredients, it was of great relevance to analyse a sample of extracts coming from the shells and not only from the chestnut shell itself, even assuming that the results would be the same. For this, the SPE method was applied to the extracts resulting from two extraction methods, UAE and SWE, without SPE optimization. After injection of the extracts, none of the pesticides under study were detected.

It can be concluded that these extracts are safe for application in cosmetic products, in relation to the 28 pesticides tested. Nevertheless, and as suggestions for future work, when the sorbent set was selected, ideally, the same extract should be optimized for all pesticides studied. Furthermore, recovery and matrix effect studies should be further explored. As for the SPE method, this should be optimised and validated similarly to what was done with the QuEChERS method, in order to ensure that it was really suitable for the intended use and that it met the specified requirements.

In this way, it is understood that the objectives proposed for this dissertation were achieved, since it was ensured, through the monitoring of some compounds present in the EU regulation, that the extracts obtained from the chestnut shells are safe in terms of OCP, PYR and OPP, for future applications in the cosmetic field. Analytical methods of extraction and analysis of contaminants were also developed and optimized, and the techniques developed were applied to the extracts under study, in order to control the presence of different contaminants.

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Appendix

Appendix A - Optimization of the QuEChERS sample preparation method

In Appendix A are the results corresponding to the tests performed for optimization of the QuEChERS method. Within this, are present, in Appendix A.1, the results of the spiking studies. In the tables there are the retention time values at which the peaks of each compound appear, their area values and the respective standard deviations and relative standard deviations, when the three sorbent sets CL1, CL2 and CL3 were used. In Appendix A.2 are the recovery and matrix effect values calculated from the areas obtained in the spiking studies.

Appendix A.1 – Spiking studies

Table A.1 Values obtained from the injection of a standard sample of OCPs and PYRs at a concentration of 50.00 µg L^{-1}.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	α-HCH	9.46	9.46	9241664	9859431	9550548	436827.13	4.57%
	HCB	9.57	9.57	6481382	7000867	6741124	367331.30	5.45%
	β-HCH	9.85	9.85	7692907	8743049	8217978	742562.32	9.04%
	Lindane	10.16	10.16	1733255	1885985	1809620	107996.98	5.97%
	δ-HCH	10.56	10.56	10120495	11430077	10775286	926014.10	8.59%
	Aldrin	11.26	11.27	32730404	36166152	34448278	2429440.92	7.05%

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Table A.1 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	IS	11.76	11.89	2203235	2298581	2250908	67419.27	3.00%
	α-Endosulfan	13.11	13.11	8830016	10535285	9682650	1205807.38	12.45%
	p.p´-DDE	13.41	13.42	10257750	12348971	11303361	1478716.09	13.08%
	Dieldrin	13.86	13.86	20100486	22043092	21071789	1373630.09	6.52%
	Endrin	14.77	14.77	1617206	1723748	1670477	75337.07	4.51%
	β- Endosulfan	15.21	15.23	33373228	39484240	36428734	4321138.34	11.86%
	p.p´-DDD	15.66	15.66	12085949	13672749	12879349	1122036.86	8.71%
	DDT	16.12	16.13	3329902	3872138	3601020	383419.07	10.65%
	Methoxychlor	18.01	18.02	3530802	3646601	3588702	81882.82	2.28%
Pyrethroids	Bifenthrin	19.76	19.85	2190585	2018972	2104779	121348.47	5.77%
	Cyhalothrin	21.39	21.39	4225206	4123621	4174413	71831.30	1.72%
	β-Permethrin	23.50	23.50	449296	490121	469709	28867.71	6.15%
	Cyfluthrin	25.18	25.18	3361283	3051679	3206481	218922.95	6.83%
	Cypermethrin	25.89	25.89	1585741	1484988	1535364	71242.71	4.64%
	α-Fenvalerate	28.45	28.46	1673361	1555304	1614333	83479.26	5.17%
	β-Fenvalerate	29.09	29.09	988768	922760	955764	46674.35	4.88%

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Table A.2 Values obtained from the injection of a sample pre-spiked with OCPs and PYRs standards at a concentration of 50.00 µg L^{-1}, using CL1 in the clean-up step (average of 2 essays).

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Pyrethroids	**Deltamethrin**	31.03	31.05	1016840	988823	1002831	19810.66	1.98%
Organochlorines	**α-HCH**	9.45	9.45	8961712	9613981	9287846	659786.06	7.68%
	HCB	9.56	9.56	5718848	6158533	5938691	380525.34	6.99%
	β-HCH	9.84	9.84	7776580	8588907	8182743	727489.91	10.80%
	Lindane	10.15	10.15	1285681	1363004	1324343	95957.75	7.83%
	δ-HCH	10.55	10.55	9801878	11049384	10425631	918590.93	9.25%
	Aldrin	11.26	11.26	29095471	31531144	30313307	3179171.82	10.97%
	IS	11.63	11.65	2589049	2701444	2645246	79475.41	9.12%
	α-Endosulfan	13.10	13.10	8363650	9656497	9010074	1018338.49	10.98%
	p.p´-DDE	13.41	13.41	10257328	11835036	11046182	1753237.11	16.39%
	Dieldrin	13.85	13.85	21801139	24430560	23115849	3317613.46	15.34%
	Endrin	14.77	14.77	2336437	2711656	2524046	528842.09	19.69%
	β-Endosulfan	15.22	15.22	34068835	43341496	38705166	6804071.54	15.47%
	p.p´-DDD	15.64	15.64	10611422	12676956	11644189	1869533.41	15.89%
	DDT	16.11	16.10	1891289	1861859	1876574	104322.33	5.84%
	Methoxychlor	18.01	18.01	3060802	3644908	3352855	514590.21	14.71%

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Tabel A.2 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Pyrethroids	Bifenthrin	19.75	19.74	5160577	6240412	5700495	988652.80	19.27%
	Cyhalothrin	21.37	21.37	4046829	4378567	4212698	554507.52	12.56%
	β-Permethrin	23.49	23.49	393728	456497	425113	44384.60	10.46%
	Cyfluthrin	25.17	25.16	3767044	3845881	3806463	507771.51	13.91%
	Cypermethrin	25.87	25.87	1716911	2058001	1887456	398386.72	20.35%
	α-Fenvalerate	28.43	28.43	1604090	1893370	1748730	259150.99	13.95%
	β-Fenvalerate	29.07	29.06	964589	1094833	1029711	92096.77	9.66%
	Deltamethrin	31.01	31.00	1047155	1250490	1148823	197554.78	16.21%

Table A.3 Values obtained from the injection of a sample pos-spiked with OCPs and PYRs standards at a concentration of 50.00 µg L^{-1}, using CL1 in the clean-up step (average of 2 essays).

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	α-HCH	9.46	9.46	10111807	9302837	9707322	839932.90	7.65%
	HCB	9.56	9.56	5408841	5416211	5412526	189866.27	3.60%
	β-HCH	9.84	9.84	9265624	8208120	8736872	929227.48	8.72%
	Lindane	10.15	10.15	1690938	1582261	1636600	126741.96	6.98%

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Table A.3 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	δ-HCH	10.56	10.56	12124555	10899066	11511810	1091574.21	8.10%
	Aldrin	11.26	11.26	31943184	30930762	31436973	2047330.53	6.31%
	IS	11.66	11.66	2977270	2725438	2851354	225259.90	6.49%
	α-Endosulfan	13.11	13.11	10321353	9959061	10140207	646471.67	6.15%
	p.p´-DDE	13.42	13.41	12286272	11543242	11914757	1003647.04	8.05%
	Dieldrin	13.85	13.85	24860861	22904927	23882894	2135960.35	8.14%
	Endrin	14.77	14.77	2782495	2782840	2782668	109962.53	3.98%
	β-Endosulfan	15.23	15.22	50847413	44612498	47729956	5620709.43	10.80%
	p.p´-DDD	15.65	15.65	14744352	13313337	14028844	1257982.77	8.10%
	DDT	16.11	16.11	2600858	2507455	2554156	317079.66	12.84%
	Methoxychlor	18.01	18.02	5597971	5325809	5461890	618296.72	11.83%
Pyrethroids	Bifenthrin	19.76	19.76	5117987	5336835	5227411	558316.10	11.66%
	Cyhalothrin	21.38	21.38	8637463	8238283	8437873	837839.02	11.21%
	β-Permethrin	23.50	23.50	838743	790297	814520	97787.00	12.50%
	Cyfluthrin	25.18	25.18	6354806	5723674	6039240	572226.87	9.46%
	Cypermethrin	25.88	25.88	3475073	3100370	3287721	273083.30	9.78%

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Table A.3 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
	α-Fenvalerate	28.45	28.45	3537704	3110347	3324026	302186.50	10.19%
	β-Fenvalerate	29.09	29.08	2127818	1746205	1937011	269841.00	13.53%
	Deltamethrin	31.03	31.03	2964157	2455054	2709605	359990.61	13.72%

Table A.4 Values obtained from the injection of a sample pre-spiked with OCPs and PYRs standards at a concentration of 50.00 µg L^{-1}, using CL2 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	**α-HCH**	9.44	9.44	6939284	6784843	6862064	109206.28	1.59%
	HCB	9.54	9.54	3583955	3695926	3639941	79175.45	2.18%
	β-HCH	9.83	9.82	5992455	5399882	5696169	419012.39	7.36%
	Lindane	10.15	10.14	1206746	1298119	1252433	64610.47	5.16%
	δ-HCH	10.54	10.53	7478135	7206694	7342415	191937.77	2.61%
	Aldrin	11.23	11.23	25895839	23582827	24739333	1635546.47	6.61%
	IS	11.60	11.58	2394654	2431588	2413121	26116.28	1.08%
	α-Endosulfan	13.07	13.06	11073773	9083441	10078607	1407377.25	13.96%
	p,p´-DDE	13.37	13.35	12868758	8544605	10706682	3057637.91	28.56%

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Table A.4 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	Dieldrin	13.81	13.80	27340036	23778501	25559269	2518385.55	9.85%
	Endrin	14.82	14.78	1126255	2086391	1606323	678918.68	42.27%
	β-Endosulfan	15.17	15.15	50597940	40331227	45464584	7259662.38	15.97%
	p,p´-DDD	15.59	15.57	13179324	13848218	13513771	472979.48	3.50%
	DDT	16.05	16.03	2380442	1775645	2078044	427656.06	20.58%
	Methoxychlor	17.91	17.86	3071606	1390919	2231263	1188425.17	53.26%
Pyrethroids	Bifenthrin	19.69	19.66	6889662	9703866	8296764	1989942.73	23.98%
	Cyhalothrin	21.30	21.25	5248953	5943080	5596017	490821.91	8.77%
	β-Permethrin	23.42	23.39	2023885	1719444	1871665	215272.30	11.50%
	Cyfluthrin	25.08	25.04	4031822	4543950	4287886	362129.18	8.45%
	Cypermethrin	25.78	25.73	2471965	2994490	2733228	369480.97	13.52%
	α-Fenvalerate	28.34	28.29	2731273	3035654	2883464	215229.87	7.46%
	β-Fenvalerate	28.96	28.92	1568356	1712075	1640216	101624.68	6.20%
	Deltamethrin	30.90	30.83	2244361	2634389	2439375	275791.44	11.31%

Table A.5 Values obtained from the injection of a sample pos-spiked with OCPs and PYRs standards at a concentration of 50.00 µg L^{-1}, using CL2 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	α-HCH	9.33	9.33	1351307	1112252	1231780	169037.41	13.72%
	HCB	9.42	9.41	92584	335415	214000	171707.45	80.24%
	β-HCH	9.71	9.71	3752793	3449033	3600913	214790.76	5.96%
	Lindane	10.02	10.02	1386132	1460625	1423379	52674.51	3.70%
Organochlorines	δ-HCH	10.40	10.40	7345987	7284540	7315264	43449.59	0.59%
	Aldrin	11.07	11.07	2043355	1680553	1861954	256539.75	13.78%
	IS	-	-	-	-	-	-	-
	α-Endosulfan	12.86	12.86	6280728	6295011	6287870	10099.61	0.16%
	p,p´-DDE	13.15	13.15	9335950	9293503	9314727	30014.56	0.32%
	Dieldrin	13.58	13.58	15042405	14595860	14819133	315755.00	2.13%
	Endrin	14.31	14.32	3406392	3781183	3593788	265017.26	7.37%
	β-Endosulfan	14.44	14.45	14133143	15074235	14603689	665452.53	4.56%
	p.p´-DDD	14.87	14.88	34068620	35136767	34602694	755293.99	2.18%
	DDT	15.28	15.29	10629323	11267738	10948531	451427.58	4.12%
	Methoxychlor	17.48	17.49	3243511	4794391	4018951	1096637.76	27.29%
Pyrethroids	Bifenthrin	19.30	19.30	2805846	2740785	2773316	46005.07	1.66%

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Table A.5 (continued)

Pyrethroids	**Cyhalothrin**	**20.95**	**20.95**	**7739079**	**8100457**	**7919768**	**255532.83**	**3.23%**
	β-Permethrin	23.15	23.15	945143	1213495	1079319	189753.52	17.58%
	Cyfluthrin	24.75	24.75	8749632	8635489	8692561	80711.29	0.93%
	Cypermethrin	25.43	25.43	4055925	7277902	5666914	2278281.79	40.20%
	α-Fenvalerate	27.89	27.89	3739698	4241996	3990847	355178.32	8.90%
	β-Fenvalerate	28.49	28.50	2581710	2953804	2767757	263110.19	9.51%
	Deltamethrin	30.37	30.36	6240299	4649849	5445074	1124617.98	20.65%

Table A.6 Values obtained from the injection of a sample pre-spiked with OCPs and PYRs standards at a concentration of 50.00 µg L^{-1}, using CL3 in the clean-up step.

Compound		**Retention time, Rt (min)**		**Peak area**			**Standard deviation, S**	**RSD**
		Essay 1	**Essay 2**	**Essay 1**	**Essay 2**	**Mean**		
Organochlorines	**α-HCH**	9.33	9.33	5686442	5707151	5696797	14643.47	0.26%
	HCB	9.42	9.42	1914980	1899261	1907121	11115.01	0.58%
	β-HCH	9.71	9.71	6423510	6405175	6414343	12964.80	0.20%
	Lindane	10.02	10.07	848092	2710999	1779546	1317274.17	74.02%
	δ-HCH	10.40	10.40	6073327	6222763	6148045	105667.21	1.72%
	Aldrin	11.07	11.07	10993114	11082615	11037865	63286.76	0.57%
	IS	11.57	11.57	2335100	2432675	2383888	68995.94	2.89%

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Table A.6 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	α-Endosulfan	12.85	12.86	6939757	6995574	6967666	39468.58	0.57%
	p,p´-DDE	13.15	13.15	8806695	8538847	8672771	189397.14	2.18%
	Dieldrin	13.58	13.58	18202543	17936074	18069309	188422.04	1.04%
	Endrin	14.31	14.31	3448276	4080260	3764268	446880.17	11.87%
	β-Endosulfan	14.45	14.45	21003940	20441779	20722860	397507.86	1.92%
	p.p´-DDD	14.88	14.88	46600657	46457712	46529185	101077.38	0.22%
	DDT	15.29	15.29	17390603	17639856	17515230	176248.49	1.01%
	Methoxychlor	17.49	17.49	5362663	5648318	5505491	201988.59	3.67%
Pyrethroids	Bifenthrin	19.31	19.31	1411088	1111637	1261363	211743.83	16.79%
	Cyhalothrin	20.95	20.95	8316957	7347176	7832067	685738.72	8.76%
	β-Permethrin	23.34	23.35	1321634	1183311	1252473	97809.13	7.81%
	Cyfluthrin	24.63	24.75	8578053	9170324	8874189	418798.84	4.72%
	Cypermethrin	25.43	25.42	2967015	10403573	6685294	5258440.59	78.66%
	α-Fenvalerate	27.89	27.90	3783757	2751627	3267692	729826.12	22.33%
	β-Fenvalerate	28.50	28.50	1902859	1537989	1720424	258002.05	15.00%
	Deltamethrin	30.22	30.24	1640585	1001048	1320817	452220.95	34.24%

Table A.7 Values obtained from the injection of a sample pos-spiked with OCPs and PYRs standards at a concentration of 50.00 µg L^{-1}, using CL3 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organochlorines	α-HCH	9,33	9,33	1772333	1672542	1722438	70562,89	4,10%
	HCB	9,42	9,42	472952	450925	461939	15575,44	3,37%
	β-HCH	9,71	9,71	4279083	4232499	4255791	32939,86	0,77%
	Lindane	10,02	10,02	1642090	1532042	1587066	77815,69	4,90%
	δ-HCH	10,40	10,40	7356611	7196208	7276410	113422,05	1,56%
	Aldrin	11.07	11.07	3315068	3238197	3276633	54356.01	1.66%
	IS	-	-	-	-	-	-	-
	α-Endosulfan	12.86	12.86	5224783	6943366	6084075	1215221.69	19.97%
	p,p´-DDE	13.15	13.15	9193011	10675971	9934491	1048611.07	10.56%
	Dieldrin	13.58	13.58	12933922	17862124	15398023	3484765.05	22.63%
	Endrin	14.31	14.32	3719376	3624283	3671830	67240.91	1.83%
	β-Endosulfan	14.45	14.45	15124937	17199640	16162289	1467036.56	9.08%
	p.p´-DDD	14.88	14.88	33748639	41420351	37584495	5424719.58	14.43%
	DDT	15.28	15.28	10834947	12868616	11851782	1438021.14	12.13%
	Methoxychlor	17.47	17.48	4828008	6666685	5747347	1300140.98	22.62%
Pyrethroids	Bifenthrin	19.30	19.29	3019589	3099661	3059625	56619.45	1.85%

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Table A.7 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Pyrethroids	Cyhalothrin	20.94	20.94	8672816	8547732	8610274	88447.74	1.03%
	β-Permethrin	-	23.15	-	1328843	1328843	-	-
	Cyfluthrin	24.75	24.75	9863066	9847844	9855455	10763.58	0.11%
	Cypermethrin	25.43	25.42	4360308	9349322	6854815	3527765.63	51.46%
	α-Fenvalerate	27.89	27.89	4294158	4292473	4293316	1191.47	0.03%
	β-Fenvalerate	28.50	28.49	2799980	2535832	2667906	186780.84	7.00%
	Deltamethrin	30.36	30.36	6151976	6165577	6158777	9617.36	0.16%

Table A.8 Values obtained from the injection of a standard sample of OPPs at a concentration of 50.00 µg L^{-1}.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.78	9.78	16767476	16965228	16866352	139831.60	0.83%
	Chlorpyrifos--methyl	13.52	13.52	1462311	1444735	1453523	12428.36	0.86%
	Parathion--methyl	13.66	13.66	1348018	1240429	1294223	76076.84	5.88%

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Table A.8 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Malathion	16.25	16.25	1317730	1270233	1293981	33585.34	2.60%
	Chlorpyrifos	16.59	16.59	1650322	1581306	1615814	48801.86	3.02%
	Chlorfenvinphos	19.63	19.63	805119	916055	860587	78443.32	9.12%
	TPP (IS)	25.80	25.33	6645791	5845686	6245738	565759.78	9.06%

Table A.9 Values obtained from the injection of a sample pre-spiked with OPPs standard at a concentration of 50.00 µg L^{-1}, using CL1 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.85	9.83	296096	446700	371398	106492.83	28.67%
	Chlorpyrifos--methyl	13.52	13.51	552847	681255	617051	90798.34	14.71%
	Parathion--methyl	13.66	13.66	448474	646104	547289	139745.02	25.53%
	Malathion	16.25	16.25	494552	670312	582432	124281.41	21.34%
	Chlorpyrifos	16.59	16.59	424722	530058	477390	74484.05	15.60%
	Chlorfenvinphos	19.63	19.63	175217	280806	228011	74662.95	32.75%
	TPP (IS)	25.53	25.53	1195596	2506596	1851096	927016.81	50.08%

Table A.10 Values obtained from the injection of a sample pos-spiked with OPPs standard at a concentration of 50.00 µg L^{-1}, using CL1 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.76	9.76	20135407	20300564	20217985	116783.71	0.58%
	Chlorpyrifos--methyl	13.51	13.51	1655378	1658281	1656829	2052.34	0.12%
	Parathion--methyl	13.65	13.65	1463606	1505682	1484644	29751.69	2.00%
	Malathion	16.25	16.25	1478461	1524085	1501273	32261.00	2.15%
	Chlorpyrifos	16.59	16.59	1765076	1787821	1776448	16083.14	0.91%
	Chlorfenvinphos	19.63	19.63	1101406	1139250	1120328	26759.78	2.39%
	TPP (IS)	25.58	25.79	4523946	5096584	4810265	404916.74	8.42%

Table A.11 Values obtained from the injection of a sample pre-spiked with OPPs standard at a concentration of 50.00 µg L^{-1}, using CL2 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.79	9.79	743635	775888	759762	22806.53	3.00%
	Chlorpyrifos--methyl	13.49	13.48	873564	859434	866499	9990.85	1.15%

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Table A.11 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Parathion--methyl	13.63	13.62	618298	868699	743499	177059.96	23.81%
	Malathion	16.22	16.22	903841	858718	881280	31906.64	3.62%
	Chlorpyrifos	16.56	16.56	613239	635217	624228	15540.51	2.49%
	Chlorfenvinphos	19.60	19.60	322463	263121	292792	41960.78	14.33%
	TPP (IS)	25.32	25.32	4383549	4245251	4314400	97791.38	2.27%

Table A.12 Values obtained from the injection of a sample pos-spiked with OPPs standard at a concentration of 50.00 µg L^{-1}, using CL2 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.87	9.86	1059206	972203	1015705	61520.41	6.06%
	Chlorpyrifos--methyl	13.46	13.46	901455	878584	890019	16171.89	1.82%
	Parathion--methyl	13.63	13.62	747299	796154	771726	34545.63	4.48%
	Malathion	16.17	16.18	978470	900380	939425	55218.11	5.88%

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Table A.12 (continued)

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Chlorpyrifos	16.52	16.52	808406	771065	789736	26403.93	3.34%
	Chlorfenvinphos	19.55	19.54	378519	329019	353769	35001.86	9.89%
	TPP (IS)	25.71	25.71	722487	711507	716997	7764.32	1.08%

Table A.13 Values obtained from the injection of a sample pre-spiked with OPPs standard at a concentration of 50.00 µg L^{-1}, using CL3 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.82	9.82	674769	834198	754483	112732.97	14.94%
	Chlorpyrifos--methyl	13.44	13.44	758920	790686	774803	22462.10	2.90%
	Parathion--methyl	13.59	13.59	597361	753090	675225	110116.89	16.31%
	Malathion	16.16	16.15	670774	762688	716731	64993.22	9.07%
	Chlorpyrifos	16.50	16.50	537934	660756	599345	86847.99	14.49%
	Chlorfenvinphos	19.54	19.54	295106	404825	349966	77583.12	22.17%
	TPP (IS)	25.70	25.70	752086	817418	784752	46196.49	5.89%

Table A.14 Values obtained from the injection of a sample pos-spiked with OPPs standard at a concentration of 50.00 µg L^{-1}, using CL3 in the clean-up step.

Compound		Retention time, Rt (min)		Peak area			Standard deviation, S	RSD
		Essay 1	Essay 2	Essay 1	Essay 2	Mean		
Organophosphorus	Dimethoate	9.88	9.86	950396	1008135	979265	40827.71	4.17%
	Chlorpyrifos--methyl	13.46	13.46	947029	876385	911707	49952.85	5.48%
	Parathion--methyl	13.63	13.61	710091	719644	714867	6754.71	0.94%
	Malathion	16.18	16.17	907025	858637	882831	34215.77	3.88%
	Chlorpyrifos	16.52	16.51	759336	776224	767780	11941.48	1.56%
	Chlorfenvinphos	19.55	19.55	395393	276941	336167	83758.14	24.92%
	TPP (IS)	25.71	25.71	763753	843361	803557	56291.00	7.01%

Appendix A.2 - Recoveries and matrix effect obtained

Table A.15 Values of recovery and matrix effect obtained for each type of sorbent sets (CL1, CL2 and CL3), for OCPs and PYRs.

Compound		Recoveries						Matrix effect					
		CL1	CL2	CL3	CL1 mean	CL2 mean	CL3 mean	CL1	CL2	CL3	CL1 mean	CL2 mean	CL3 mean
Organochlorines	α-HCH	100%	557%	331%	90%	152%	168%	1.02	0.13	0.18	1.09	0.98	1.08
	HCB	110%	1701%	413%				0.80	0.03	0.07			
	β-HCH	99%	158%	151%				1.06	0.44	0.52			
	Lindane	85%	88%	112%				0.90	0.79	0.88			
	δ-HCH	95%	100%	84%				1.07	0.68	0.68			
	Aldrin	98%	1329%	337%				0.91	0.05	0.10			
	α-Endosulfan	91%	160%	115%				1.05	0.65	0.63			
	p,p´-DDE	93%	115%	87%				1.05	0.82	0.88			
	Dieldrin	98%	172%	117%				1.13	0.70	0.73			
	Endrin	90%	45%	103%				1.67	2.15	2.20			
	β-Endosulfan	84%	311%	128%				1.31	0.40	0.44			
	p.p´-DDD	85%	39%	124%				1.09	2.69	2.92			
	DDT	73%	19%	148%				0.71	3.04	3.29			

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Table A.15 (continued)

Compound		Recoveries						Matrix effect					
		CL1	CL2	CL3	CL1 mean	CL2 mean	CL3 mean	CL1	CL2	CL3	CL1 mean	CL2 mean	CL3 mean
Organochlorines	**Methoxychlor**	62%	56%	96%				1.52	1.12	1.60			
Pyrethroids	**Bifenthrin**	111%	299%	41%	60%	74%	84%	2.48	1.32	1.45	2.13	2.84	3.01
	Cyhalothrin	50%	71%	91%				2.02	1.90	2.06			
	β-Permethrin	52%	173%	189%				1.73	2.30	1.41			
	Cyfluthrin	63%	49%	90%				1.88	2.71	3.07			
	Cypermethrin	58%	48%	98%				2.14	3.69	4.46			
	α-Fenvalerate	53%	72%	76%				2.06	2.47	2.66			
	β-Fenvalerate	53%	59%	64%				2.03	2.90	2.79			
	Deltamethrin	42%	45%	21%				2.70	5.43	6.14			

Table A.16 Values of recovery and matrix effect obtained for each type of sorbent sets (CL1, CL2 and CL3), for OPPs.

Compound		Recoveries						Matrix effect					
		CL1	CL2	CL3	CL1 mean	CL2 mean	CL3 mean	CL1	CL2	CL3	CL1 mean	CL2 mean	CL3 mean
Organophosphorus	Dimethoate	2%	3%	77%	27%	32%	87%	1.20	0.06	0.06	1.17	0.48	0.46
	Chlorpyrifos--methyl	37%	44%	85%				1.14	0.61	0.63			
	Parathion--methyl	37%	44%	94%				1.15	0.60	0.55			
	Malathion	39%	48%	81%				1.16	0.73	0.68			
	Chlorpyrifos	27%	30%	78%				1.10	0.49	0.48			
	Chlorfenvinphos	20%	22%	104%				1.30	0.41	0.39			

Appendix B – Validation of the analytical method

Annex B presents the calibration curves obtained for OCPs, PYRs (in matrix) and OPPs (in matrix and in solvent (*n*-hexane)), based on which the modified QuEChERS method was validated.

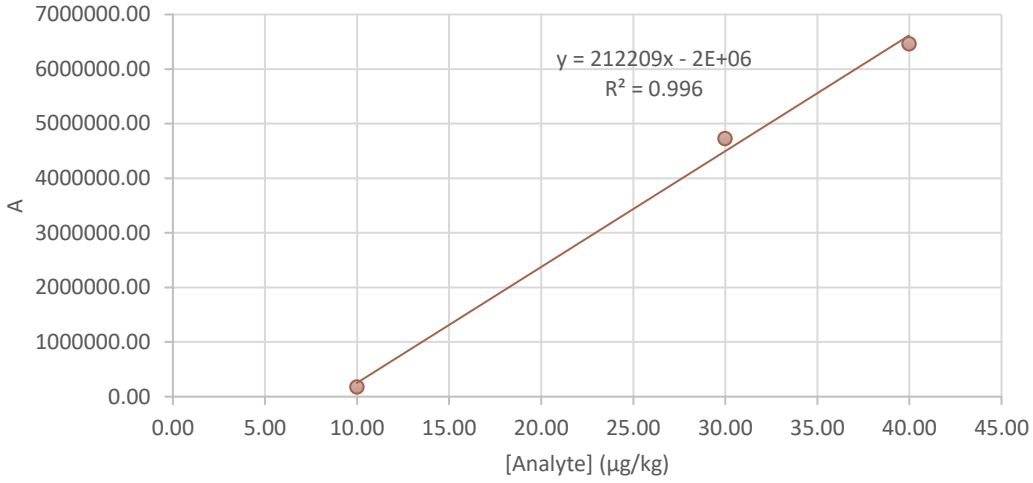

Figure B.1 Calibration curve obtained for α-HCH in matrix

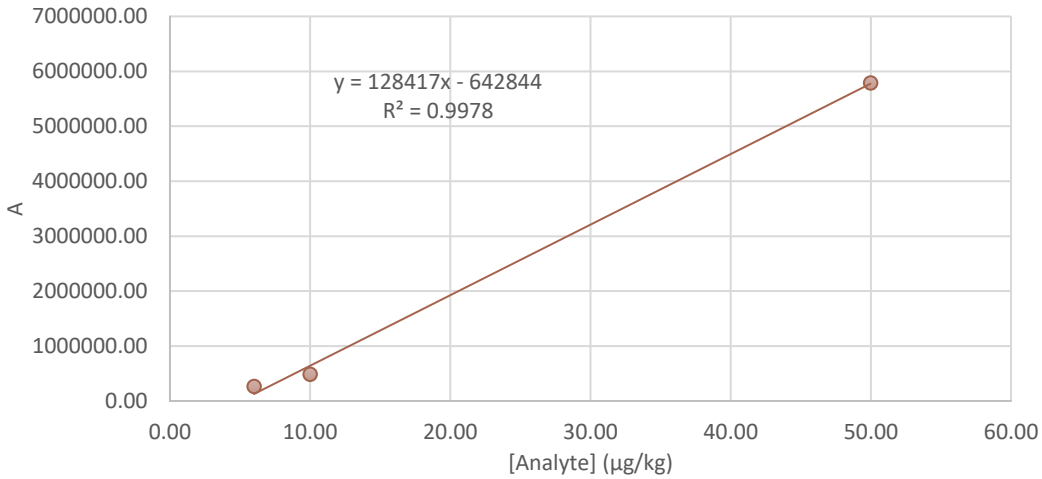

Figure B.2 Calibration curve obtained for HCB in matrix.

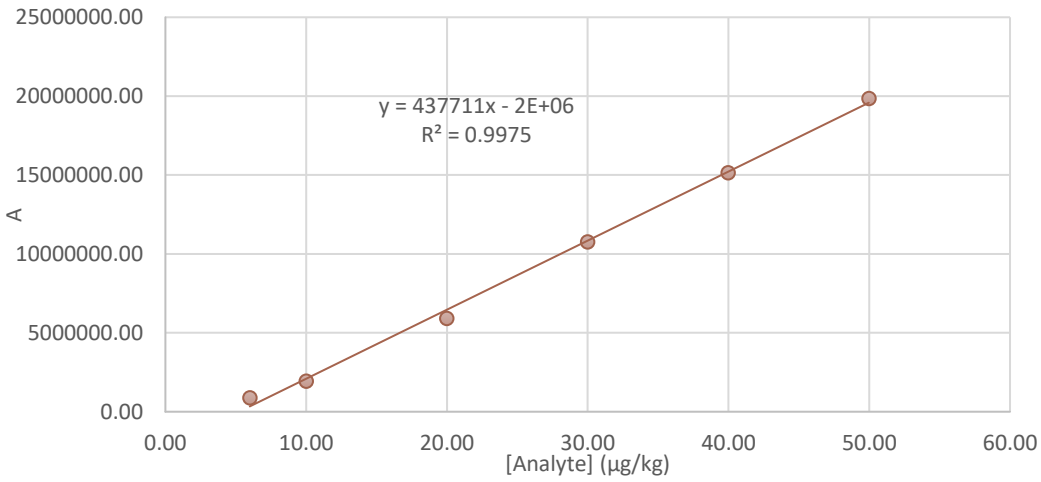

Figure B.3 Calibration curve obtained for β-HCH in matrix.

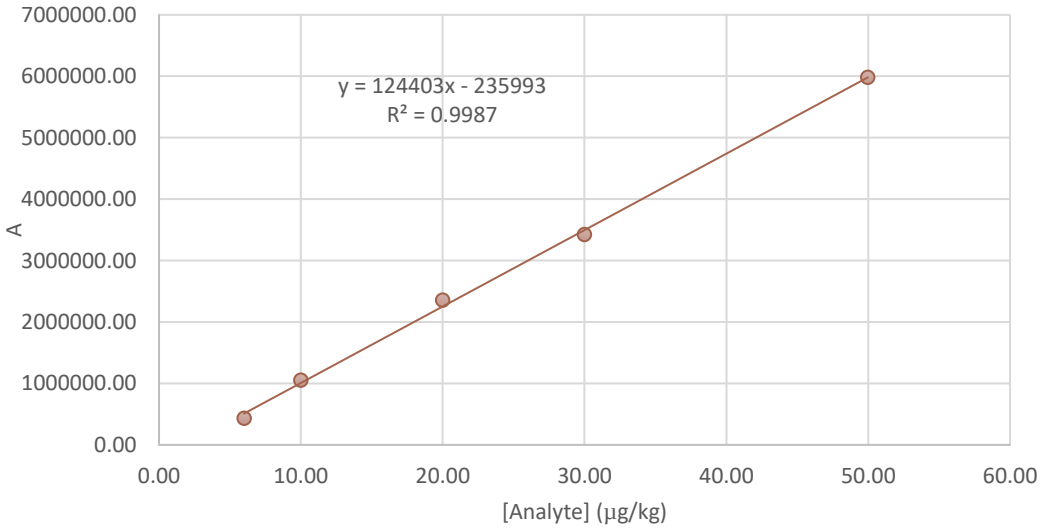

Figure B.4 Calibration curve obtained for lindane in matrix.

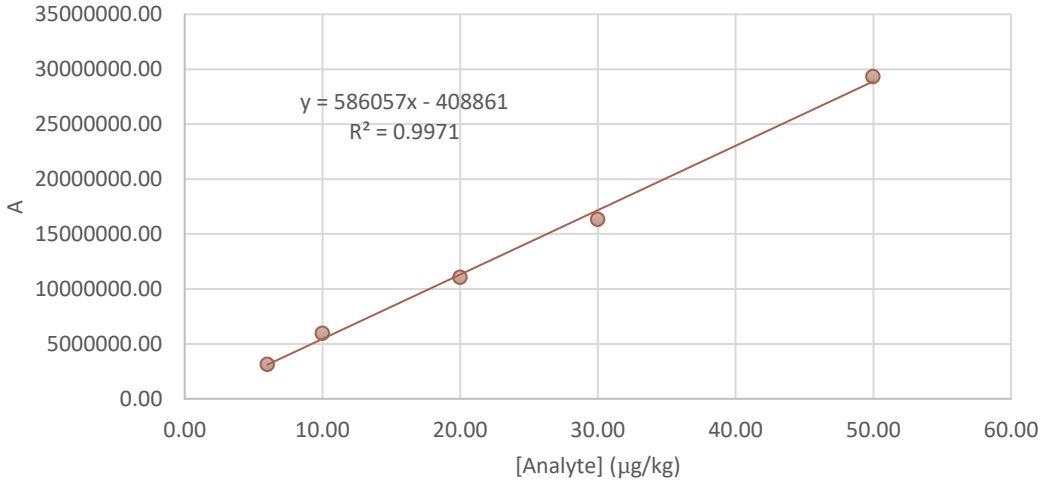

Figure B.5 Calibration curve obtained for δ-HCH in matrix.

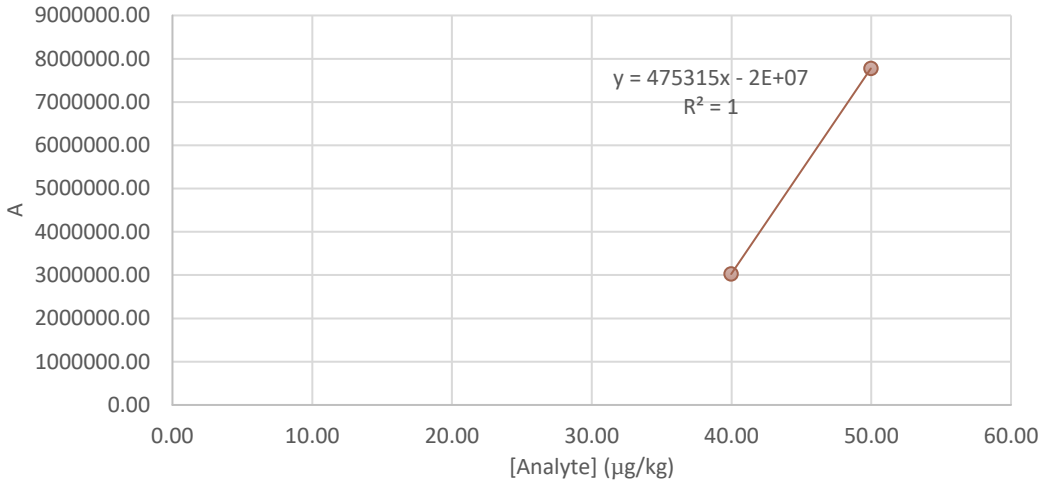

Figure B.6 Calibration curve obtained for aldrin in matrix.

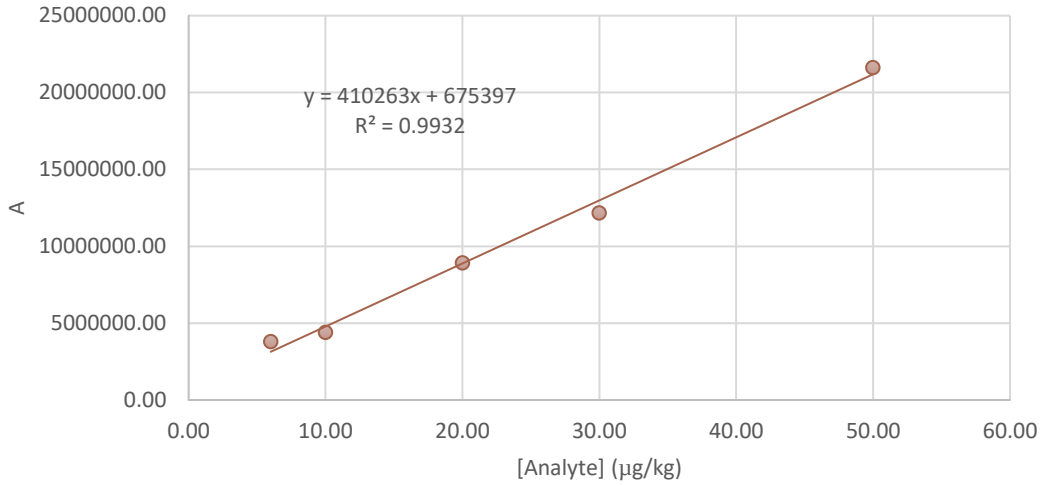

Figure B.7 Calibration curve obtained for α-end in matrix.

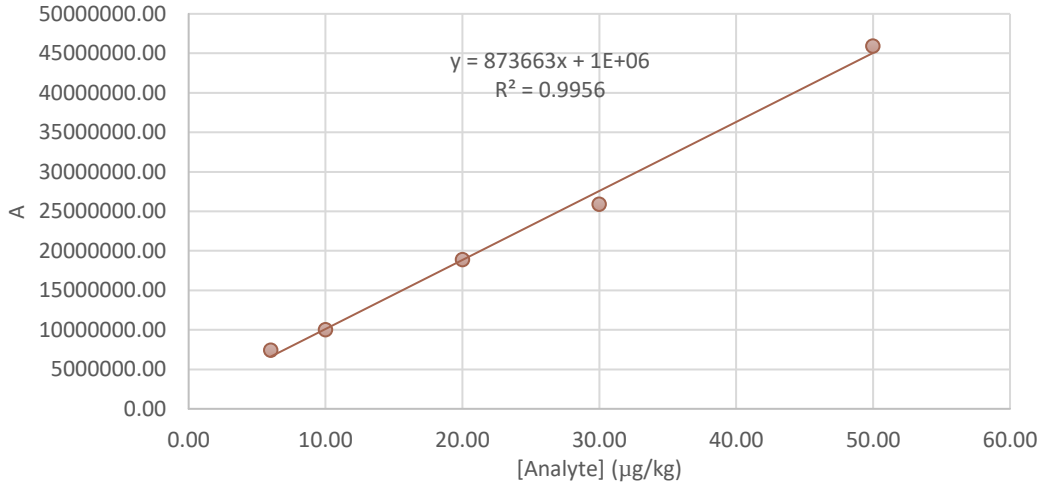

Figure B.8 Calibration curve obtained for p.p´-DDE in matrix.

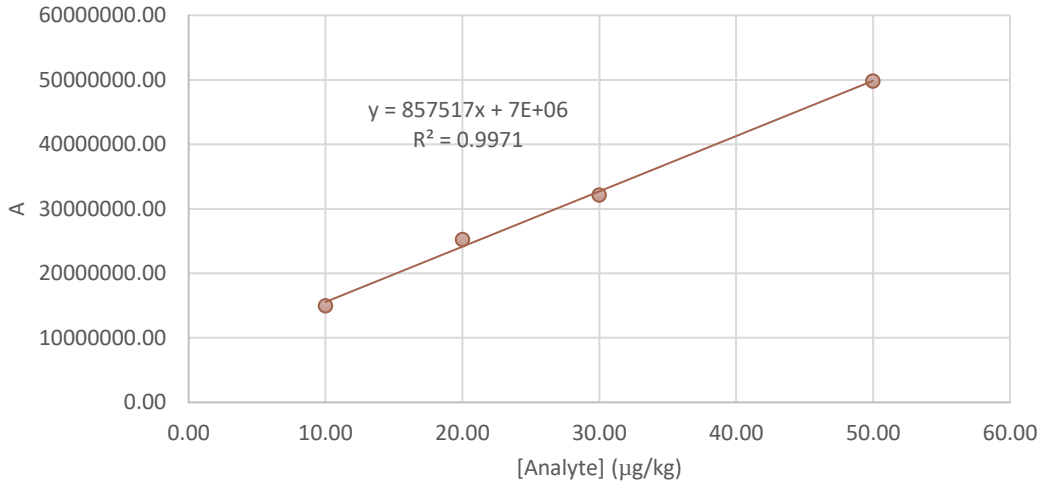

Figure B.9 Calibration curve obtained for dieldrin in matrix.

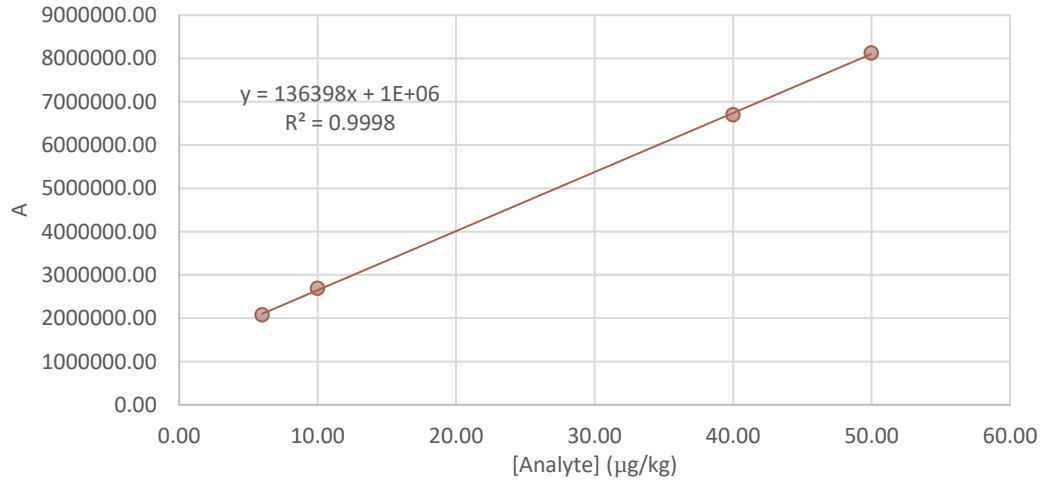

Figure B.10 Calibration curve obtained for endrin in matrix.

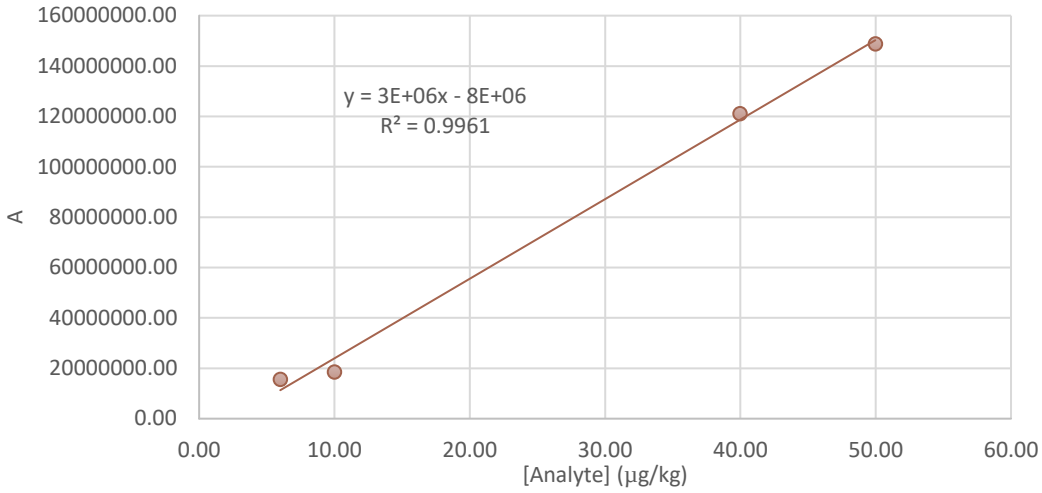

Figure B.11 Calibration curve obtained for β-end in matrix.

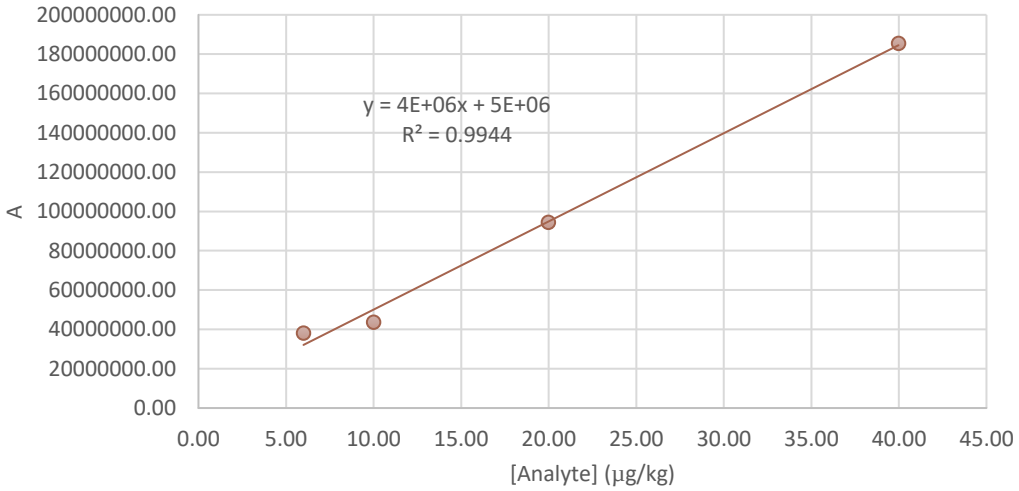

Figure B.12 Calibration curve obtained for p.p´-DDD in matrix.

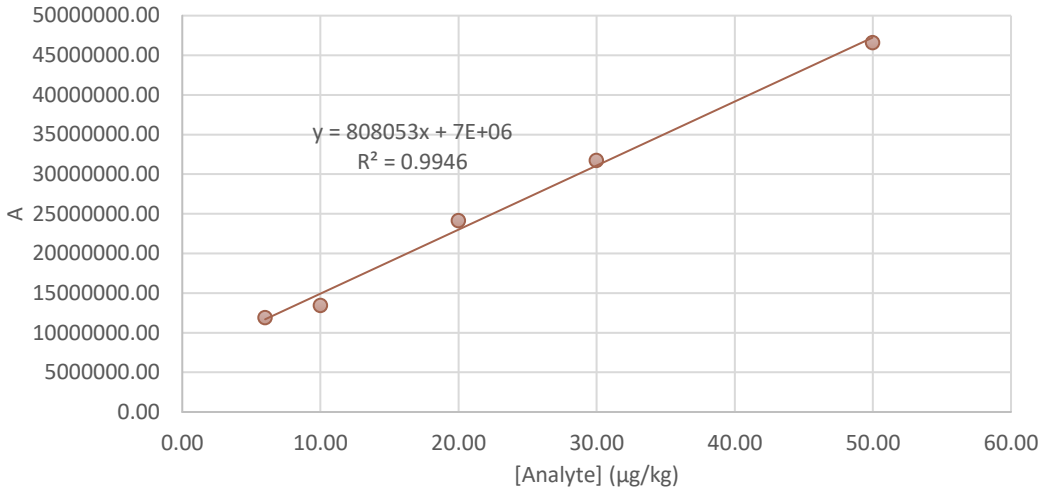

Figure B.13 Calibration curve obtained for DDT in matrix.

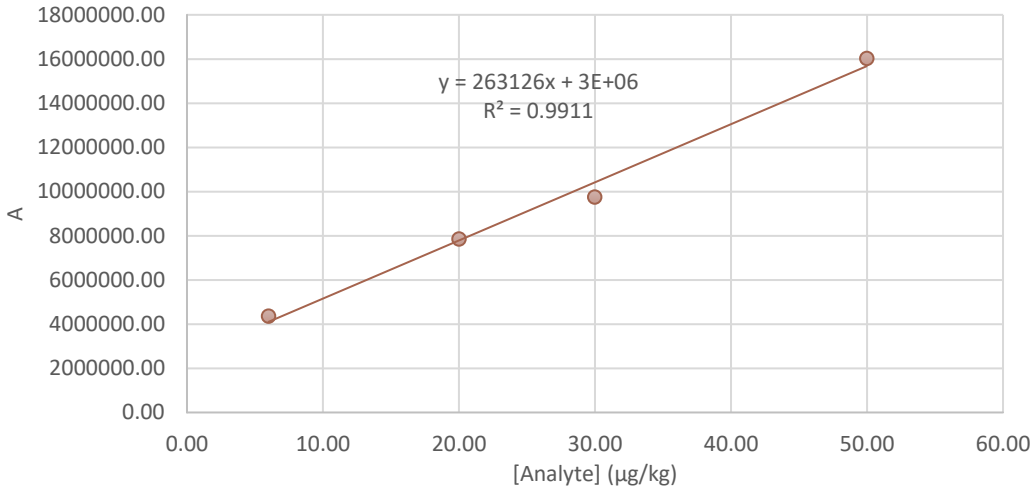

Figure B.14 Calibration curve obtained for methoxychlor in matrix.

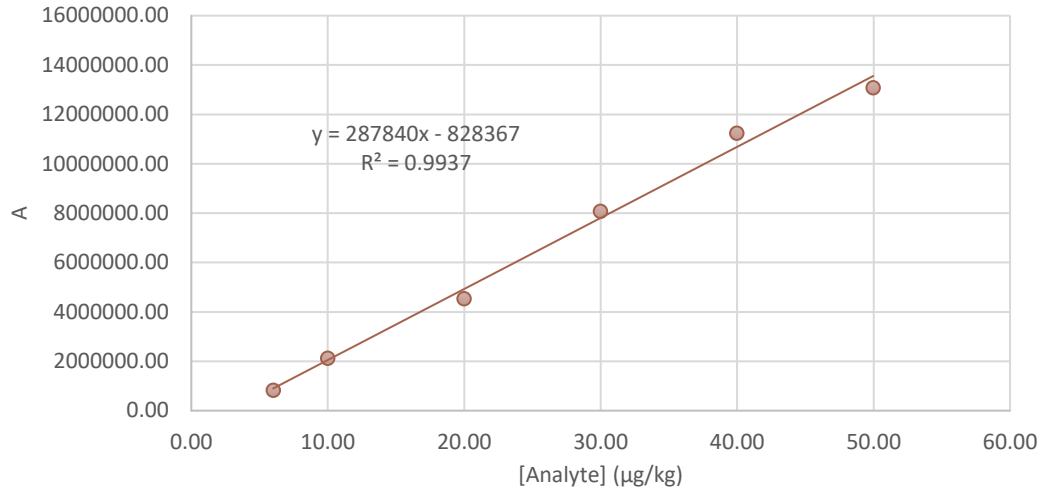

Figure B.15 Calibration curve obtained for bifenthrin in matrix.

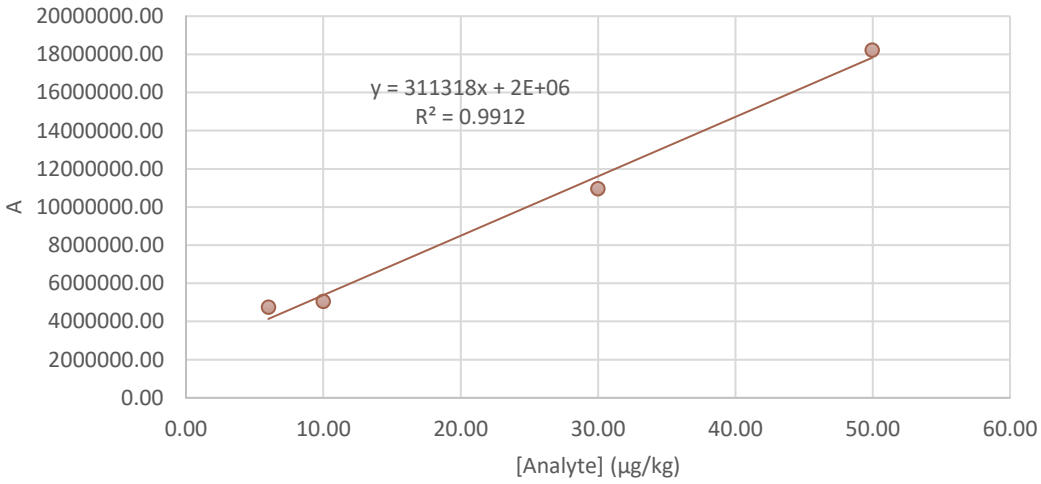

Figure B.16 Calibration curve obtained for cyhalothrin in matrix.

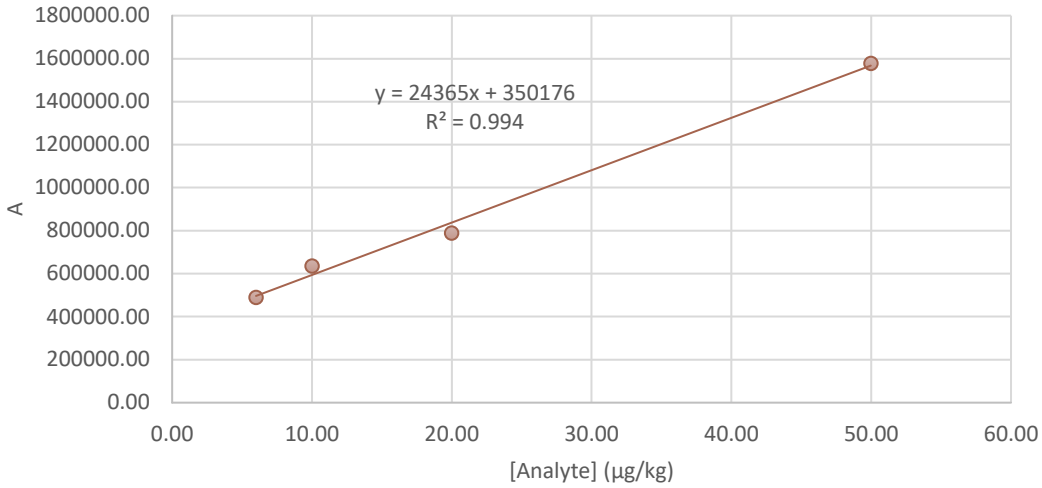

Figure B.17 Calibration curve obtained for permethrin in matrix.

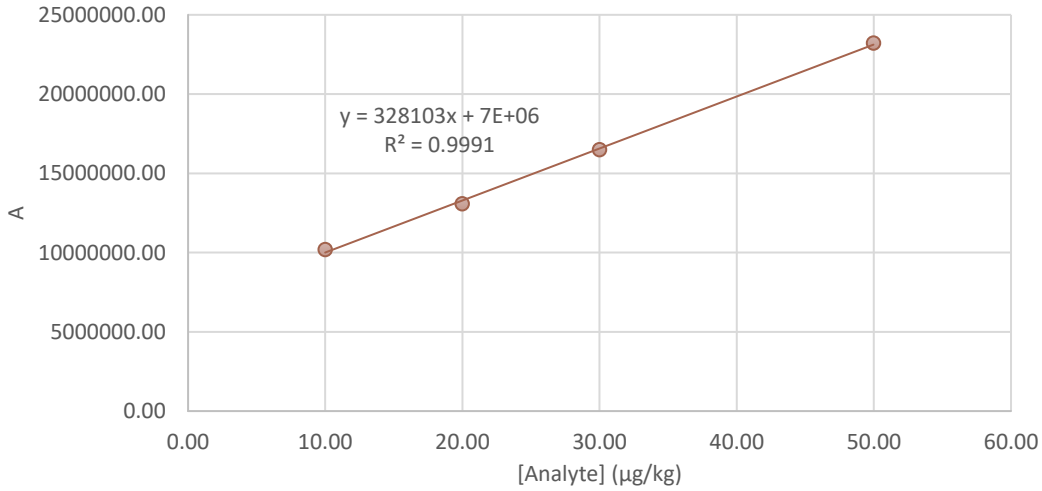

Figure B.18 Calibration curve obtained for cyfluthrin in matrix.

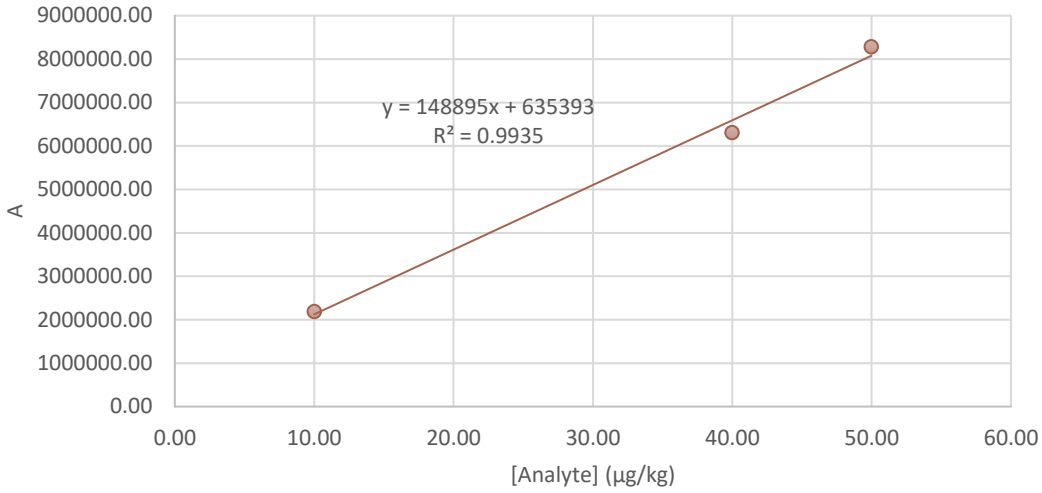

Figure B.19 Calibration curve obtained for cypermethrin in matrix.

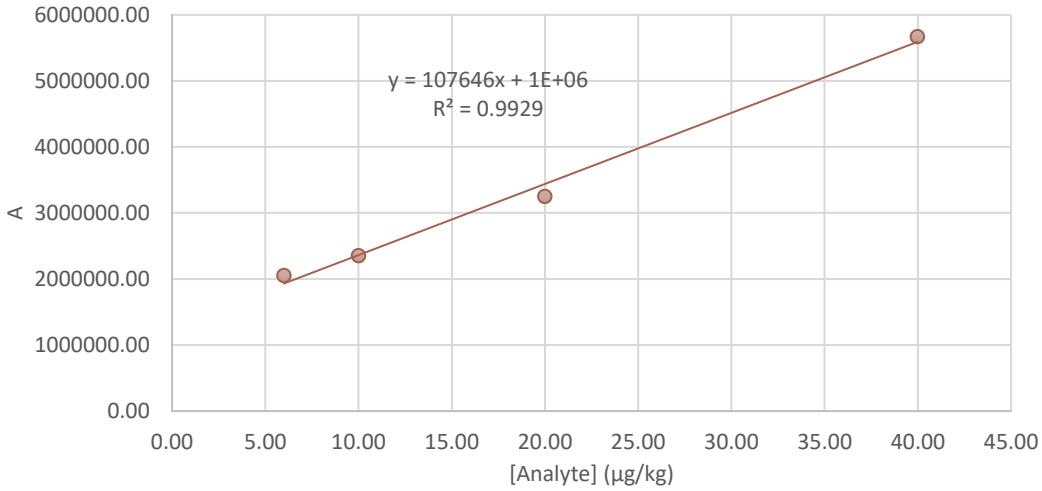

Figure B.20 Calibration curve obtained for α-fenvalerate in matrix.

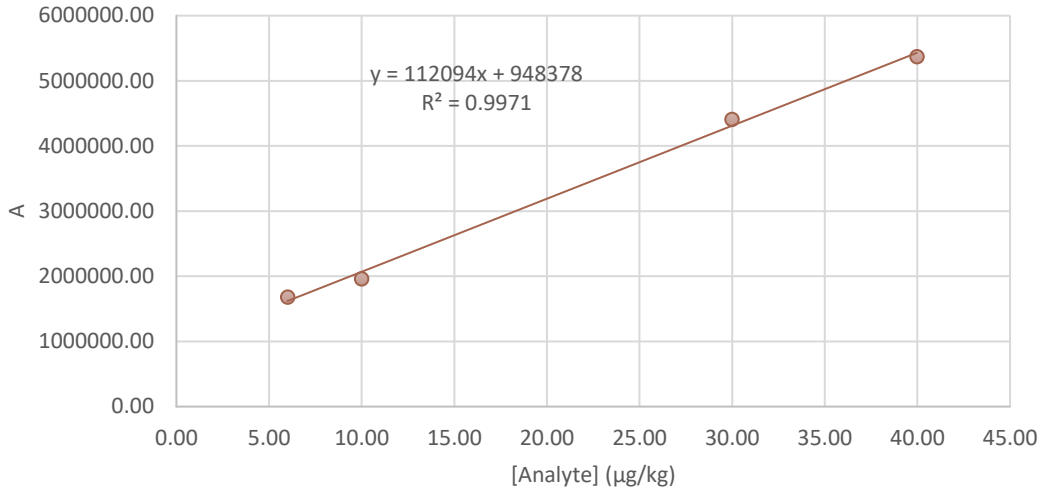

Figure B.21 Calibration curve obtained for β-fenvalerate in matrix.

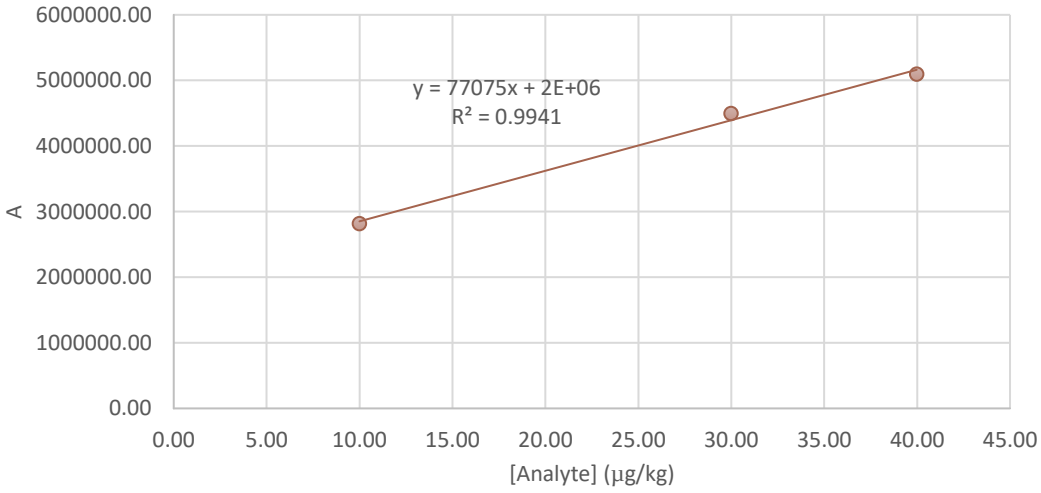

Figure B.22 Calibration curve obtained for deltamethrin in matrix.

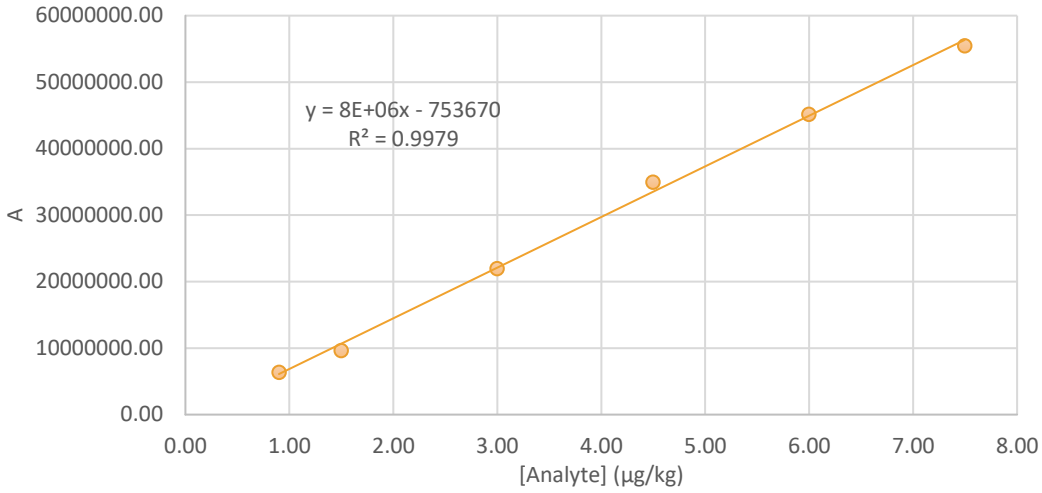

Figure B.23 Calibration curve obtained for dimethoate in matrix.

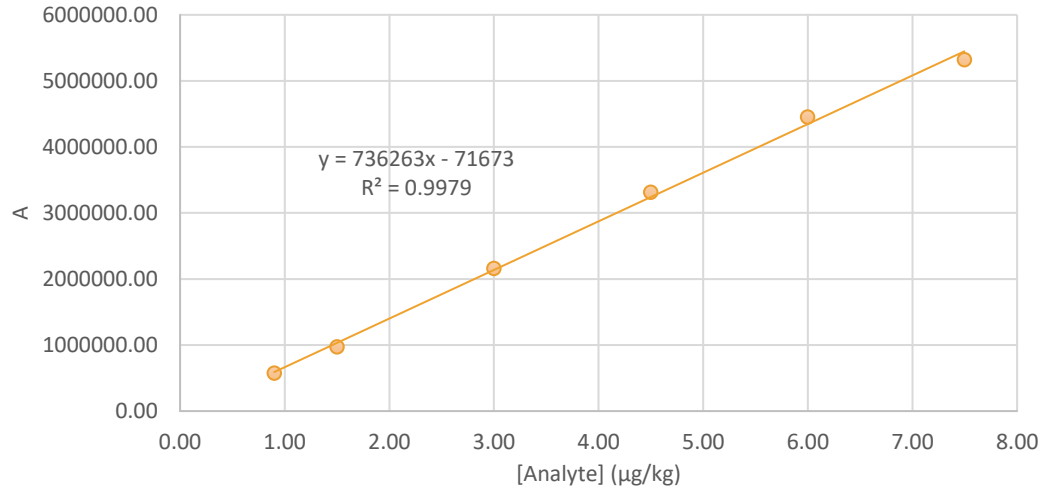

Figure B.24 Calibration curve obtained for chlorpyrifos-methyl in matrix.

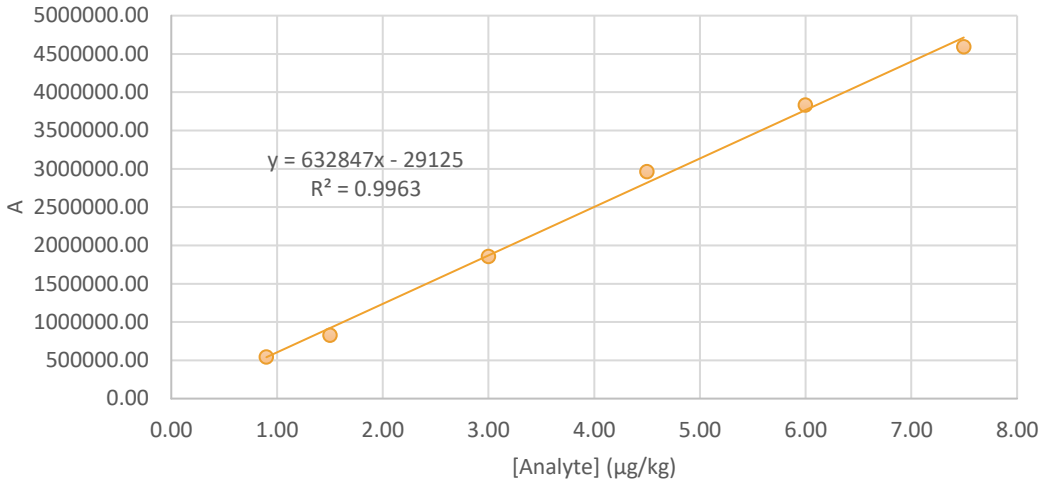

Figure B.25 Calibration curve obtained for parathion-methyl in matrix.

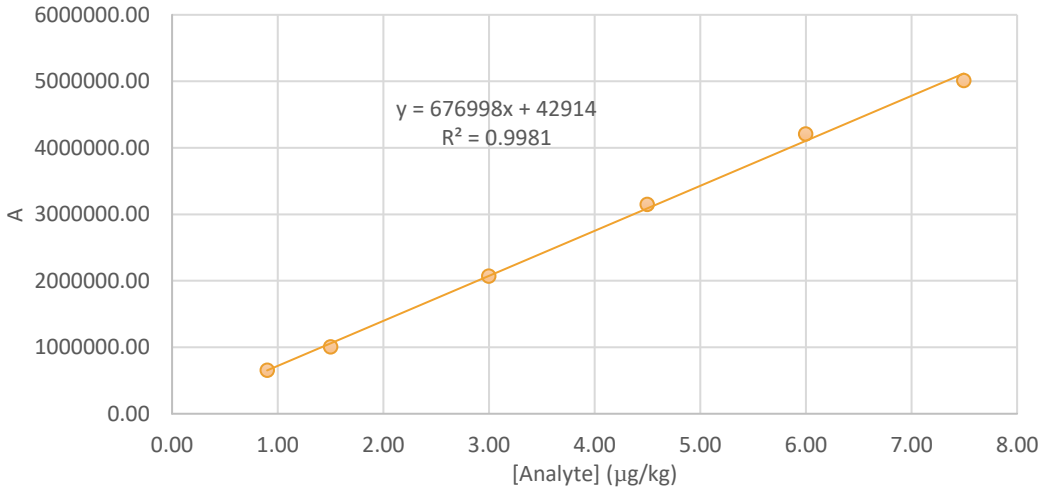

Figure B.26 Calibration curve obtained for malathion matrix.

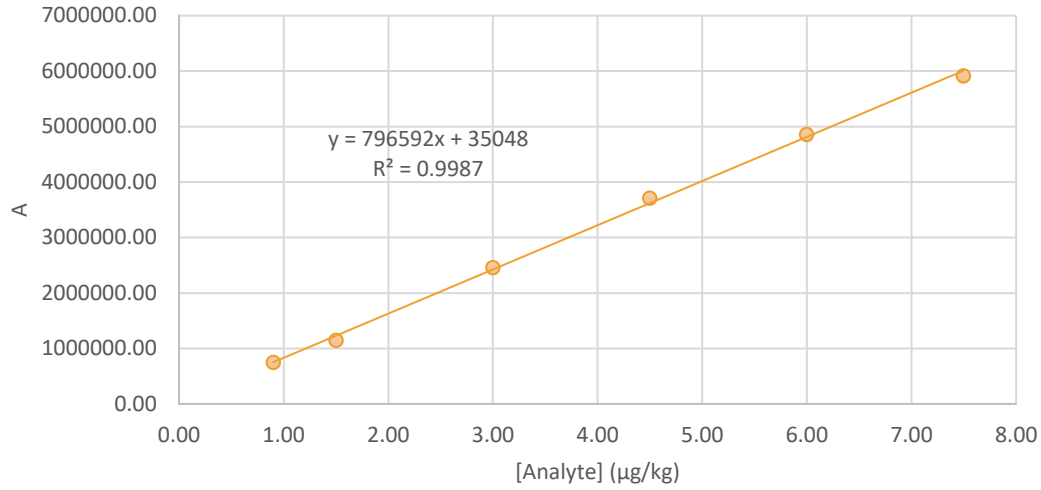

Figure B.27 Calibration curve obtained for chlorpyrifos in matrix.

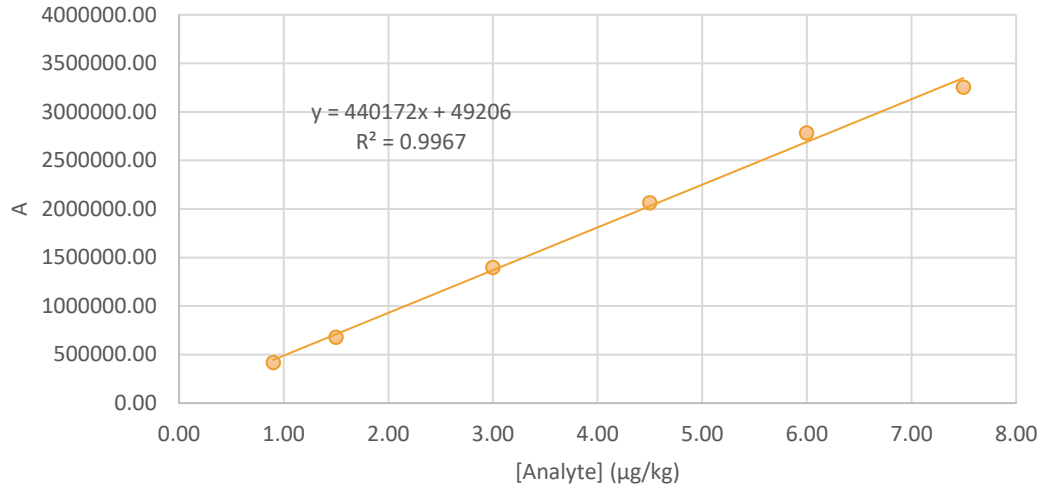

Figure B.28 Calibration curve obtained for chlorfenvinphos in matrix.

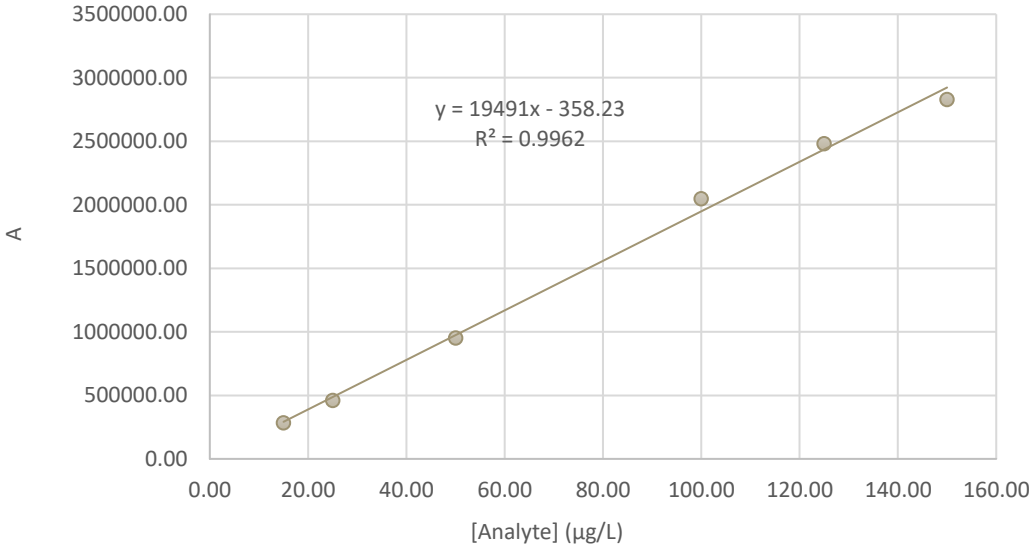

Figure B.29 Calibration curve obtained for dimethoate in n-hexane.

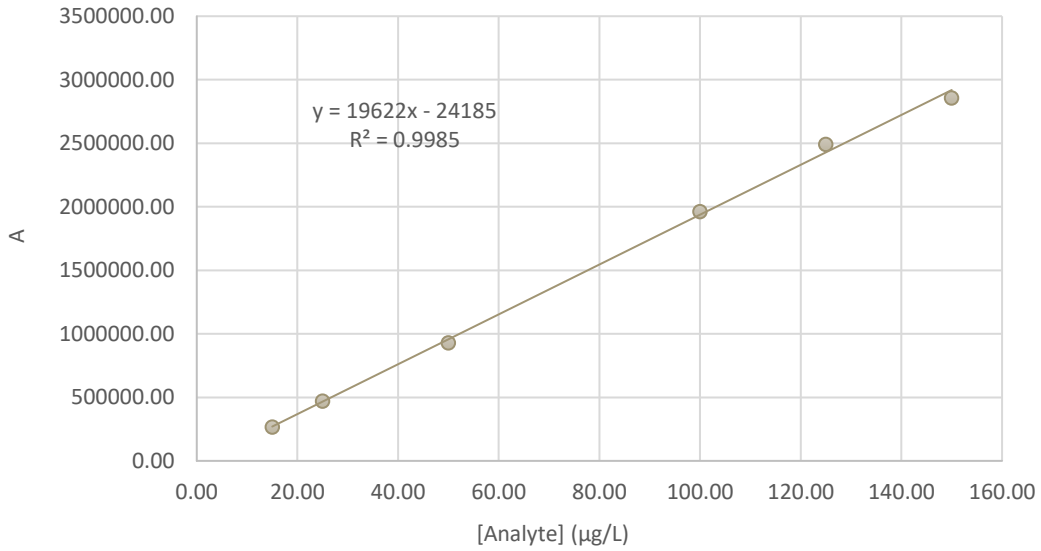

Figure B.30 Calibration curve obtained for chlorpyrifos-methyl in n-hexane.

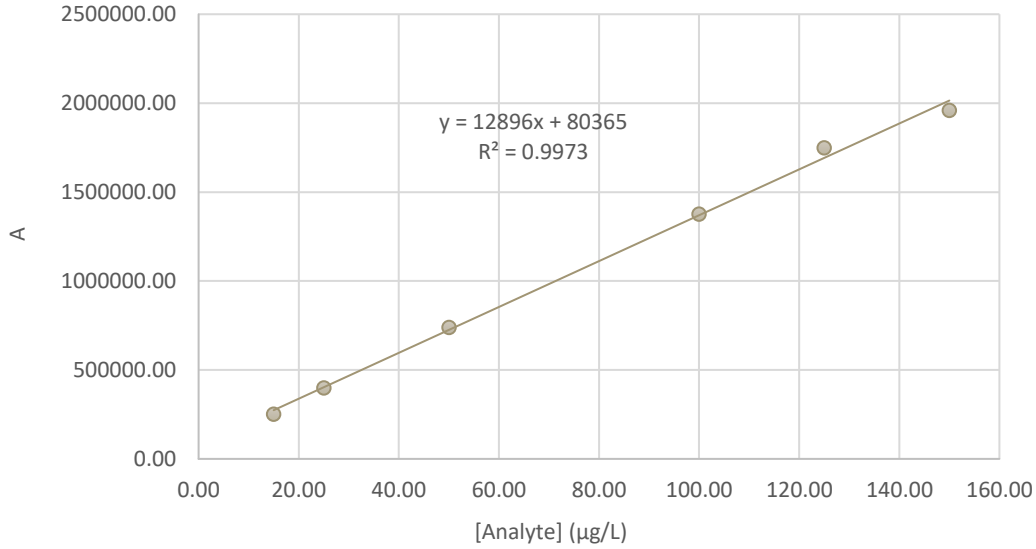

Figure B.31 Calibration curve obtained for parathion-methyl in n-hexane.

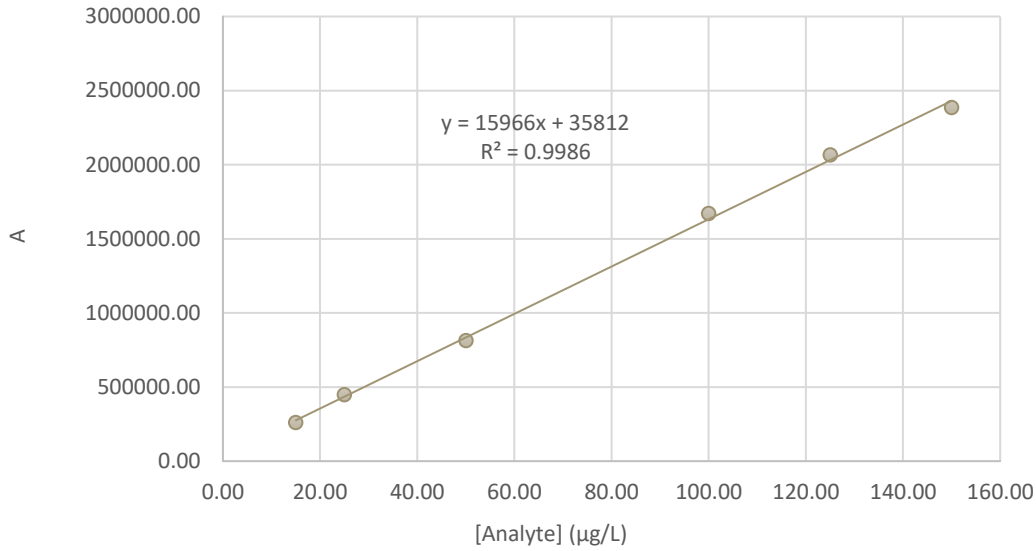

Figure B.32 Calibration curve obtained for malathion in n-hexane.

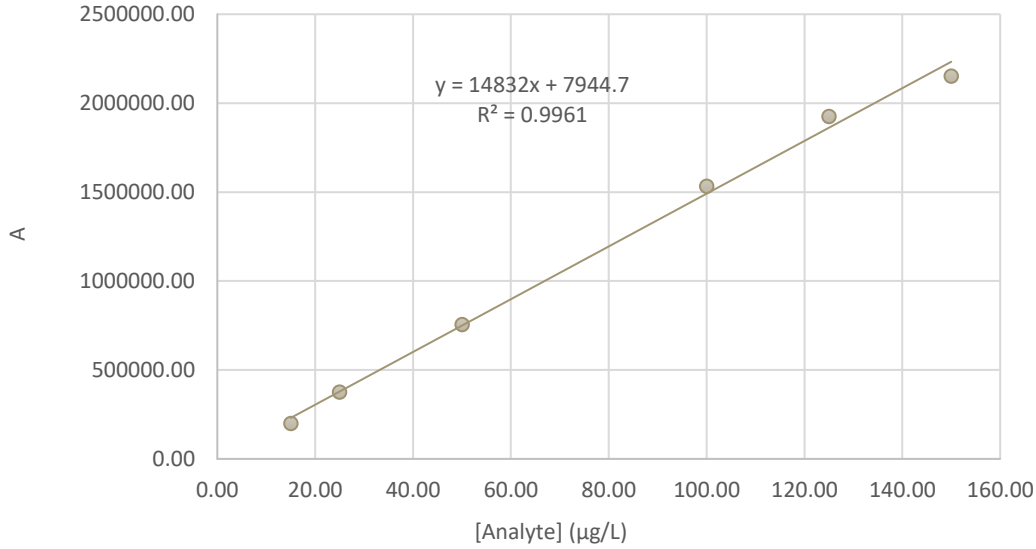

Figure B.33 Calibration curve obtained for chlorpyrifos in n-hexane

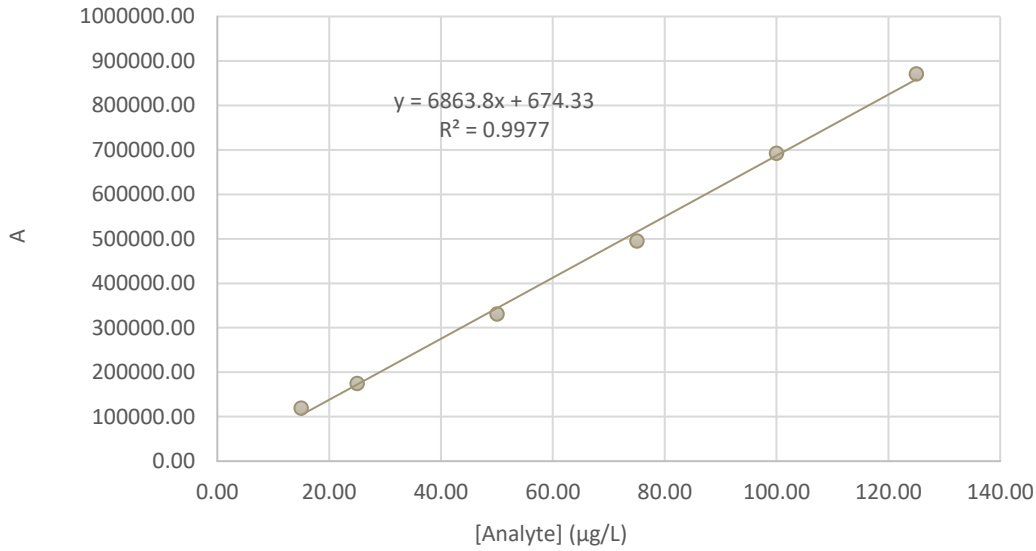

Figure B.34 Calibration curve obtained for chlorpyrifos in n-hexane.