NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF ENVIRONMENTAL SCIENCES AND ENGINEERING

ANALYSIS OF PESTICIDE RESIDUES IN SOIL

NEW APPROACHES FOR ENVIRONMENTAL MONITORING

JOÃO EDUARDO RODRIGUES SEDAS BRINCO

Master in Bioorganic Chemistry

DOCTORATE IN ENVIRONMENT AND SUSTAINABILITY

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New Approaches for Environmental Monitoring

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Santuṭṭhiparamaṃ dhanaṃ
Contentment is the highest wealth

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Preface

The present thesis was produced as a requirement for the doctoral degree in Environment and Sustainability from NOVA School of Science and Technology, NOVA University Lisbon. The PhD work was conducted between February 2021 and January 2025 at the university.

Integrated into the thesis are four articles, reproduced exactly as in the published versions. These are interspersed with introductory and explanatory text in order to hopefully provide a structured and easier reading experience. The references for each of the articles are self-contained. At the end of this thesis, only the references outside the articles are presented, from the entire document. The lists of figures and tables likewise refer only to the ones outside the articles. Chapters 5 and 6 are entirely composed of new results not yet published.

Fundação para a Ciência e a Tecnologia is acknowledged as the main funder of this work, through the PhD scholarship UI/BD/150867/2021, under collaboration with the R&D unit CENSE- Center for Environmental and Sustainability Research. This work was also supported by national funds from Fundação para a Ciência e a Tecnologia through the Research units CENSE- Center for Enviromental and Sustainability Research (PTDC/CTAAMB/6587/2020) and LAQV/REQUIMTE (UID/QUI/50006/2020). This work was anchored at RESOLUTION LAB, an infrastructure at NOVA School of Science and Technology.

The thesis was done without the use of any type of generative artificial intelligence. Permission to reproduce the article "Monitoring pesticides in post-consumer containers by GC/TOFMS and HPLC/DAD after the triple rinse method" has been granted from the publisher.

Abstract

Environmental monitoring is a very important part of dealing with soil contamination problems. As an essential support for life on land, soil should be protected and monitored. This dissertation is centered on the development of methodologies for pesticide residues analysis in soil, which are of great relevance due to being widely applied on agricultural soils and by having known detrimental effects.

The first work presented comprises a method development and monitoring campaign of empty pesticide packaging from Portugal in order to determine their hazardousness. The compounds most found in this monitoring along with those frequently reported in the relevant literature as being present in European Union (EU) soils were used to construct a list of priority analytes to focus on. This list contained thirteen currently used pesticides and one degradation product; of these, the herbicide glyphosate is the most challenging in analytical terms.

A literature review on currently used methods for pesticide residue analysis in soil was conducted, along with a side-by-side comparison of their properties. QuEChERS was found to be the most widely used and promising extraction method currently employed. However, none of the methodologies studied seemed particularly fitted to extract "multiclass" pesticides along with glyphosate, and thus a new form of extraction using semi-disposable Solid-Phase Microextraction (SPME) fibers on an aqueous soil slurry was developed, and combined with solid-liquid extraction followed by chemical derivatization in order to permit this analysis in a single run employing Gas Chromatography coupled to tandem Mass Spectrometry (GC-MS/MS).

The same semi-disposable SPME fibers were also applied to the development of a new "on-the-fly" detection method for pesticides in soil which forgoes traditional sampling and extensive lab-work. The fibers are inserted directly into the soil, after which they are retro-extracted onto a suitable solvent and analysed through GC-MS/MS. The proposed method was tested for monitoring on an electrokinetic remediation experiment at the lab scale. Results show that this new methodology cannot be used for accurate quantification, but may be employed in qualitative analysis for detection of contaminants above a certain confidence threshold.

Throughout the work different pieces of software were written in the Python language to perform calculations, mostly for experimental design and quantification/validation. These were compiled and documented in a package called Chromapy, which is now freely available for use and modification.

Keywords: Environmental monitoring, soil pesticide analysis, solid-phase microextraction, free-software development.

Resumo

A monitorização ambiental é uma parte muito importante na abordagem dos problemas de contaminação do solo. Enquanto suporte essencial para a vida terrestre, o solo deve ser protegido e monitorizado. Esta dissertação centra-se no desenvolvimento de metodologias para a análise de resíduos de pesticidas no solo, os quais são de grande relevância devido à sua aplicação generalizada nos solos agrícolas e aos seus conhecidos efeitos prejudiciais.

O primeiro trabalho apresentado envolve o desenvolvimento de um método e uma campanha de monitorização de embalagens vazias de pesticidas em Portugal, com o objetivo de determinar a sua perigosidade. Os compostos mais encontrados nesta monitorização, juntamente com aqueles frequentemente encontrados nos solos da União Europeia na literatura relevante, foram utilizados para construir uma lista de analitos prioritários a focar. Esta lista contém treze pesticidas atualmente utilizados e um produto de degradação; destes, o herbicida glifosato é o mais desafiante em termos analíticos.

Foi realizada uma revisão da literatura sobre os métodos atualmente utilizados para a análise de resíduos de pesticidas no solo, juntamente com uma comparação das suas propriedades. O método de extração QuEChERS foi identificado como o mais amplamente utilizado e promissor. No entanto, nenhuma das metodologias estudadas se revelou particularmente adequada para extrair várias classes de pesticidas juntamente com o glifosato. Assim, foi desenvolvido um novo método de extração utilizando fibras semi-descartáveis de Microextração em Fase Sólida (SPME) a partir de uma suspensão aquosa de solo, combinado com extração sólido-líquido seguida de derivatização química, permitindo a análise numa única corrida de Cromatografia Gasosa acoplada a Espectrometria de Massas em Tandem (GC-MS/MS).

As mesmas fibras semi-descartáveis de SPME foram aplicadas no desenvolvimento de um novo método de deteção "on-the-fly" para pesticidas no solo, que dispensa a amostragem tradicional e o extenso trabalho de laboratório. As fibras são inseridas diretamente no solo, sendo posteriormente retroextraídas para um solvente adequado e analisadas através de GC-MS/MS. O método proposto foi testado para monitorização numa experiência de remediação eletrocinética em escala laboratorial. Os resultados mostram que esta nova metodologia não pode ser utilizada para quantificação exata, mas

pode ser empregada na análise qualitativa para deteção de contaminantes acima de um determinado limite de confiança.

Ao longo do trabalho, foram desenvolvidos diferentes módulos de software na linguagem Python para realizar cálculos, maioritariamente de desenho experimental e quantificação/validação. Estes foram compilados e documentados num pacote chamado Chromapy, que agora está disponível para utilização e modificação.

Palavras-chave: Monitorização ambiental, análise de pesticidas no solo, microextração em fase sólida, desenvolvimento de software livre.

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Acronyms

2,4-D 2,4-dichlorophenoxyacetic acid *(pp. 21, 41, 43, 55, 72, 75)*

AMPA aminomethylphosphonic acid *(pp. 3, 21, 41, 43, 55, 68, 69, 72, 75, 80, 95)*

DART Direct Analysis in Real Time ionization source *(pp. 79, 82, 96)*
DI-SPME Direct-Immersion Solid-Phase Microextraction *(pp. 4, 23, 43, 55, 95)*

EK Electrokinetic *(pp. 71–75, 80–82)*
EU European Union *(pp. ix, 2, 3, 7, 95)*

Fmoc-Cl fluorenylmethyloxycarbonyl chloride *(p. 41)*

GC Gas Chromatography *(pp. 3, 41, 55, 93, 96)*
GC-MS/MS Gas Chromatography coupled to tandem Mass Spectrometry *(pp. ix, 41)*
GC-TOFMS Gas Chromatography coupled to Time-Of-Flight Mass Spectrometry *(pp. 7, 87)*
GPL GNU General Public License *(pp. 85, 93)*

HPLC High-Performance Liquid Chromatography *(pp. 41, 93)*
HPLC-DAD High-Performance Liquid Chromatography coupled to Diode Array Detection *(p. 7)*

LC-MS High-Performance Liquid Chromatography coupled to Mass Spectrometry *(pp. 21, 95)*

MCPA 2-methyl-4-chlorophenoxyacetic acid *(pp. 21, 41, 43, 55, 72, 75)*
MRM Multiple-Reaction Monitoring mass spectrometry *(pp. 68, 78)*
MTBSTFA N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide *(p. 41)*

PAH polyaromatic hydrocarbons *(p. 95)*
PCA Principal Component Analysis *(pp. xv, 86–89)*
PLS Partial Least Squares regression *(pp. xv, 86–89)*

SPME Solid-Phase Microextraction *(pp. ix, 4, 5, 23, 42, 43, 69, 96)*

TFAA trifluoroacetic anhydride *(p. 41)*
TFE 2,2,2-trifluoroethanol *(p. 41)*

1

General Introduction

The present work is centered around analytical methods for the determination of pesticide residues in soil. More generally, it can be inserted into a subset of analytical chemistry whose main application is environmental monitoring. Currently, there is an undeniable interest and concern with environmental contaminants by the scientific community, policy makers, and a certain subset of the general population. Naturally, one of the extremely important aspects of dealing with environmental contamination is monitoring, for which adequate, sustainable and economical methods are required.

Since the dawn of the industrial age, several contaminants have been released onto the environment in large quantities, of which pesticides are only a fraction. The achievements and problems of this industrial civilization (and more broadly of all civilizations) is a widely discussed and contentious issue [2] which need not be addressed here. Regardless, it is evident that our civilization's present state is entirely dependent on the availability of extremely abundant, concentrated and fungible sources of energy, namely fossil fuels. This, along with certain important intellectual and psychological changes has put our civilization in a particular, and somewhat unprecedented situation. Nevertheless, it is crucial to note that above all our existence is, and will remain, dependent on the natural world and the myriad natural phenomena (*e.g.* climate, natural energy flows, photosynthesis), and these cannot be replaced by surrogate human activities on any meaningful scale, regardless of energy availability: an assertion which can be proved by the work of Costanza *et al.* [3], who quantified the economic value of selected ecosystem services in financial terms and found them to be larger than the global gross domestic product.

One of the crucial facets of terrestrial ecosystems is soil. As an essential support for life on land, it is often overlooked. Indeed, the connection between the decline of several previous civilizations and their soil quality is a very interesting and seldom discussed fact [4]. It is now well established that environmental factors played a major role in the fall of the Roman Empire [5]. Furthermore, soil degradation has been a recurrent consequence of past civilizations in the Mediterranean region as well as a major catalyst of their demise [4]. Thus, soil health should not be taken as a simple problem which can be easily dealt with if only the political will can be mustered, but as a serious threat to a

civilization's economic stability.

Since soil is an integral part of terrestrial ecosystems and intimately connected to all facets of the environment, soil contamination is not something which can be compartmentalized and segregated. Soil contaminants can eventually mobilize and possibly affect other aspects of the environment [6, 7], not to mention the adverse effects on the soil itself. In this respect, pesticides are a very important class of soil contaminants, being the most commonly applied synthetic compounds onto agricultural soils (with the possible exception of chemical fertilizers).

The term "pesticide" simply describes a compound or mixture used to deter pests. Since this comprises an extremely diverse group, a qualification is necessary: this work is focussed on organic compounds used mainly as plant protection products, mostly (though not necessarily) of synthetic origin. The terms "organic" and "organic compound" are used in the chemical sense rather than that of "organic agriculture". Thus, an "organic pesticide" is one who's active compound is an organic molecule or mixture thereof. Also, the terms "pesticide" and "pesticide residues" are used interchangeably although they are not exactly the same: "pesticide residues" are the compounds present after the "pesticide" application (*e.g.* in the soil), be those the original compound and/or degradation products. When talking about soil monitoring, what is being assessed is always the residues. Most of these can be classified as contaminants since they would not be naturally present in the soil [8].

It is evident that the use of pesticides in agriculture has greatly contributed to increased productivity and food security. Nevertheless, several pesticides have been proven to be not only environmental contaminants, but pollutants (*i.e.* by having a detrimental effect [8]). Serious toxicity towards non-target organisms or, more generally, ecotoxicity affecting ecosystem health are well known effects of a large number of currently used pesticides [9, 10] (though not all). The very well known detrimental effects of pesticide usage, including on human health [11] have led to strict governmental regulation, and sometimes banning of certain compounds for agricultural uses, as was the case with DDT [12]. More recently, the European Commission has published a proposal for a Soil Monitoring Law (COM/2023/416), which is aimed at addressing the major soil threats in the EU, but is yet to be implemented [13]. As a testament to the importance placed on soils, the proposal opens as follows: "Soil is a vital, limited, non-renewable and irreplaceable resource." Among other things, it specifies "soil descriptors" which should be monitored, including "Concentration of a selection of organic contaminants" which is likely to include certain pesticides. Thus, the determination of pesticide residues in soil is not only certain to remain relevant, but likely to become more so in the future [13]. However, the large expense and cumbersome nature of this type of analysis remain obstacles to the implementation of more ambitious monitoring programs. Furthermore, laboratory analysis themselves often generate large amounts of pollutants and consume significant quantities of non-renewable resources either directly or through their high energy requirements. Traditionally, optimization in this regard has not been a priority, but

recently new frameworks such as green analytical chemistry [14, 15] have been developed in an attempt to tackle such environmental issues. Green analytical chemistry, in particular, is an approach to the development and evaluation of analytical methods which aims to reduce their negative environmental impact and increase operator safety. This is further explained in Section 3.1.

The objectives of this work were as follows:

1. To determine which currently used pesticides are most relevant as soil contaminants in the EU.

2. To develop an analytical method for quantification of those contaminants in a sampled soil, which is aligned with green analytical chemistry principles and is as environmentally benign as possible, without forgoing metrological performance.

3. To attempt to create a new method for on-line monitoring of multiple pesticides in soil without recourse to extensive sampling and extraction, having the same design goals as point 2 above.

4. To develop the software required for calculations throughout the work and compile it into a package for free distribution.

The main objective of this work has been to contribute to the development of analytical methodologies in support of environmental monitoring. As well as attempting to work towards sustainability (mostly through the green analytical chemistry framework), analysis cost was also a concern, which is important for the method's viability. But naturally, metrological performance (repeatability, detection limits, etc.) is always paramount, as it is unlikely that a worse-performing method will be adopted unless it has very significant advantages in other areas.

After the initial survey of priority pesticides to focus on (described in Chapter 2), it was found that the ionic compound glyphosate and its main degradation product aminomethylphosphonic acid (AMPA) where extremely common contaminants in EU soils [16]. Since glyphosate and AMPA cannot be extracted and analysed along with most other currently used pesticides due to being of a different chemical nature, their separate analysis becomes costly and cumbersome. Interestingly, very few articles report values for glyphosate in soil, even though it is one of the most commonly applied herbicides. This has led to the interest in developing a single methodology which could analyse glyphosate and AMPA along with other currently used pesticides that are typically determined using Gas Chromatography (GC). The results of this effort are presented in Chapter 4.

Another important objective of this work was to develop a portion of the software needed to run its calculations rather than using existing proprietary programs, with the aim of eventually uniting it in a single package for distribution as free software. Scripts to perform experimental design development and analysis as well as calculations relating to the calibration and quantification of the analytes, among others, were developed and

used throughout the work, and eventually turned into a Python package called Chromapy, which is now freely available.

This work has led to the publishing of four articles, which are included in the thesis:

- **J. Brinco**, A. B. Ribeiro, J. Cardoso and M. G. da Silva (2024). *Monitoring pesticides in post-consumer containers by GC/TOFMS and HPLC/DAD after the triple rinse method.* in International Journal of Environmental Analytical Chemistry [17]. **Article 1**.
- **J. Brinco**, P. Guedes, M. G. da Silva E. P. Mateus and A. B. Ribeiro (2023). *Analysis of pesticide residues in soil: A review and comparison of methodologies.* in Microchemical Journal [14]. **Article 2**.
- **J. Brinco**, R. Carvalho, M. G. da Silva, P. Guedes, A. B. Ribeiro and E. P. Mateus (2024). *Extraction of pesticides from soil using direct-immersion SPME LC-Tips followed by GC–MS/MS: Evaluation and proof-of-concept.* in Journal of Chromatography A [18]. **Article 3**.
- **J. Brinco**, P. Guedes, M. G. da Silva, E. P. Mateus and A. B. Ribeiro (2024). *Simultaneous determination of glyphosate and 13 multiclass pesticides in agricultural soil by direct-immersion SPME followed by solid–liquid extraction.* in Applied Sciences [19]. **Article 4**.

The remaining document is comprised of six chapters, namely:

Chapter 2

Presents the results of a monitoring campaign of pesticide concentrations in empty packaging (**Article 1**). It also describes the selection of analytes for the main study.

Chapter 3

A review of currently existing methods for multiclass pesticide residue analysis in soil (**Article 2**) as well as some considerations on the selection of analytical methods for the present work.

Chapter 4

Describes the main analytical methodologies developed during this work, firstly employing Direct-Immersion Solid-Phase Microextraction (DI-SPME) extraction for ten currently used pesticides (**Article 3**), and secondly a combined method employing DI-SPME along with solid-liquid extraction aiming to simultaneously determine the aforementioned pesticides along with others of ionic nature, notably glyphosate (**Article 4**).

Chapter 5

Presents the development and validation of another method using SPME for direct extraction of pesticides from soil without requiring extensive lab-work, by inserting

the SPME fiber directly onto the soil. It also contains the results of an electrokinetic remediation experiment used partly to validate this new method.

Chapter 6

Describes a new free software package for the analysis of chromatographic data which is a compilation of different programs designed and used during this work.

Chapter 7

Some conclusions and outcomes of the work, as well as avenues for further exploration.

2

Pesticide Monitoring and Priority Analytes

2.1 Introduction

The main work of this thesis was prefaced with a project commissioned by Valorfito [20], a Portuguese system which is in charge of recovering and managing used pesticide packaging in line with national and EU regulations. Valorfito wished to establish a monitoring scheme for quantifying the leftover pesticides in the empty packaging returned by the farmers. This coincided with their campaign to implement the triple rinse process, whereby the farmer will wash the empty pesticide package three times with clean water, a technique which has been proven to render empty pesticide packaging non-hazardous in most circumstances, though not all [21]. Valorfito hoped that the adoption of this technique by Portuguese farmers would render their waste non-hazardous according to the EU regulation (described in detail in the paper below). However, they required a way to quantify the presence of hazardous compounds in the packaging waste, and thus an analytical method was developed using solid-liquid extraction followed by Gas Chromatography coupled to Time-Of-Flight Mass Spectrometry (GC-TOFMS) and High-Performance Liquid Chromatography coupled to Diode Array Detection (HPLC-DAD).

The results of the Valorfito monitoring campaigns were taken as rough proxies of pesticide usage in Portugal, and used as a secondary source to construct the list of priority analytes for the remaining thesis work. The main source for this list was a review of recent papers quantifying pesticides in EU soils, in order to ascertain those most commonly found.

Section 2.2 of this Chapter shows the published work of pesticide quantification in used packaging, whereas Section 2.3 describes the analyte selection for the main work.

2.2 Article 1

Monitoring Pesticides in Post-Consumer Containers by GC/TOFMS and HPLC/DAD After the Triple Rinse Method

João Brinco, Alexandra B. Ribeiro, João Cardoso, Marco Gomes da Silva

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Monitoring pesticides in post-consumer containers by GC/TOFMS and HPLC/DAD after the triple rinse method

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ABSTRACT

Plant protection products are commercially available both in solid form and liquid formulation solutions. In Portugal, the volume of pesticides sold in 2019 translates into almost 800 tons of packaging material. Most of these materials are plastic containers, the remainder being mainly cardboard, mixed materials and metal. After use, the containers may still have relatively high amounts of toxic pesticides, which render them hazardous. The triple rinsing practice has been highly suggested to reduce the amount of pesticides in the empty containers, in order to allow further recycling. In Portugal there is an ongoing campaign to encourage the triple rinsing, aiming to make this type of waste non-hazardous. Thus, a way to monitor the concentration of leftover pesticides in the containers is necessary. This work describes the development of a methodology for the determination of 32 pesticides in used containers, and a monitoring campaign carried out between April 2018 and February 2021. The method involves grinding the material to a small particle size (<0.5 mm), followed by ultrasound-assisted liquid extraction with a tetrahydrofuran solution and analysis by GC/TOFMS and HPLC/DAD. The recoveries obtained were between 71–116% for all compounds except captan (62%), triclopyr (40%) and mesotrione (32%). The limits of detection and quantification were between 2.6–53.6 mg/kg for GC/TOFMS and 8.1–162.5 mg/kg for HPLC/DAD. The first three collections of containers (containing several materials from plastic to metal) showed high values for the sum of all analytes (1661 to 4332 mg/kg), whereas the last five collections (only plastic materials) presented a lower content (180 to 521 mg/kg), which reflects the effectiveness of the campaign promoting the triple rinsing practice of plastic containers.

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KEYWORDS
Empty plant protection products packaging; triple rinsing; pesticides; plastic packaging; GC/TOFMS; HPLC/DAD

1. Introduction

It is well known that pesticides carry health and environmental hazards. These compounds have very strict rules regarding their use, which are aimed at protecting the consumer from potentially toxic amounts of pesticides. Also, farmers themselves have safety guidelines intended to protect them, since improper handling causes exposure to

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high levels of these compounds, which have been linked to a host of human diseases [1–3]. The effect on the environment, however, is not so easily bypassed. Pesticides can contaminate natural systems through mechanisms such as runoff and leaching, causing an adverse effect to many non-target species including plants, insects, birds and mammals, thus presenting a danger to ecosystem´s resilience [2]. Recently, there have been works published on the toxicity of pesticides in bees [4,5]. The worldwide decline in the number of bees is a serious ecological and economic problem, especially because of these insect´s role in pollination. Although this problem is not caused entirely by pesticides, it is mostly agreed that they have an important contribution [6]. Although the term 'pesticide' may carry a negative connotation, it encompasses a very large number of compounds, and it is important to note that some will be more toxic than others. Potassium salts of fatty acids, for example, are used as insecticides and acaricides. These are some of the lesser toxic pesticides [7]. Government bodies are in charge of regulating the use of pesticides and may withdraw the usage of a particular pesticide. Recently, the European Union has removed the approval of the commonly used herbicide Diquat, for example, as its application was found to present a high risk for bystanders and residents [8]. Moreover, due to their nature, plant protection products need a particular attention concerning their use, in order to not pose a risk for the operator or the environment. With this regard, in 2013 the European commission approved the Safe and sustainable use directive of plant protection products (on the implementation of Directive 2009/128/EC on the sustainable use of pesticides. 2017/2284(INI)), on which are retained the best practices for the correct use of pesticides. Among them is the triple rinsing and the correct empty container management. Besides, the same regulatory framework, induced a gradual replacement of active substances of higher hazardous content to less harmful substances.

Pesticides are sold in a variety of different formulations. These are extremely important, because when the farmer dilutes the product for application, it must be assumed that the mixture is homogeneous – there are also other considerations, such as storage stability and ability to penetrate biological surfaces. Pesticide formulations are either presented in liquid or dry forms [9]. Although there are many different variants (depending on the chemical properties and mode of action), most of these are packaged in plastic containers, although cardboard and metal are also used. In Portugal, plastic accounts for an average 73% of usage in all pesticide containers, 4% for cardboard and 1% for metal, while the remainder is composite of several materials [10]. After use, pesticide containers must be disposed of in a dedicated container management system. Since they may still have residues of pesticides, these cannot be treated as regular household wastes, and improper disposal can lead to serious damage to environmental systems and human health [11]. Most European countries have a container management system organisation charged with the collection and proper disposal of this specific type of agricultural by-product, and farmers are encouraged to return the empty containers. In 2019, the sum of pesticides sold in Portugal amounted to almost 800 tons in packaging material [10].

Most plastic pesticide containers are made from High density Polyethylene (HDPE) or Polyethylene terephthalate (PET). These materials are both solid, and have a somewhat high plasticity. Other materials might include aluminium foil, cardboard, and different plastic polymers. In the European Union (EU), there has been an effort to implement a viable system for collection and disposal of used agrochemical containers [12]. In Portugal, there is a system for collection and disposal of empty pesticide containers,

named Valorfito. Indeed, there are over 1000 return points scattered through the country for the recovery of this type of waste. Currently, the empty containers collected by Valorfito are processed, and used mostly for co-incineration. This method of disposal uses the chemical energy present in the plastic polymers (by combustion), and if performed correctly can eliminate all organic contaminants. However, because co-incineration can be used as a solution in the disposal of many different types of industrial wastes, there may be a problem of supply and demand. Since co-incineration always needs a main fuel (such as fossil fuels), the quantity of waste that can be burned is limited, and the supply can easily outgrow the demand. Therefore, other venues for treatment of wastes must be pursued as well. In the case of pesticide containers, the most reasonable approach is to make the waste less toxic, so that it can be repurposed in other ways, such as recycling. This is achieved by reducing the amount of leftover pesticide in the empty containers. Pesticide consumers are hence encouraged to follow the 'triple rinse' technique, which entails washing the empty containers three times with water and mixing the rinse with the product. This method has been proven to reduce the leftover pesticide amount in the containers to levels considered non-hazardous [13]. The Portuguese agencies in charge of Valorfito have been campaigning since 2018 to incentivise farmers, to perform the triple rinse method in all empty rigid plastic pesticide containers, when mentioned on the product label. However, since there is no way of proving that farmers performed the triple rinse on containers they returned, the waste must be analysed.

For the waste to be classified as non-hazardous, it must comply with several complementary European directives. The first step is to determine the type of waste, according to the European Residue List [14]. This classification depends on the area of activity from which the waste comes (mining, agriculture, etc.), and the specific type of waste. Some entries are considered absolutely non-hazardous (e.g. animal tissues), while others are considered absolutely hazardous (e.g. synthetic hydraulic oils). Wastes that fall into any of these categories do not require any further evaluation, as they have already been labelled appropriately. Empty pesticide containers are a 'mirror' entry in the European residue list, meaning they can be given two different codes, depending on the level of dangerous substances present, i.e. they can be on the 150,110* category if they are considered dangerous, or 150,101, in case they are not dangerous waste. Therefore, this type of waste must be characterised, which consists of gathering all possible information about it, in order to identify dangerous substances present. If any such substances are detected (or suspected), these should be analysed individually regarding their level of toxicity and concentration. The European Union stipulates a maximum concentration of 0.1% (weight/weight) for the sum of the most dangerous compounds present, classified according to their hazard statement codes (some compounds might have a higher threshold) [15]. Furthermore, the waste must not contain persistent organic pollutants (POPs), as defined by the EU Regulation [16]. However, because of the traceable origin and use of post-consumer pesticide packaging, there is no suspicion that POPs might be present in this waste. Therefore, if the sum of hazardous substances in the material is under 0.1%, then the waste may be classified as non-hazardous.

When considering a solid polymeric material such as pesticide containers, there are several extraction approaches that can be employed to quantify the compounds present, although they all follow the same general path: homogenisation (milling) to increase surface area and improve repeatability, followed by extraction with an organic solvent or

mixture, using a variety of possible methods, and analysis. The use of heat and/or pressure is not recommended for thermo-labile pesticides. Agitation and mixing should be used instead [17–19]. Solvent choice is very important not only for extraction efficiency, but also because many compounds degrade easily in solution. Mastovská and Lehotay reported that captan and folpet entirely degraded in certain acetonitrile lots at room temperature, while being stable in others even after 5 days (initial concentration of 0.5 µg/mL, full degradation after 24 hours), which might have been caused by residual water [20].

The instrumental analysis of pesticides is mostly done by either capillary gas-liquid chromatography (GC) or High-performance liquid chromatography (HPLC). The performance of both techniques in pesticide analysis has been compared for many commercial pesticides, and neither one nor the other is ideal for every compound [21,22]. In terms of detection, generally multi-residue methods employ tandem MS, because this yields the best selectivity and lowest limits of detection. Even though high-resolution MS can give better results, the increased cost prevents it from being used in routine analysis [23].

To our knowledge, works on quantification of pesticides in plastic materials have been very sparse [17,18]. The purpose of the present work was to develop a methodology for the quantification of the main hazardous substances found in the empty pesticide containers, that can be analysed by GC/MS and LC/DAD, using a common extraction procedure for all target pesticides. The pesticides analysed in this work were selected based on their usage in Portugal, throughout all seasons and regions. Since waste toxicity classification is based on the sum of all dangerous compounds present, it is only necessary to quantify those that make up the highest percentage in that waste. This method aims to be implemented in routine analysis, thus providing a proper evaluation of the waste according to EU regulations, as there is no way of proving that the farmers have performed the triple rinse for a particular batch of containers. The aimed method is quick, cheap, easy to perform, and gives timely reliable results for routine analysis.

2. Materials and methods

2.1. Reagents

Pesticides standards, namely: acetamiprid, bromoxynil octanoate, captan, chlorpyrifos, clorothalonyl, deltamethrin, diflufenican, dimethoate, fenpiroximate, fluazifop-p-butyl, folpet, indoxacarbe, iprodione, linuron, methiocarb, metribuzin, oxadixyl, penconazole, s-metolachlor, tebuconazole, terbuthylazine, thiametoxame, λ-cyhalothrin, abamectin, bentazone, bromoxynil, chlorantraniliprole, mesotrione, penoxsulame, spinosade, thiacloprid, triclopyr were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO, USA). Bromoxynil butyrate was purchased from Chemspace (Riga, Latvia). Solvents were HPLC or LC/MS grade and were purchased from Honeywell (Charlotte, NC, USA). The water used in all experiments was distilled and purified by a milli-q system (millipore, Bedford, MA, USA). All solutions were filtered through Whatman 0.45 µm PTFE membranes filters (Buckinghamshire, UK) and de- gassed before use.

Stock solutions were prepared by weighing 5 ± 0.1 mg of each analyte and adding 20 mL of solvent, making 250 µg/mL. Captan was weighed under a nitrogen atmosphere. Solutions for GC were prepared in tetrahydrofuran (THF), while those for HPLC were initially dissolved in methanol, and then in a mixture of equal parts eluent A and B (as

explained below). For GC: The compounds were separated into two groups, because they exhibited different sensitivities. Some were calibrated between 0.2–1.4 µg/mL (named group α), while the others were calibrated between 0.7–2.5 µg/mL (group β). Oxadixyl was added to all solutions before GC analysis, with a concentration of 0.5 µg/mL, to be used as internal standard. For HPLC: The analytes were calibrated between 0.5–5 µg/mL. All solutions were kept at −18°C until analysis and protected from sunlight. For recovery experiments: A high-density polyethylene pesticide container taken from the production line before being filled was milled as explained below, 2 g were weighed, then 1.6 mL of each stock solution was added, and the solvent was evaporated under a very gentle nitrogen stream, so that the sample had 200 mg/kg of each analyte.

2.2. Sampling

Post-consumer pesticide packaging samples were taken from the waste processing facilities, which changed from 2018 to 2021. The first facility (A) had an industrial milling machine which produced very roughly milled material, and samples were taken by hand from big-bags, with most being still semi-intact containers. The second facility (B) mixed the pesticide container with other wastes, such as paper bags, cloth rags and empty pesticide containers from other sources, and then milled them. The third facility (C) produced a very homogeneous milled material with sizes of around 0.5–2 mm. Samples were taken from the milled material. two collections were made from facility A, in April and July 2018, entitled 1 and 2, respectively, one from facility B in October 2019, entitled 3, and five from facility C from February 2020 to February 2021 entitled 4 to 8, as indicated in Table 1 (in results and discussion section more details are given). From each collection, two samplings were made from material taken at different times in the milling process, or from different bags (X and Y). Material from collections 1, 2 and 3 were further cut to a size of about 1–2 mm. All samples were stored at −18°C before extraction. Each sample (X and Y) was extracted in triplicate, to a total of six extractions per collection.

2.3. Extraction

Milling was performed immediately after removing the sample from the freezer, at −18°C, in a ZM-1 centrifugal mill (Retsch GmbH, Haan, Germany), first with a 1 mm mesh, then 0.5 mm. Finally the milled material was sieved with a hand mesh of 0.4 mm. Ultrasound-assisted liquid extraction took place by weighing 0.15 g of sample into a test tube. 2 mL of

Table 1. Collection dates for each sample and plant from 2018 to 2021.

Collection	Date	Facility
1	April 2018	A
2	July 2018	A
3	October 2019	B
4	February 2020	C
5	June 2020	C
6	October 2020	C
7	January 2021	C
8	February 2021	C

THF was added, vortexed for 10 seconds and sonicated for 15 minutes. The liquid phase was removed with a Pasteur pipette, and the process was repeated with 2 mL of the solution described elsewhere [9,16]. Both extracts were mixed and filtered with a 0.45 µm syringe filter (PTFE). About 1 mL of THF was used for cleaning the syringe and filter and added to the extracted solution, so that the final volume was exactly 5 mL. For HPLC determination, 0.5 mL of sample extract was dried under a stream of nitrogen and resuspended with 1 mL of a 50/50 (v/v %) mixture of eluent A and B (indicated below). Whenever necessary, the solution was submitted to an ultrasound bath (keeping it at room temperature) to promote complete dissolution.

2.4. Chromatographic analysis

GC-TOFMS: Analyses were performed with an Agilent 7890B (Palo Alto, California, USA) gas chromatograph equipped with a split/splitless injector. An Agilent HP-5 MS UI fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm df – film thickness) was used for all separations. 1 µL of sample was injected via a LECO L-PAL3 autosampler fitted with a 10 µL syringe. The injector was operated at 260°C in pulsed splitless mode at 25 psig for 60 seconds, then purged with 20mLmin-1. 99.9999% Helium was used at a constant flow rate of 1.2 mLmin-1. The oven programme was as follows: 80°C for 1 min, then 15°C/min until 130°C, then 3°C/min until 200°C, and finally 8°C/min up to 300°C and maintained for 5 min, for a total run time of 45 min. plus cooling. Detection was performed with a LECO Pegasus BT Time-of-Flight mass spectrometer (Saint Joseph, Michigan, USA). The transferline was at 300°C. The MS was operated with the ion source at 250°C, electron ionisation at 70 eV, acquisition from m/z 40 to 550, 10 spectra per second and an acquisition delay of 10 minutes. Data acquisition, system control and spectra deconvolution were performed using LECO ChromaTOF version 5.40. NIST MS Search Program Version 2.3 g was used for spectra matching (NIST, 2015). Linear retention index (LRIs) values for sample peaks were calculated by analysing the commercial alkane standard solution C8 – C40, using the aforementioned chromatographic conditions [24].

HPLC/DAD: The system comprised an Agilent Quaternary Pump and vial sampler (Infinity II, 1260 Series), coupled to an Agilent Diode Array Detector (1100 Series). Chromatographic separation was performed on a Phenomenex kinetex C18 100 × 4.6 mm, 2.6 µm particle size (Torrance, CA, USA), maintained at 36°C and fitted with a pre-column. The chromatographic separation was carried out using as mobile phase eluent A: H2O/Acetonitrile/formic acid (94.5/5/0.5, % v/v) and eluent B: H2O/Acetonitrile/formic acid (5/94.5/0.5, % v/v). The program was: 60% eluent A for the first 2 min, then to 5% A at 5 min, 1% A at 7.5 min, and again 60% A at 8 minutes, with a constant flow of 0.9 mL/min. Quantification was performed at two wavelengths: 245 and 290 nm. The vial sampler was kept at 20°C, and an injection of 20 µL was performed.

2.5. Method validation

Recovery values were calculated by extracting the spiked material in triplicate, and then injecting each extract three times. No outliers were removed (including real samples). Limits of detection and quantification (LOD and LOQ) were calculated from the calibration curves, according to Hubetska et al., and adjusted to account for recoveries [25]. Each

extract (six per collection, as explained in Section 2.2.) was injected three times into the chromatographic system. However, it was found that instrumental errors (deviation between injections of the same extract) were meaningless compared to deviations between extracts. Therefore, these were only used to check for errors in instrument operation and the obtained triplicate values were averaged, resulting in six values per collection, one per extract.

3. Results and discussion

Samples were collected from three types of facilities, as described above, between 2018 and 2021, according to Table 1.

The full list of analytes was selected according to the pesticide's representativeness in the Portuguese professional agricultural market (unpublished data from Valorfito, 2018).

The extraction procedure was adapted from a previous method [12,19], with modifications. The solvent used for the second batch extraction can be found in the original method [12,19]. Sample grinding followed by ultrasound-assisted liquid extraction is a standard procedure for this type of material. The ratio of sample mass to extraction solvent must be kept low, as pesticides may be highly adsorbed to the polymeric material [17]. Three different sample masses had been previously tested for this extraction: 0.1, 0.15 and 0.2 g. Results showed that for the volume of solvent used (2 mL, twice), from 0.15 g of milled material provided the best compromise between good recovery, precision and method detection limit.

It is important to emphasise that the samples from facility A and B (Table 1) were intended to refine the methodology of sample preparation being constituted by a variety of materials from paper, plastic metal, among others, which do not represent the target matrix of the study – plastic – but which were considered in this work for the refinement of the analytical method (representativeness and contamination/presence of materials not targeted of the study). Facility C samples are in fact the samples that represent the reality of the recycling preparation process.

Because of the small sample size, milling to a fine powder before extraction is of major importance. Three different milling methods were tested: Ball impact cryo milling, ultra-centrifugal milling with the sample at sub-ambient temperature and ultra-centrifugal milling with the sample cryogenically frozen with liquid nitrogen. A Retzsh Cryomill was tested, whose principle of operation is to cool the sample using liquid nitrogen inside a small chamber with ceramic balls, and then using a very vigorous motion to break the sample via impact. Before milling, the samples had been cut to pieces of approximately 1 cm. This method proved very inefficient for this material, due to several reasons: the sample chamber only allowed a very small amount, so it would be difficult to ensure representability of the lot; each sample took several hours to mill, both because of the milling itself, and also because of the time required to get the sample chamber back to ambient temperature; furthermore, the milling yielded a very heterogeneous material (even after nine 15 min cycles). A Retzsh ZM1 ultra-centrifugal mill was tested in two ways: with the sample frozen in liquid nitrogen, and at sub-ambient temperature. The experiment using liquid nitrogen proved unsuccessful, because the cold sample condensed a lot of atmospheric water. Ideally, a centrifugal mill with a cryogenic accessory should have been used, because it would ensure that the analytes remained stable throughout the

process and would also have improved milling [19]. Finally, samples were stored in a freezer at −18°C, and immediately milled. This method proved the most successful and economic. After milling with a 1 and 0.5 mm mesh, the material still had some large pieces (1–0.5 mm), that probably escaped through the mesh due to the material's plasticity, so it was found that hand sieving with a 0.4 mm mesh greatly helped in lowering deviation in replicate samples.

The extraction solvent follows the original method [12,19]. THF is a moderately polar solvent, compatible with GC and also HPLC, if dilution in initial eluent mixture is performed. Direct injection of the undiluted filtered extract had been previously tested, and significant peak distortion caused by differing viscosity and strength between the mobile phase and THF was observed, as expected [26]. Dilutions of up to 1:4 (% v/v) with mobile phase still yielded poor peak shapes, and therefore, in order to maintain an acceptable limit of detection, the solvent was evaporated, and the dry extract redissolved. However, it was found that for full dissolution of the dry extract, a higher amount of aqueous solvent had to be used, at least twice as much as the evaporated volume, resulting in a minimum dilution of 1:2 (% v/v) compared to if the extract had been injected directly.

GC/TOFMS analysis was performed for all GC-amenable analytes. The time-of-flight mass spectrometer allowed for full-scan analysis while retaining good detection limits. In this way, target analytes can be adequately quantified (in agreement with the maximum allowed concentration), and a characterisation of the sample is simultaneously performed through identification of unknown peaks by comparison with the NIST spectra database [27] and LRIs. Furthermore, each sample can be retroactively searched at a later date for some compound that may become relevant. For quantification, oxadixyl was used as internal standard, as it was not found in any sample. Merit parameters for analytes detected by GC/TOFMS are presented in Table 2.

Table 2. Merit parameters for all compounds analysed by GC/TOFMS. Relative standard deviation (RSD) refers to the recovery.

Compound	Recovery %	RSD %	Calibration Range	R^2	LOD mg/kg	LOQ mg/kg	Quantifier m/z (Da)	Qualifiers m/z (Da)
Bromoxynil Butyrate	94	3	α	0.9923	4.1	12.6	71	88, 277
Bromoxynil Octanoate	92	4	α	0.9969	2.7	8.2	127	67, 88
Captan	62	3	α	0.9969	4.0	12.5	79	77, 149
Chlorothalonil	106	3	α	0.9919	3.8	11.4	266	264, 268
Chlorpyrifos	95	5	α	0.9922	4.1	12.5	97	197, 199
Deltamethrin	94	1	β	0.9879	7.4	22.7	181	253, 172
Diflufenican	97	4	α	0.9960	2.9	8.8	266	394, 101
Dimethoate	103	3	α	0.9962	2.6	8.1	87	93, 125
Fluazifop-p-Butyl	89	3	α	0.9950	3.6	10.8	282	254, 383
Folpet	75	3	α	0.9958	3.9	11.6	104	130, 260
Indoxacarb	97	3	β	0.9889	7.0	21.1	203	59, 150
Iprodione	99	3	β	0.9914	6.0	18.1	314	58, 316
Linuron	99	4	β	0.9853	7.9	23.7	187	61, 46
Methiocarb	97	3	β	0.9873	7.4	22.6	168	153, 109
Metolachlor	93	3	α	0.9955	3.2	9.7	162	238, 240
Metribuzin	93	4	α	0.9966	2.9	8.7	198	103, 144
Penconazole	94	4	α	0.9919	4.3	13.0	159	161, 248
Tebuconazole	93	4	α	0.9965	2.8	8.6	125	250, 127
Terbuthylazine	101	3	α	0.9824	5.8	17.8	214	173, 68
Thiamethoxam	86	2	α	0.9921	4.5	13.8	132	182, 212
λ-Cyhalothrin	100	4	β	0.9895	6.6	19.9	181	197, 208

Table 3. Merit parameters for all compounds analysed by HPLC/DAD.

Compund	Recovery %	RSD %	R^2	LOD mg/kg	LOQ mg/kg	Quantification Wavelength nm
Abamectin	93	1	0.9992	21.8	66	245
Acetamiprid	89	4	0.9998	11.6	35.2	245
Bentazon	76	6	0.9978	43.9	133.1	245
Bromoxynil	71	1	0.9992	29.1	88.3	245
Chlorantraniliprole	75	6	0.9989	30.9	93.7	245
Fenpyroximate	95	7	0.9976	53.6	162.5	245
Mesotrione	32	8	0.9997	37.6	114	290
Penoxsulam	83	1	0.9974	37.7	113.9	290
Spinosyn A	116	11	0.9980	25.9	78.6	245
Thiacloprid	91	4	0.9996	15.6	47.4	245
Triclopyr	40	5	0.9994	41.1	124.5	290

Because some of the analytes in the list could not be analysed by GC (due to either low volatility or thermal instability), HPLC-DAD was also employed. The chosen quantitation wavelengths and the merit parameters are presented in Table 3. In the case of Spinosad, only spinosyn A (the major component) was quantified, as spinosyn D (the minor component) presented insufficient signal at working concentrations.

Quantification results for all collections are displayed in Table 4. In each collection (1–8) two samples were taken (X and Y, each extracted in triplicate), as explained above. The highest value obtained from the two samples (X and Y) of each collection, per pesticide, is displayed. In total each pesticide amount translates the results

Table 4. Quantification results for all collections. Only the highest value from each sample (per analyte) is displayed. The maximum sum was calculated considering the compound respective LOQ, whenever the analyte was below this value. Analytes not shown were not detected in any sample.

Compound	1	2	3	4 mg/kg	5	6	7	8
Acetamiprid	-	<LOQ	-	-	-	-	-	-
Bromoxynil Butyrate	-	-	-	-	19	-	-	-
Bromoxynil Octanoate	-	-	-	-	-	10	-	-
Chlorantraniliprole	<LOQ	-	-	-	-	<LOQ	-	-
Chlorthalonil	-	-	-	<LOQ	-	30	<LOQ	<LOQ
Chlorpyrifos	18	19	164	<LOQ	-	13	-	<LOQ
Deltamethrin	-	33	45	-	<LOQ	-	-	-
Diflufenican	77	289	130	18	9	82	20	23
Dimethoate	-	<LOQ	<LOQ	<LOQ	<LOQ	-	<LOQ	-
Fenpyroximate	<LOQ	<LOQ	258	-	-	-	-	-
Fluazifop-p-Butyl	32	39	124	-	<LOQ	-	-	-
Folpet	-	-	15	-	-	-	-	-
Indoxacarb	41	419	211	<LOQ	<LOQ	-	-	-
Linuron	<LOQ	<LOQ	<LOQ	<LOQ	-	<LOQ	<LOQ	<LOQ
Methiocarb	1209	174	2144	<LOQ	23	<LOQ	-	<LOQ
Metolachlor	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	173	120	151
Metribuzin	127	26	818	29	<LOQ	<LOQ	<LOQ	<LOQ
Penconazole	<LOQ	<LOQ	<LOQ	<LOQ	-	-	<LOQ	<LOQ
Penoxsulam	-	<LOQ	-	-	-	-	-	-
Spinosyn A	149	-	-	-	-	-	-	-
Tebuconazole	-	-	9	-	10	22	-	-
Terbuthylazine	-	-	<LOQ	-	<LOQ	22	<LOQ	<LOQ
Thiacloprid	<LOQ	<LOQ	104	-	-	-	-	-
Thiamethoxam	17	80	70	-	-	-	-	.
Triclopyr	-	<LOQ	<LOQ	-	-	-	-	-
λ-Cyhalothrin	<LOQ	43	43	<LOQ	<LOQ	<LOQ	-	-
Sum	2041	1661	4332	189	180	521	223	284

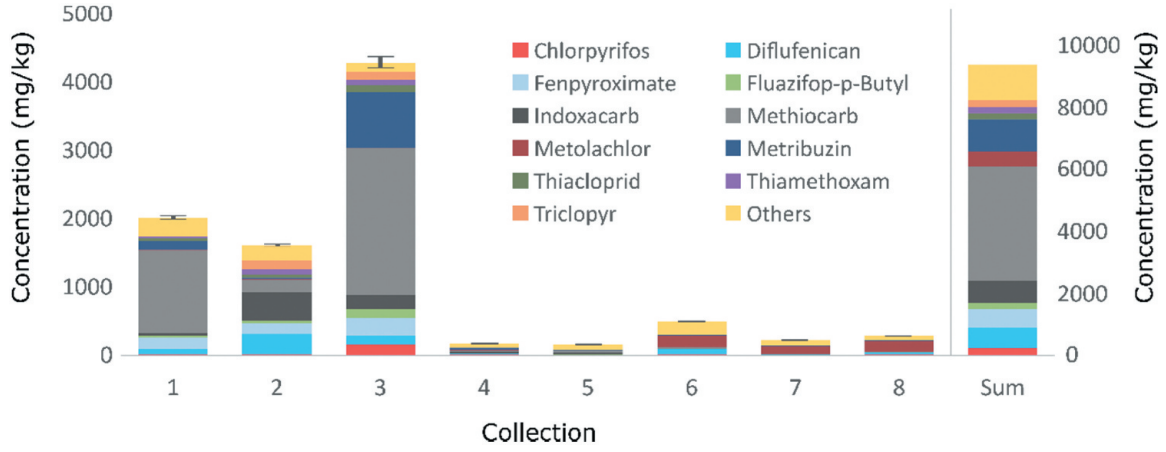

Figure 1. Graph representing the evolution in pesticide concentration for collections 1–8, left scale. Error bars were calculated from the square root of the sum of variances, representing the standard deviation of the total amount for each collection. The 'Sum' bar shows the overall presence of each pesticide in all collections, right scale.

obtained for 3 replicates, where the highest average value for each pesticide was chosen. The overall pesticide concentration is deliberately overestimated since the results for each pesticide are obtained from sample X or Y, according to which presented the highest average value for a particular pesticide. The relative standard deviation of replicates from the same sample (method relative standard deviation) was at most 14%, and typically around 1–5% (data not shown).

However, between the two samples in each collection, the relative standard deviation of the sum of all analytes was much higher, as much as 28% for collection 1. Therefore, the sampling appears to be the most critical step in the methodology, and ideally the material should be industrially milled to as fine a particle size as possible before sampling, to improve homogeneity and representability. The sum values for each collection can be seen in Figure 1.

The decline in pesticide concentrations in the last five collections suggests that the triple rinse campaign by Valorfito has been successful, and that the later materials (collections 4–8) might be classified as non-hazardous. However, it should be noted that the samples have concentration values aggregated according to the plant where they were processed: collections 1 and 2 presented similar values (both from plant A). Collection 3 has a value above any other (plant B), and collections 4 to 8 also have similar values (plant C). The material from collections 1 and 2 was obtained at the beginning of the awareness campaign on triple washing, so the highest concentration of plant protection products is expected. Regarding collection 3, the processing plant B does not have a pre-milling sorting, so the material may be mixed with others from different sources, possibly not subject to the triple rinse. The analytes quantified do not represent all pesticides that are present in the residue. There were also compounds not monitored which were tentatively identified in the sample, by comparison with the NIST database, and linear retention indexes (maximum deviation allowed = 50). The ones with higher detected signal can be seen in Table 5 which will be considered for monitorization in a future work.

Table 5. Compounds identified by GC/TOFMS with NIST spectra matching and LRIs. X denotes the presence in that collection.

		Collection							
Compound	CAS	1	2	3	4	5	6	7	8
2,4-Dichlorophenoxyacetic acid (Ester) [a]	25168–26-7				X			X	
Azoxystrobin	131860–33-8	X	X	X		X	X		
Cypermethrin (Isomers)	52315–07-8	X							
Dimethomorph	110488–70-5	X	X	X					
Metazachlor	67129–08-2		X						
Oxyfluorfen	42874–03-3	X	X	X	X	X	X		
Pronosulfocarb	52888–80-9					X	X	X	X
Pyraclostrobin	175013–18-0		X	X					
Tetraconazole	112281–77-3	X	X	X					

[a]No LRI value found in literature.

4. Conclusion

The method developed, combining GC/TOFMS and HPLC-DAD, allowed the analysis of post-consumer pesticide packaging samples according to EU regulations. Since the minimum mass limit of the sum of all the most dangerous compounds is 0.1% (w/w), which corresponds to 1000 mg of analyte per kg of material, the LOD and LOQ of the method are suitable. If it is desirable in the future to reduce this value, it will be necessary to use LC/ MS/ MS, since the HPLC-DAD analysis yielded LODs and LOQs considerably higher than GC/ TOFMS. The amount of plant protection products in the material decreased in a non-linear manner between collections, which seems to indicate the success of the triple wash campaign. The 4th, 5th, 6th, 7th and 8th collections, which indeed represent the target matrices, have concentrations below the strictest limit of 0.1% (w/w). For future analyses, the analytes list should be modified to include other relevant compounds found in the samples.

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Disclosure statement

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2.3 Analyte Selection

After the method described above was developed, yearly monitoring campaigns were performed until 2024, with all waste being classified as non-hazardous (based on the tested analytes). The results of these monitorings were taken as a rough proxy for pesticide usage in Portugal, and the most prevalent compounds were added to the analyte list for the remainder of the work.

As stated previously, the main source of information to construct the analyte list were recently published papers reporting on pesticide residues in EU soils (and to a lesser extent, water bodies). Besides being commonly used and found as environmental contaminants, two other considerations were taken when constructing the analyte list: whether the compounds could be analysed by gas chromatography (including through derivatization) and whether they had interesting/worrying properties in terms of soil persistence and/or ecotoxicology. This data was obtained from the pesticide properties database [22]. Compounds such as insecticidal soaps, for example, are of little interest in the context of this work.

The analyte list was published in one of the articles of this thesis (Table 1 in Section 4.2), which includes structures and references to the works reporting its presence in EU soils. The original compounds are boscalid, diflufenican, epoxiconazole, indoxacarb, metalaxyl, metolachlor, metribuzin, penconazol, tebuconazole and terbutylazine. The analytes requiring derivatization were not added to that publication, which are: glyphosate and its degradation product AMPA [16, 23, 24], 2,4-dichlorophenoxyacetic acid (2,4-D) [25, 26] and 2-methyl-4-chlorophenoxyacetic acid (MCPA) [23, 26]. This was the original list of 14 compounds focussed in this work. Figure 2.1 shows the chemical structures of the four analytes which require derivatization. It should be noted that 2,4-D and MCPA can already be analysed through High-Performance Liquid Chromatography coupled to Mass Spectrometry (LC-MS) without derivatization, and thus the most important analytes of that group were glyphosate and its degradation product.

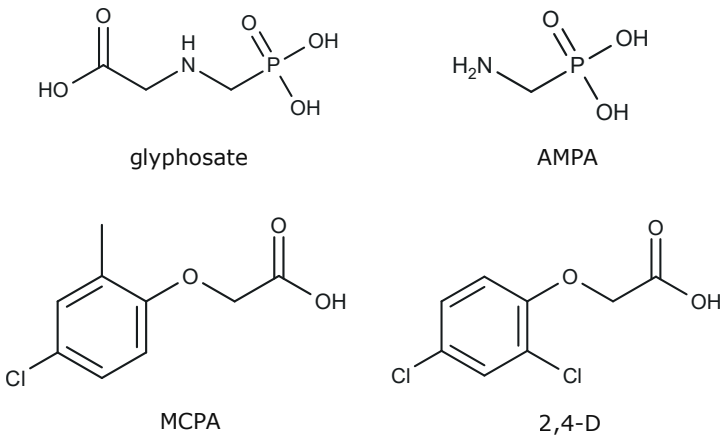

Figure 2.1: Simplified chemical structures of glyphosate, aminomethylphosphonic acid (AMPA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA).

3

A Review of Methods for Pesticide Analysis in Soil

Not many review articles have been published specifically on multiclass pesticide analysis in soil (to my knowledge). This is expected given that it is a somewhat small area of environmental monitoring. One can rightly argue that pesticide monitoring in food or drinking water is much more valued because it is consumed directly by humans. Thus, in both economic and legislative terms, pesticide analysis is more pressing for these commodities, something which can be proven by the great amount of literature on them. Nevertheless, comprehensive reviews have been published specifically on the analytical challenges and accomplishments of soil pesticide analysis, notably by Andreu *et al.* in 2004 [7] and Tadeo *et al.* in 2012 [27]. Recent works are generally more focussed on specific subjects such as microextraction techniques [28], QuEChERS [29] or a specific pesticide class such as neonicotinoids [30]; or else they bundle "environmental samples" such as soil and water with foodstuffs [31] which, although useful, does not permit an in-depth treatment of each sample type since they are different from an analytical perspective. A single recent review, by Orazbayeva *et al.* [32] focuses specifically of chromatographic determination of pesticides in soil.

Section 3.1 presents the published review paper on multiclass pesticide analysis in soil, as well as a comparison in terms of Green Analytical Chemistry, cost and time. This was obviously done in order to support the remainder of the work, and the comparison was especially relevant since none was found in the recent literature related to this specific analysis. The review also describes the novel SPME fiber configuration called "LC-Tips" which became central to the remainder of the work. Section 3.2 describes the selection of the analytical process for the present work (notably the combination of "multiclass" analytes with glyphosate), the different routes theorized, and why a novel DI-SPME extraction using the LC-Tips had to be developed.

3.1 Article 2

Analysis of Pesticide Residues in Soil: A Review and Comparison of Methodologies

João Brinco, Paula Guedes, Marco Gomes da Silva, Eduardo P. Mateus, Alexandra B. Ribeiro

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Review Article

Analysis of pesticide residues in soil: A review and comparison of methodologies

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ABSTRACT

This work reviews recently developed methodologies for multiclass pesticide residue analysis in soil and evaluates them under the focus of Green Analytical Chemistry principles, cost and time. Different extraction, clean-up and determination techniques are highlighted. QuEChERS was found to be the dominant form of extraction reported, although extractions using pressurized fluid, ultrasound and simple solid–liquid partitioning are still widely employed. GC–MS and LC-MS remain the standard analytical techniques, with the latter becoming more prevalent due to its greater versatility in analysing different chemical classes of pesticide residues, namely poorly volatile compounds. A selection of twelve representative methods was compared using the analytical eco-scale and AGREE metrics, as well as in terms of instrumental and operational cost, and time. The analysis shows that the choice of reagents and other operational parameters are more important towards the greenness of a method than the extraction and determination techniques used, but cost and time are more dependent on the techniques themselves.

1. Introduction

Soil is an invaluable resource to life on earth. Since it is not considered renewable within the time frame of a human life, soil degradation is an important and currently pressing matter [1]. For this reason, several policy frameworks address soils. Among the UN Sustainable Development Goals (SDGs), at least seven goals directly (SDG 3, 13 and 15) or indirectly (SDG 2, 6, 11 and 12) cannot be achieved without having soil as a relevant factor [2]. In this respect, contamination from anthropogenic compounds is a significant driver in the reduction of soil quality. Among them, pesticides are some of the most important contaminants in agricultural soils, both for being widely applied and for their potential harm to various organisms (including humans) [3,4].

Pesticide residue analysis in soil is an important feature of environmental monitoring. Several recent studies have pointed out the high level of contamination from currently used pesticides in European agricultural soils [5–7]. The latent presence of some pesticides in soil after use and their degradation products is a serious environmental concern. These can contaminate the food chain, promote biomagnification, be a source of adverse health effects, negatively impact microbial communities and migrate through mechanisms such as leaching and runoff to other environmental compartments, such as water. In humans, acute pesticide toxicity (high level of exposure over a short time) is rarely a concern, but chronic exposure (small dosages over a long period) is a growing problem both for farm workers and the general population [8]. Therefore, pesticide monitoring in soil is very important from a human health perspective: to identify the source of contamination and be able to address it. Nevertheless, some pesticide residues might have low direct human toxicity, but still be very harmful to environmental systems.

Generally, contaminated soils tend to have several different hazardous compounds present [7]. These result from the broad spectrum of compounds applied and from the many degradation products that can be formed from different pesticides decomposition. Consequently, analytical methods should be able to extract and determine a wide variety of compounds. Furthermore, these contaminants are often present in very low concentrations, and a portion might be strongly bound to the soil

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Table 1
Quantitative methods for multiclass pesticide residues analysis in soil published since 2010.

Analytes	Analytical Procedure	Extraction Method	Clean up and solvent shift	Instrumental Determination	%Recovery range (%RSD range)	LOD (μg/kg)	LOQ (μg/kg)	REF
5 triazine and organophosphorous pesticide residues	SLE-HS-SPME-GC–MS	SLE with Methanol/Acetone (1:1, v/v). Centrifuged and evaporated to dryness.	Resuspended in Acetone and diluted 1:50 with 25 % (wt/v) NaCl in Water. Then HS-SPME.	GC–MS (EI-Q) with USP G27 column. 36 min runtime.	70.2–104.5 (7.0–12.8) [30 μg/kg spike]	0.08–3.14	–	[31]
10 Organophosphorous pesticide residues and Buprofezin	QuEChERS (d-SPE)-GC-NPD	QuEChERS with Acetonitrile (no Water). Then $MgSO_4$, NaCl and citrate buffer. Mixture was sonicated.	d-SPE with PSA, sonicated. Dried with rotary evaporator, redisolved in cyclohexane and filtered.	GC-NPD with USP G27 column. 44 min runtime.	45–96 (1–15) [Different spike levels, except for two analytes (poor recovery)]	0.48–12.5	1.61–41.6	[24]
98 multiclass pesticide residues	**PLE-GC–MS/MS**	PLE: two extraction cycles with ethyl acetate/Methanol (3:1, v/v) at 85 °C and 1500 Psi. Dried and redisolved in ethyl acetate.	Injected directly.	GC–MS/MS (EI-QqQ) with USP G27 column. 26 min runtime. Large volume injection.	72–108 (4–25) [10 μg/kg spike]	0.2–1.7	0.3–3.3	[32]
28 multiclass pesticide residues	**PLE-UPLC-MS/MS**		Dried under nitrogen and redisolved in 50/50 mix (v/v) of mobile phases.	UPLC-MS/MS (ESI-QqQ) with C18 column. 9.5 min runtime.	77–121 (5–27) [10 μg/kg spike]	0.2–5	0.3–10	[32]
7 organophosphorous pesticide residues	SFE-DLLME-GC-FID	Supercritical CO_2 with small ammount of Methanol modifier at 60 °C and 150Bar. Collected in Acetonitrile.	DLLME with CCl_4 as extractor (added to Acetonitrile extract), dispersed in Water. Removed and injected CCl4 phase.	GC-FID with USP G27 column. Helium as carrier gas.	80–100 (3.6–12.1) [200 μg/kg spike]	1–9	–	[33]
31 multiclass pesticide residues	SFE-GC-μECD-NPD	Supercritical CO_2 with around 15 % Methanol (w/w), at 15 MPa and 318 K, four static extractions of 10 min.	Evaporated and redisolved in ethyl acetate.	GC with flow divider connected to USP G27 column for μ-ECD, and USP G3 column for NPD	44–109 (1–24) [different spiked concentrations]	4–105100	7–17710	[34]
70 multicass pesticide residues	MAE-GC–MS	Microwave extraction with Hexane:Acetone (1:1, v/v) at 100 °C for 10 min.	Centrifuged, decanted and concentrated under nitrogen.	GC–MS (EI-Q) with USP G27 column. 42 min runtime.	75.7–119.2 (2.8–18.6) [100 μg/kg spike]	–	0.001–0.4225 mg/L (Instrumental)	[35]
7 multiclass pesticide residues	QuEChERS-LC-MS/MS	Alkaline QuEChERS with Acetonitrile and Water saturated with Ca(OH)2. Then $MgSO_4$ and NaCl, mixed. Then neutralized with Hcl, following formate buffer.	Dried Acetonitrile phase with $MgSO_4$, filtered and injected.	LC-MS/MS (ESI-QqQ) with C18 column. 20 min runtime.	72.5–113.8 (1–16.3) [2–100 μg/kg spike]	0.1–0.6	0.4–2	[36]
25 multiclass pesticide residues	PLE-UPLC-MS/MS	PLE with Acetonitrile/Water (2:1, v/v) at 100 °C and 1500 Psi.	Dried and redisolved in acidified Methanol/Water (1:1, v/v).	UPLC-MS/MS (ESI-QqQ) with C18 column. 8 min runtime	65.1–122.2 (1.7–23.4) [50 μg/kg spike]	–	0.1–2.9	[37]
25 multiclass pesticide residues	QuEChERS (d-SPE)-UPLC-MS/MS	QuEChERS with Acetonitrile and Water. Then $MgSO_4$, NaCl and citrate buffer.	d-SPE with C18.	UPLC-MS/MS (ESI-QqQ) with C18 column. 8 min runtime	79.4–113.3 (1.0–12.2) [50 μg/kg spike]	–	0.1–2.9	[37]
17 multiclass pesticide residues	**SLE-LC-MS**	Agitation for 24 h at 20 °C with Methanol/Acetone (1:1), or Methanol/water (1:1) (depending on analyte)	If Water was used, SPE with Oasis HLB. Evaporated, redisolved in Methanol and filtered. For LC-MS, diluted in Water (1:1, v/v).	LC-MS (ESI) with Luna FPF2 column. 32 min runtime.	78.7–112.2 (0.1–7) [100 μg/kg spike]	0.1–0.4	0.22–0.65	[38]
14 multiclass pesticide residues	**SLE-GC–MS**			GC–MS with USP G3 column. 32 min runtime.	74.5–105.1 (3–9) [100 μg/kg spike]	0.2–0.4	0.26–0.51	[38]

(*continued on next page*)

Table 1 (*continued*)

Analytes	Analytical Procedure	Extraction Method	Clean up and solvent shift	Instrumental Determination	%Recovery range (%RSD range)	LOD (μg/kg)	LOQ (μg/kg)	REF
12 multiclass pesticide residues	PLE-GC-ECD	PLE with Dichloromethane/ Acetone (1:1, v/v) at 100 °C and 1500 Psi.	Evaporated and redisolved in acetone.	GC-ECD with USP G27 column. 39 min runtime.	77–106 (11–27) [different spiked concentrations]	0.9–8	3–7	[39]
18 multiclass pesticide residues	**UAE-LC-MS/MS**	Shaken for 1 h with Acetonitrile/Water (25:5, v/v), then sonicated. Centrifuged.	Diluted with Water and formic acid, filtered.	LC-MS/MS (ESI-QqQ) with C18 column. 15 min runtime.	50–134 (2–10) [50 μg/kg spike]	0.1–3.9	50	[40]
50 multiclass pesticide residues	QuEChERS (d-SPE)-LC-MS/MS	QuEChERS with Acetonitrile and Water. Then $MgSO_4$, NaCl and citrate buffer.	d-SPE with PSA and C18.	LC-MS/MS (ESI-QqQ) with C18 column. 16 min runtime.	40–92 (1–17) [50 μg/kg spike]	–	0.06–10	[29]
5 imidazolinone herbicides	SLE-UPLC-MS/MS	Shaken with basic solution (ammonium acetate 0.5 M in Water) and centrifuged.	Aqueous extract subjected to d-SPE with PSA, filtered, acidified with HCl and diluted with Water.	UPLC-MS/MS (ESI-QqQ) with C18. 3 min runtime.	70–93 (9–17) [different spiked concentrations]	1.5	5	[41]
29 multiclass pesticide residues	PLE-QuEChERS (d-SPE)-LC-HRMS/MS	PLE first with Acetone/Ethyl Acetate (30:70, v/v) at 80 °C, then Acetone/1% phosforic acid in Water (70:30, v/v) at 120 °C. Evaporated organic phase.	QuEChERS: To aqueous phase (after evaporation) added Acetonitrile, $MgSO_4$ and NH_4Cl. Collected Acetonitrile and performed d-SPE with PSA, C18 and GCB. Evaporated partially, added Methanol and filtered.	LC-HRMS/MS (ESI-LIT-Orbitrap) with C18. 27 min runtime.	70–245 (2–32)	–	0.7–25	[42]
216 multilcass pesticide residues	**QuEChERS-GC–MS/MS**	QuEChERS with Acetonitrile and Water. Then $MgSO_4$, NaCl and citrate buffer.	Evaporated with two drops of dodecane as keeper. Then redisolved in n-Hexane/Acetone (9:1 v/v) and filtered.	GC–MS/MS (EI-QqQ), with USP G27 column.	60–120 (1–15) [spike at LOQ level]	–	5–10	[20]
30 multiclass pesticide residues	**SLE-SPE-LC-MS/MS**	SLE with Methanol shaken for 4 h. Centrifuged and removed Methanol phase. Then extracted the same soil with Water for 12 h and centrifuged.	Methanol extract was evaporated. Water extract was subjected to SPE with OASIS HLB cartridge, eluted with Methanol/ Acetonitrile (1:1, v/v). Extracts were combined, evaporated to dryness and resuspended in Acetonitrile.	LC-MS/MS (ESI-QqLIT) with C18 column. Runtime: 33 min for + ESI, 17 min for -ESI.	70–106 (1–19) [10 μg/kg spike]	–	1	[30]
73 contaminants, of which 3 pesticides	QuEChERS (d-SPE)-UPLC-MS/MS	QuEChERS with Acetonitrile acidified with 1 % Acetic acid, and Water. Then $MgSO_4$ and Sodium Acetate.	d-SPE with C18.	UHPLC-MS/MS (ESI-QqLIT) with C18 column. 18 min runtime.	26–141 (0–29) [20 μg/kg spike]	–	0.1–5	[43]
46 multiclass pesticide residues	**QuEChERS-UPLC-MS/MS**	QuEChERS with Acetonitrile acidified with 1 % Ac. acid, and Water. Then $MgSO_4$ and NaAc.	Diluted with Water and acidified Acetonitrile, filterd.	UPLC-MS/MS (ESI-QqQ) with C18 column. 14 min runtime.	70–120 [5–250 μg/kg spike]	–	10	[6]
28 multiclass pesticide residues	**QuEChERS (d-SPE)-GC-HRMS/MS**		d-SPE with PSA and C18.	GC-HRMS/MS (EI-Q-Orbitrap), with TraceGOLD™ TG-OCP I (proprietary) column. 26 min runtime.		–	5	[6]

(*continued on next page*)

Table 1 (*continued*)

Analytes	Analytical Procedure	Extraction Method	Clean up and solvent shift	Instrumental Determination	%Recovery range (%RSD range)	LOD (μg/kg)	LOQ (μg/kg)	REF
Glyphosate and AMPA (with isotopically labeled IS)	SLE-LC-MS/MS	SLE with KOH 0.6 M in Water, shaken for 60 min and centrifuged.	Acidified extract with HCl, then borate buffer and derivatization with FMOC-Cl for 30 min. Added formic acid, vortexed and analysed.	LC-MS/MS (ESI-QqQ) with C18. 14 min runtime.		–	50	[6]
15 organochlorine pesticide residues	QuEChERS (Magnetic d-SPE)-GC–MS	QuEChERS with Acetonitrile and Water. Then $MgSO_4$ and NaCl.	d-SPE with Fe_3O_4/Triton (magnetic) and GCB.	GC–MS (EI-Q) with USP 2 column. 47 min runtime.	86–106 (2.5–8) [spike at LOQ level]	0.11–1.85	0.34–5.45	[44]
10 organochlorines and trifluralin.	QuEChERS (d-SPE)-HS-SPME-GC–MS	QuEChERS with Acetonitrile and Water. Then $MgSO_4$, NaCl and citrate buffer.	d-SPE with PSA. Dried under nitrogen, resuspended in Methanol / aqueous NaCl solution (1:100, v/v) for HS-SPME.	HS-SPME-GC–MS (EI-Q) with PDMS/DVB SPME fiber and TraceGOLD™ TG-XLBMS (proprietary) column. 60 min runtime (SPME extraction)	67.8–169.3 (1.2–21.8) [50 μg/kg spike]	0.001–1.48	0.004–4.93	[45]
10 organochlorines and trifluralin.	**SLE-HS-SPME-GC–MS**	SLE with acetone/petroleum ether (1:1, v/v) shaken for 30 min and centrifuged. Then re-extracted with petroleum ether for 30 min.	Mixed extacts, added Acetonitrile as keeper, and evaporated to Acetonitrile volume. Then added aqueous NaCl solution for HS-SPME.		65.8–180.9 (1.3–22.4) [50 μg/kg spike]	0.005–1.16	0.02–3.85	[45]
13 multiclass pesticides and 14 other analytes	**QuEChERS (d-SPE)-UHPLC-MS/MS**	QueChERS with Water and 0.5 % formic acid in Acetonitrile. Then $MgSO_4$, NaCl and Citrate buffer.	d-SPE with C18. Evaporated and re-disolved in Acetonitrile/Water (1:9 v/v).	UHPLC-MS/MS (ESI-QqQ) with C8 column. 9.5 min runtime.	47–87 (1–20) [10 μg/kg spike]	–	0.05–0.5	[46]
12 multiclass pesticide residues	QuEChERS (d-SPE)-UHPLC-MS/MS	QuEChERS with Acetonitrile with 1 % Ac. acid, and Water. Then MgSO4 and Sodium Acetate.	Acetonitrile extract frozen at −18 °C overnight "for precipitation of the wax". Then d-SPE with PSA.	UHPLC-MS/MS (ESI-QqQ) with C18 column. 15 min runtime.	65.9–89.5 [50 μg/kg spike]	10–20	–	[47]
13 multiclass pesticides, 12 pharmaceuticals and 5 transformation products	QuEChERS (d-SPE)-UHPLC-MS/MS	QueChERS with Water and 0.5 % formic acid in Acetonitrile. Then $MgSO_4$, NaCl and Citrate buffer.	d-SPE with C18. Evaporated and re-disolved in Acetonitrile/Water (1:9 v/v).	UHPLC-MS/MS (ESI-QqQ) with C18 column. 18 min runtime.	3–99 (1–14) [50 μg/kg spike]	–	0.05–0.5	[21]
38 multiclass pesticide residues and 28 other analytes	QuEChERS-LC-MS/MS	QuEChERS with Acetonitrile acidified with 1 % Acetic acid, and Water. Then $MgSO_4$ and Sodium Acetate.	Filtered.	LC-MS/MS (ESI-QqLIT) with Core-Shell C18 column. 16 min runtime.	32–143 (6–35.5) [10 μg/kg spike]	0.01–8.15	0.04–33	[3]
34 multiclass pesticide residues	**PLE-LC-HRMS/MS**	PLE with Methanol at 80 °C and 150 Bar, in two cycles of 5 min each.	Added dodecane as keeper, evaporated, redisolved in Methanol and filtered.	LC-HRMS/MS (ESI-Q-TOF) with C18. 20 min runtime.	72–126 (1–21) [40 μg/kg spike]	–	0.01–1.25 μg/L (Instrumental)	[19]
51 multiclass pesticide residues	QuEChERS-GC–MS/MS	QuEChERS with 2.5 % formic acid in Acetonitrile (no Water). Then $MgSO_4$ and sodium citrate, sonicated and shaken.	Filtered.	GC–MS/MS (EI-QqQ) with USP G27 column. 21 min runtime.	63.4–130.7 (1.3–14.3) [20 μg/kg spike]	0.024–3.125	0.5–20	[48,49]
167 multiclass pesticide residues	QuEChERS-LC-MS/MS		Filtered and dilluted with Water.	LC-MS/MS (ESI-QqQ) with C18 column. 18 min runtime.	60–128.7 (0.9–26.6) [20 μg/kg spike]	0.024–6.25	0.5–20	[48,49]
31 multiclass pesticide residues	QuEChERS (d-SPE)-LC-MS/MS	QuEChERS. 0.1 M EDTA in Water, sonicated. Then Acetonitrile and citrate buffer. Then re-	Extracts combined then d-SPE with PSA/C18. Added DMSO as keeper, and evaporated.	LC-MS/MS (ESI-QqQ) with phenyl-hexyl column. 13.5 min runtime.	66.5–118 (3.3–27.5) [20xLOQ spike]	0.01–3	0.01–5.5	[50]

(*continued on next page*)

Table 1 (*continued*)

Analytes	Analytical Procedure	Extraction Method	Clean up and solvent shift	Instrumental Determination	%Recovery range (%RSD range)	LOD (μg/kg)	LOQ (μg/kg)	REF
		extracted with more Acetonitrile.	Redisolved in Water/Methanol (8:1).					

matrix, making their extraction difficult or even impossible [9,10]. The fact that the soil itself is a very complex and variable matrix also increases the difficulty of the analysis, as it generates complex extracts that may frequently demand the use of sample clean-up procedures. All these factors play a role in the choice and development of a suitable analytical methodology.

Metrological performance and number of compounds analysed are the factors most valued and optimized for in method development studies. However, there are also other very important variables that will be explored in this review, namely the cost and time taken per sample and accordance with Green Analytical Chemistry principles. The first two factors are pragmatic in the sense that cheaper and less time-consuming analyses would allow for better environmental monitoring. Also, instrument and qualified personnel requirements are other important variables in this category. The third factor, Green Analytical Chemistry, is a relatively new framework aimed at guiding the development and implementation of analytical methodologies in the light of Green Chemistry principles, which in general aim to eliminate or reduce the usage and generation of hazardous substances, and to be as waste and energy efficient and sustainable as possible [11].

The present study intends to review techniques for multiclass analysis of pesticide residues in soil published since 2010. Twelve representative methods employing different extraction and determination techniques were selected and compared in terms of cost, time and Green Analytical Chemistry principles.

2. Pesticide analysis in soil

Soil is a complex media with a widely varied composition. The amount of organic matter (OM) is generally between 1 and 5 % (weight) in agricultural soils, but can reach nearly 100 % for organic soils, whereas minerals of different chemical and physical composition make up the remaining bulk of the solid phase [12]. The most common soil parameters evaluated when developing and validating an analytical method for pesticides are the soil's pH, OM percentage and texture [13–16]. Both organic and mineral colloids have a profound influence on pesticide adsorption and subsequent extraction efficiency [17,18].

2.1. Extraction

Soil sampling is usually performed by hand with a trowel or an equivalent tool [3]. The sampled soil depth is normally between 0 and 20 cm [19–22], related to the depth of ploughing. After collection, the disturbed samples are typically sieved through a 2 mm mesh, separating the coarse elements from the fine earth (<2 mm) [23], which is known as the "active" part of soil. Afterwards, samples are commonly dried without direct sunlight at room temperature [24–27] or at 30–40 °C [20,21,28], but lyophilization can also be employed [27,29,30]. Table 1 presents methods for the analysis of multiclass pesticide residues in soil published since 2010. Each method was given an acronym for ease of identification.

Pesticide sorption in soil is most dependent on the solid phase, both organic and inorganic [18]. For most forms of extraction, an exact amount of water is added to the sample before the extraction solvent; It helps as co-extractant of relatively polar pesticides and competes for soil sorption sites, favouring non-polar pesticide's extraction as well [51,52]. Soil pH is also an important factor in the extraction step, especially for ionizable pesticides [53]. Often, sample clean-up techniques have to be used, especially in soils with high OM content, as matrix components can seriously hamper instrumental performance and longevity [54]. Because of soil's inherent composition variability, recoveries and other merit parameters change significantly in dependence on factors such as soil pH, OM content and texture [13]. Consequently, even a standardized procedure may need to be verified among different soil types.

The mandatory determination of recovery efficiency is troublesome in soil analysis. Due to the complexity of soil systems, varying environmental conditions and chemical nature of the pesticides, they can, over time, bind to the soil matrix through several interaction mechanisms. Some of these (especially covalent bonding to soil humus) leads to stable chemical species [9], not easily overcome by solvent extraction. Thus the simple addition of pesticide standard to a blank sample may not mimic real samples for some analytes. This issue was adequately treated in a previous review [55]. Some studies report the aging of spiked samples before extraction to better emulate real conditions, generally from 3 to 20 days, but in some cases up to 2 years [28,55]. However, no perfect way exists of addressing this analytical issue.

The European Commission's guideline SANTE/2020/12830 [56] recommends that analytical methods for pesticide analysis in soil have quantification limits (LoQ) not over 50 μg/kg. Indeed, most recovery studies for method validation use concentrations equal or under this value (Table 1). However, the same document also states that relevant ecotoxicological concentrations for the most vulnerable non-target terrestrial organism be taken into account (*e.g.* median lethal dose, no observed effect concentration) [56]. If this value is lower than the expected soil concentration for a certain application rate, then the LoQ must also be lowered.

2.1.1. Solid-liquid extraction

Solid-liquid extraction (SLE) with some form of shaking is a popular technique, which is still in use today. The simplicity and low requirements in terms of equipment make it an interesting alternative to more sophisticated extraction methods. Usually, a suitable solvent or solvent mixture is added to the sample and shaken for several hours, followed by centrifugation to remove the extract [38,57]. When water is used as extraction solvent, the extract will commonly undergo subsequent solid-phase extraction (SPE) whose function is concentration, solvent shifting (for chromatographic analysis) and clean-up [30]. However, it is costly and time intensive, especially if no automated system is used.

One of the greatest advantages of SLE is its versatility. It can be used for difficult analytes such as glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), which cannot be extracted along with other pesticides in multi-residue methods due to their ionic nature [6,57]. The choice of solvent, modifiers (particularly pH control) and extraction conditions make this technique, above all others, the most adaptable. In multi-residue methods however, the high extraction time, solvent volume and frequent need for clean-up and solvent evaporation make it less desirable when compared to other techniques.

2.1.2. Pressurized liquid extraction

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction or pressurized fluid extraction, has been widely applied in the analysis of pesticide residues in soil. It is an extraction technique which uses organic or aqueous solvents at increased temperature and pressure (in the range of 40–200 °C and 35–200 Bar) [58]. This

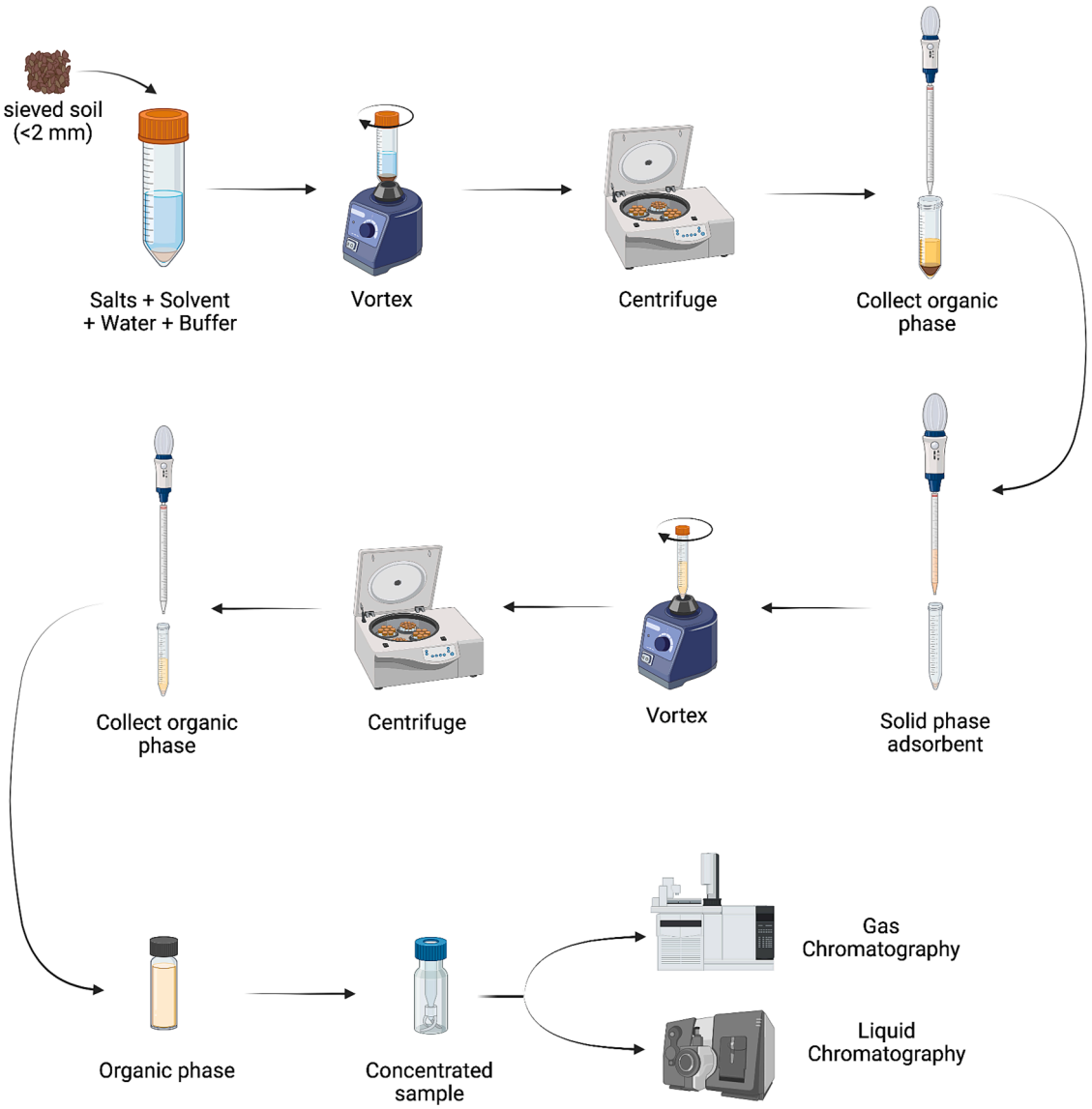

Fig. 1. Simplified scheme for QuEChERS methodology used for pesticides extraction from soil (Created with BioRender.com).

technique has seen wide acceptance due to its ease of use, speed, and effectiveness. However, equipment and operative costs remain high, and the extreme conditions may not be favourable for the extraction of some thermo-labile and sensitive analytes [59].

Pesticide extraction from soil has been performed with a variety of different solvents such as acetone, methanol and acetonitrile, but is commonly executed with a combination of solvents [19,29,32,37]. These can also be moderately acidified for better extraction and analyte stability [21]. Besides the use of organic solvents, the sample is commonly mixed with diatomaceous earth prior to PLE for drying and preventing the pressure-induced aggregation of sample particles [32]. Although this adds extra waste, diatomaceous earth itself is not toxic. The combination of low solvent volumes, semi-automation and green solvent choices make PLE a good choice for the future development of greener extraction methodologies.

2.1.3. *QuEChERS*

QuEChERS (short for Quick Easy Cheap Effective Rugged and Safe) is the commercial name given to a family of extraction methods proposed by Anastassiades *et al.* [59]. The original methodology had the purpose of overcoming limitations of routine pesticide monitoring in foodstuffs, for which a simple, cheap and fast method is essential. Since then, the method has been widely accepted by the scientific community and used in the extraction of several different analytes from various materials [60]. A general diagram of QuEChERS for soil analysis is presented in Fig. 1.

The basic method involves extraction with acetonitrile followed by the addition of salts (most commonly magnesium sulphate and sodium chloride) and centrifugation to separate the organic, aqueous and solid phases, taking advantage of the inherently high volume of water in most food samples. For soil extraction, water is added along with the acetonitrile and then partitioned in the same way [3,37,43,46]. Subsequently, the same authors and others improved the technique with the addition of a buffer to the salt mixture during the salting-out stage, to maintain the pH at around 5, which was found to be the best compromise, reducing degradation of pH sensitive analytes [61]. This has also become standard in QuEChERS for soil pesticide residue analysis, with works reporting good results with both acetate and citrate buffers.

After an aliquot of the separated organic phase is removed, it is usually subjected to a clean-up step by dispersive solid-phase extraction (d-SPE). The most commonly used adsorbents are primary-secondary amine (PSA) [24], end-capped C18 [37], or a combination of both [29], depending on the co-extractives present and the chemical nature of the analytes. Chiaia-Hernandez *et al.* reported improved recoveries for

some analytes when using a combination of PSA, end-capped C18 and graphitized carbon black (GCB), probably due to reduced ion suppression in electrospray ionization [42]. d-SPE is most commonly used for QuEChERS but has also been employed in combination with other extraction techniques such as ultrasound-assisted extraction (UAE) [21,62], and solid–liquid extraction [41].

One of the biggest advantages of QuEChERS is its low equipment requirements and cost when compared to other extraction techniques. In essence, the core method only requires a homogeneously milled or otherwise porous sample and a centrifuge. Vortex mixers are widely employed in the extraction step, but not always required [63,64]. In contrast to techniques such as supercritical-fluid extraction (SFE) or PLE, the cost and knowledge barriers to applying this methodology in routine analysis are low.

Several authors have conducted studies comparing different extraction techniques for pesticide analysis in soil (PLE, UAE, SLE and QuEChERS) and despite no technique being ideal for every analyte class, QuEChERS was always reported to be generally superior in terms of metrological parameters (especially recovery) [21,29,37,65]. Valverde *et al.* [21] tested PLE, UAE and QuEChERS for the extraction of 13 pesticides along with 17 other contaminants of emerging concern (mostly pharmaceuticals) and found PLE (no d-SPE) to be the fastest method. However, QuEChERS (with d-SPE) performed better in terms of recovery for all analytes (mean recovery of 79 % versus 46 % with PLE, 50 ng/g spike). UAE also performed well in terms of recovery (mean 62 %), but the method was tedious and labour intensive [21]. Masiá *et al.* [29] compared PLE and QuEChERS for the extraction of 50 pesticides (only the latter employing d-SPE). QuEChERS performed slightly better for soil, with a mean recovery of 76 % versus 68 % for PLE (100 ng/g spike). In total, 8 compounds had recoveries under 50 % with PLE, whereas only 3 with QuEChERS. These results seem to support the decision that given current knowledge, QuEChERS should be the first option when developing a method for multi-residue pesticide analysis in soil. However, aqueous SLE or PLE must still be used for particularly polar and ionic pesticides, not easily extracted by QuEChERS [6].

2.1.4. Other extraction methodologies.

2.1.4.1. Ultrasound-assisted extraction. The use of high-frequency mechanical waves to accelerate and improve extraction is a well-established and studied method. Ultrasound-assisted extraction (UAE) can be performed using a high-power probe, which typically allows for a more fine-grained control of ultrasound application [21,65], but is commonly performed by inserting the sample vial with the extraction solvent into an ultra-sound bath [66–68]. The application of ultrasound vibrations facilitates solvent penetration through the medium and solid–liquid mass transfer, as well as dispersing soil aggregates [69,70].

UAE is commonly performed with an organic solvent like ethyl acetate, followed by evaporation and resuspension [66]. It has also been used for extracting highly polar or ionizable pesticides with aqueous solvent mixtures, which are then *retro*-extracted via solid-phase extraction (SPE) or solid-phase micro-extraction (SPME) [68,71].

Valverde *et al.* [21] used UAE with a mixture of water and acidified acetonitrile, followed by centrifugation and subsequent dispersive-solid-phase-extraction (d-SPE) for sample clean-up, in a procedure very similar to QuEChERS, but foregoing the salting-out step. However, this method was found to be less efficient than QuEChERS itself for a variety of pesticides and pharmaceuticals [21] Other authors have used sonication with QuEChERS itself to assist in the mixing of the organic, aqueous and solid phases [24,48,72,73].

As a standalone extraction method, UAE is outperformed by other currently used methodologies because it requires several batch extractions from the same sample to obtain a good recovery, resulting in a tedious and solvent-consuming method [66].

2.1.4.2. Supercritical fluid extraction (SFE). The use of SFE in soil analysis appears to be sparse. The extraction with supercritical CO_2 usually features a certain amount of a modifier solvent (generally methanol), to improve the extraction efficiency of polar analytes [74]. Changing the pressure and temperature of the fluid can greatly modify its solvation properties. Therefore, with proper method development, SFE can exhibit great extraction selectivity [75]. Gonçalves *et al.* [28] developed and optimized an SFE-GC–MS/MS methodology to extract 20 pesticides of different chemical classes from soil, with excellent recoveries (80.4106.5 %) and good intermediate precision (4.2–15.7 %, n = 18, time span unspecified) [28]. Naeeni *et al.* [33] combined SFE with dispersive liquid–liquid microextraction (DLLME) to concentrate the sample without the time-consuming and polluting evaporation step: after SFE extraction, the collection solvent (acetonitrile) was dispersed in water and the analytes were *retro*-extracted with 17 μL of carbon tetrachloride, followed by centrifugation, after which the carbon tetrachloride layer was directly injected into the chromatographic system [33]. Although analytically relevant, this approach is complicated and labour intensive and does not address the main disadvantages of SFE in analytical extraction as a whole: it presents high cost and poor throughput when compared to techniques like QuEChERS [59]. Nevertheless, other than CO_2, SFE uses only a small volume of modifier solvent (usually) and collection solvent, which results in little waste generation.

2.1.4.3. Dispersive liquid–liquid microextraction (DLLME). This technique is most suited for the extraction of relatively hydrophobic compounds from aqueous solutions. However, it has been used for solvent shifting and concentration in soil extraction. It consists in adding a mixture of a dispersive solvent (*e.g.* acetonitrile or methanol) and an extraction solvent (*e.g.* dichloromethane, chloroform) to the aqueous solution. Through mixing or sonication, fine droplets of the extraction solvent are formed, resulting in a high contact area and efficient extraction. After centrifugation, the dense extraction solvent can easily be removed from the bottom phase [33].

Watanabe and Seike [13] have used DLLME in conjunction with solid–liquid extraction. The method involved extraction of soil with an aqueous solution for 24 h, followed by dispersion of a 6:1 (v/v) solution of dichloromethane/acetonitrile into the aqueous extract and centrifugation. This could be a viable alternative to costly and time-consuming SPE, also providing concentration and solvent shifting. However, it is not as versatile as SPE, due to having very strict requirements in terms of solvent choice, and also being a time-consuming process, involving several steps. Furthermore, most analytes extracted by aqueous SLE would not be readily soluble in the dichloromethane phase, making this technique's scope limited. Also, the use of toxic chlorinated solvents should be mitigated.

2.1.4.4. Microwave-assisted extraction (MAE). MAE has been used for the extraction of several pesticide classes from soils [22,35,76,77] and has proven to be fast and efficient, albeit extracts tend to require some form of clean-up due to the frequent presence of interferents [22]. Microwave irradiation increases both temperature and pressure in a controlled manner inside a static, sealed vial, which reduces the volume of solvent needed. Extracts are generally removed manually after cool-down, unlike PLE, for example, which collects the extract automatically. MAE requires a polar solvent to receive the energy from the microwave radiation, although a new form of proprietary stir-bar can circumvent this problem by receiving the microwave energy itself [78]. Several different solvent mixtures have been used, such as hexane–acetone (1:1, v/v) [35,76] and acetonitrile [77]. Fuentes *et al.* used water-acetonitrile and water-methanol mixtures along with hexane in the extraction cell for the analysis of different pesticide classes, which allowed for a simple removal of the hexane layer once the extraction was completed [16,22]. Furthermore, they used only 1 g of soil sample and 6 mL of extraction solvent, obtaining very good recovery RSD's, which

highlights MAE's potential for miniaturization [22]. However, this particular application only seems suitable for non-polar pesticides. Zhang *et al.* compared MAE against PLE, UAE and Soxhlet for the extraction of 70 multiclass pesticide residues and found it to perform comparably well [35].

2.1.4.5. Solid-phase microextraction (SPME). In general, SPME is not the most suitable technique for environmental pesticide analysis except for aqueous samples, where the fibre can be immersed [79]. As most pesticides are not sufficiently volatile, headspace sampling is only possible for a small subset of compounds and direct immersion in soil slurry is often impracticable, due to fibre degradation. Doong and Liao [80] used Headspace-SPME (HS-SPME) followed by gas-chromatography and electron capture detection (GC-ECD) for the determination of 18 organochlorine pesticides, by making a slurry of soil and water and extracting from the headspace. This approach is considered green: having a small number of analytical steps and being fully automatable, advantages not shared by most other extraction techniques. However, it could not be used for the analysis of most currently used pesticides, due to their low volatility. HS-SPME for quantitative purposes is also controversial among the scientific community [81].

SPME can also be used in combination with solid–liquid extraction or UAE as an alternative to SPE for concentration and solvent elimination. Lambropoulou [71] performed UAE with a 95:5 (v/v) mixture of water/acetone, followed by immersion SPME. However, the contact with organic solvents (even in small concentrations) is known to reduce the fibre lifetime. Yet, this approach could be viable for some analytes. Đurović *et al.* [31] extracted soil with a mixture of methanol-acetone, followed by evaporation to dryness, resuspension in acetone and dilution (1:50) in water with 25 % (wt/v) NaCl and performed HS-SPME with a polydimethylsiloxane (PDMS) fibre, thus avoiding extended contact with the organic solvent. However, this approach still suffers from the need for analyte volatility.

2.2. Instrumental determination

There are many factors which permitted the fast increase in the sophistication of analytical instruments witnessed in the last decades, most important of which is the advance of computational power. Whereas in the 1970′s a chromatogram was directly printed on thermal paper from which little information was obtainable, nowadays a chromatograph coupled to a high-resolution mass spectrometer can acquire upwards of 20 spectra *per* second, allowing for subsequent isolation of *m/z* fragmentation traces with four or more decimal places of precision. Furthermore, as instrument prices drop, mass spectrometers have become prevalent in both academia and industry. Nowadays many analyses must use mass spectrometry as no other detector will reach the limits of detection and confirmatory identification required by law [82,83].

2.2.1. Gas chromatography

The first pesticides to raise widespread environmental concern were non-polar organochlorines (DDT, aldrin, etc.), which are easily determined by standard capillary gas chromatography (GC) and indeed literature can be found as far back as the year 1964 [84] describing pesticide analysis in soil by GC. The increased use of highly polar and ionic herbicides, such as glyphosate and 2,4-D as well as the development of high-performance liquid chromatography coupled to mass spectrometry (LC-MS) have gained this technique wider use in pesticide analysis. However, GC remains the standard method for analysis of semi-volatile and non-polar pesticides.

Nearly all recent studies employing GC for pesticide analysis in soil use (5 %-phenyl)-polymethylsiloxane stationary phases (USP G27), or their equivalents (DB-5 ms, for example) [20,32,64]. These phases are known for their robustness, repeatability, and better retention of moderately polar functional groups than 100 % polydimethylsiloxane, although its use has also been reported [44]. More polar phases, such as (50 %-phenyl)-polymethylsiloxane [38] or (14 % cyanopropyl-phenyl)-polymethylsiloxane [27] have been used and may be a good choice for difficult separations of polar pesticides. Proprietary columns of undisclosed phase chemistry, specific for pesticide (and related molecules) have also been reported [45].

Comprehensive multidimensional gas chromatography has been used sparsely for analysing organic compounds in soil. The vastly improved chromatographic resolution is especially useful in non-targeted analysis, where it can be coupled to a high-duty cycle mass analyser (Time-of-Flight) to resolve incredibly complex mixtures and identify unknown contaminants [85]. The technique has also been used for targeted analysis [86,87], but the increased cost and operative expertise necessary prevent it from being commonly used, as one-dimensional high-resolution gas chromatography coupled to tandem mass spectrometry tends to be sufficient in targeted analysis.

Many detectors have been used in combination with GC for soil pesticide analysis, such as electron capture (ECD), nitrogen-phosphorous (NPD) or flame ionization (FID) [20,33]. Although still used in routine analysis, most of these detectors have been phased out by the scientific community in favour of mass spectrometers. The ECD has been extensively used in the past as it has very good sensitivity for most pesticides. However, it does not provide confirmatory identification as tandem mass spectrometry [88,89]. Furthermore, the use of a radioactive material presents disposability problems for end-of-life instruments. The only downsides of MS as opposed to these detectors are the higher costs and instrument complexity, as well as being more difficult to operate, although in some cases the ECD could obtain detection limits comparable to tandem mass spectrometry [88].

High-resolution hybrid tandem mass spectrometers (Q-Orbitrap and Q-ToF) have also been applied in soil analysis [6,90]. These provide a more sensitive and accurate drop-in replacement for every function the triple quadrupole performs, whilst also introducing more sophisticated techniques, especially relevant in nontargeted analyses [91]. These new mass spectrometers generally outperform the triple quadrupole in terms of identification, but not necessarily quantification limits. Belarbi *et al.* [92] reported some improvements in LoQ when using a GC-Q-Orbitrap versus GC-QqQ for 86 of 100 pesticides analysed. The authors also noted a strong tendency for matrix-induced analyte suppression rather than enhancement in the GC-Q-Orbitrap method [92]. Due to their much higher price and comparatively slight advantages these instruments are not expected to offer a cost benefit that promotes the replacement of triple-quadrupoles in targeted pesticide analysis anytime soon.

2.2.1.1. Sample preparation and analytical considerations. Matrix effects, especially matrix-induced response enhancement, are a major concern in GC analysis which can lead to serious over-estimation of the real values within a sample [93]. As internal standards, isotopically labelled analogous for each analyte are not generally employed due to their high cost (especially in multiresidue methods), although one analyte's labelled standard is commonly used as surrogate to check if recoveries are according to those determined during validation [21,43,87]. Other compounds, such as isotopically labelled caffeine and triphenyl phosphate have been used as volumetric internal standard added just before GC analysis [20,32]. Still, the most used technique to mitigate inaccuracy is matrix-matched calibration [28,43,67]. Analyte protectants have also been used in soil pesticide analysis, albeit scarcely [22,94]. These are polar compounds (usually sugars or vegetable oils) added to the sample before chromatographic analysis, masking active sites and thus enhancing analyte response [95]. Analyte protectants are injected at high concentrations (commonly between 0.1 and 1 mg/mL) and they must have sufficient volatility to move through the column and elute (as extremely broad peaks, not detected in tandem mass-spectrometry experiments). This technique is essentially an artificial form of matrix-

Table 2
Comparative analysis of selected methods in regard to the analytical eco-scale and AGREE metrics as well as cost and.

			Instrumental Cost		Operational Cost			
Method Acronym	Analytical Eco-scale	AGREE	Extraction	Determination	Extraction	Determination	Operator Time	Ref
PLE-GC–MS/MS	74	0.46	+++++	+	+++	+	+	[32]
PLE-LC-HRMS/MS	68	0.44	+++++	+++++	+++	+++	+	[19]
PLE-UPLC-MS/MS	68	0.44	+++++	+++	+++	+++	+	[32]
QuEChERS(d-SPE)-GC-HRMS/MS	79	0.41	+++	+++++	+++++	+++	+++	[6]
QuEChERS(d-SPE)-UHPLC-MS/MS	73	0.38	+++	+++	+++++	+++	+++	[46]
QuEChERS-UPLC-MS/MS	75	0.44	+++	+++	+++	+++	+	[6]
QuEChERS-GC–MS/MS	68	0.35	+++	+	+++	+	+	[20]
SLE-HS-SPME-GC–MS	79	0.46	+++	+	+	+	+++	[45]
SLE-GC–MS	81	0.46	+++	+	+	+	+++	[38]
SLE-LC-MS	72	0.44	+++	+++	+	+++	+++	[38]
SLE-SPE-LC-MS/MS	58	0.29	+++	+++	+++++	+++	+++++	[30]
UAE-LC-MS/MS	71	0.42	+++	+++	+++	+++	+++	[40]

induced response enhancement, which is controllable and reproducible. The use of analyte protectants in GC has recently been thoroughly reviewed [96]. It appears that this technique has not been widely accepted even though it is very effective at homogenizing matrix-induced response enhancement: this may be attributed to the perceived higher strain that the technique puts on instruments, due to the high concentrations used and possible contaminations from the standards (especially vegetable oils).

2.2.2. *High-Performance Liquid Chromatography*

HPLC has become critically important in pesticide analysis due to the agrochemical industry's interest in developing pesticides which are more polar, have lower volatility and are easily degradable. This development aims to mitigate some of the environmental problems that the earlier pesticides presented, such as bioaccumulation, persistence and long-range transport [97].

From an analytical perspective, polar and non-volatile compounds are not easily analysed by GC, since an expensive and time-consuming derivatization may be compulsory [98], which is not in line with green analytical chemistry principles. Reverse-phase HPLC can be used for most pesticides and a wide variety of chemical classes can be analysed in the same run [99]. Nearly all published studies use C18 column chemistry (Table 1), with either methanol/water [37,41] or acetonitrile/water [30,36,47] and formic acid modifier as mobile phases. Polar pesticides can also be analysed by hydrophilic interaction columns (HILIC), using the same mobile phases [100,101]. Especially difficult analytes such as glyphosate are often derivatized (most commonly with fluorenylmethoxycarbonyl chloride, FMOC-Cl) prior to HPLC injection in order to improve column retention [102,103]. Botero-Coy *et al.* [104] published a method for the analysis of glyphosate without derivatization by using a highly polar column. However, they also noted this column's poor robustness and rapid degradation [104].

Ultra-high performance liquid chromatography (UPLC or UHPLC) instruments have become quite common, and several studies have reported their use [6,32,37]. The higher operating pressures allow for columns with sub 2 μ*m* particles. These are generally 2.1 mm internal diameter (i.d.) and have significantly improved chromatographic efficiency than traditional HPLC columns (over 2 μ*m* particles). Furthermore, the small internal diameter also results in less mobile phase flow, which drastically reduces the cost and waste produced by LC-MS instruments.

For pesticide analysis, LC-MS with an electrospray ion source (ESI) is by far the most used [21,26,40,100]. Simpler detectors such as the diode-array (DAD) and fluorescence (FLD) have largely been replaced in most applications.

High resolution hybrid mass spectrometers coupled to LC have also been used in soil analysis [19,42]. As with GC-High Resolution MS, the advantages of these instruments in target analysis over QqQ are not always obvious [105,106]. However, their use allows for non-targeted (or "screening") analysis of environmental contaminants [42,91]. Q-q-TOF and Q-q-Orbitrap instruments enable the acquisition of full collision induced dissociation spectra from ESI generated precursors and thus allow tentative identification of unknowns. There has been some discussion in the scientific community as to whether non-targeted analysis is reproducible [107–109], but several studies have found it a very useful way to diagnose environmental contamination [110].

2.2.2.1. *Sample preparation and analytical considerations.*

HPLC can be used in conjunction with virtually all extraction methods for pesticide analysis in soil presented above. When the final extract is dissolved in an organic solvent (such as acetonitrile in QuEChERS) the extract is either dried under a stream of nitrogen, following re-suspension in an aqueous solvent [19,21,32,43], or diluted with water [40,64] and injected, for compatibility with reversed-phase LC eluents. Direct dilution and injection results in a simpler and greener method (as no solvent evaporation or energy expenditure is involved), but some methods require the concentration step to reach lower detection limits.

LC-MS ionization sources are prone to analyte signal suppression due to matrix co-elutants [111]. This is true even in highly selective multiple-reaction-monitoring (MRM) experiments. In order to reduce these phenomena, an adequate clean-up step is commonly employed. The use of isotopically labelled internal standards is common [48,50], as is matrix-matched calibration [36,47].

3. Multi-criteria comparison of methodologies

The wide range of techniques that evolved in environmental pesticide analysis is a sign of its importance and interest to the scientific community. However, they can also be a source of some perplexity and create a difficulty of choice. Metrological performance (Trueness, limit of quantitation, *etc.*) is often the most important factor when choosing a technique and optimizing a methodology [112], yet it is also fitting to compare them in terms of other criteria such as monetary cost, time expended and accordance with green analytical chemistry principles.

Twelve exemplary methods were selected for comparison based on a mix of currently used techniques both for extraction and determination. The methods are summarily described in Table 1 highlighted in Bold (Analytical Procedure column). Table 2 presents the results of each method for the criteria used. Methods for calculation and conventions are explained in the Supplementary Info.

3.1. *Green Analytical Chemistry*

The concept of Green Chemistry emerged in the 1990′s as a framework for the development of better chemical practices. It aims to promote a chemistry which is sustainable, safe for human health and the environment [113]. Paul Anastas, one of the field's founders, also preempted to the fact that analytical chemistry is an area that could be

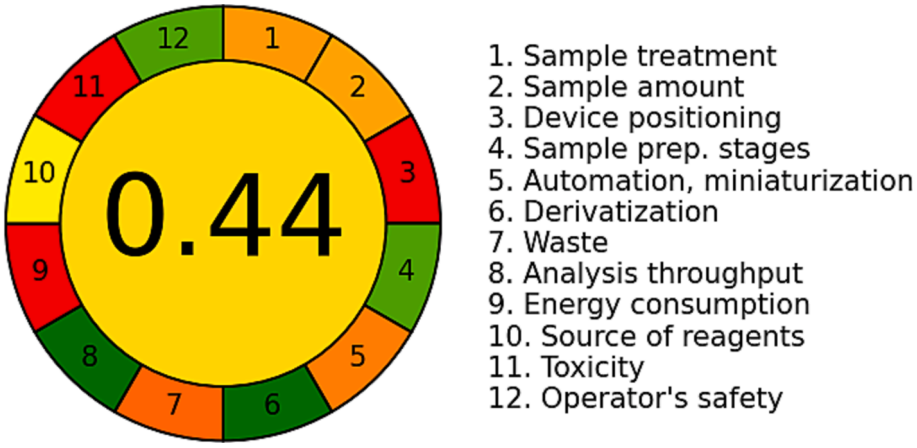

Fig. 2. Graphical representation of the AGREE metric [131].

(and has been) benefiting from the introduction of Green Chemistry Principles [113], as large amounts of hazardous solvents were often used in analytical methods [114,115]. The framework was later improved upon by the adaptation of the 12 principles of Green Chemistry to green analytical chemistry [116].

A greener method is safer for the operator and environment, faster, more efficient and often cheaper (though not necessarily related to metrological performance) [117]. In order to gauge the "greenness" of analytical methods, several methods have been developed [118,119], some of which are pictogram-based, such as the Green Analytical Procedure Index (GAPI) [120], whilst others are quantitative, providing a numerical value. Both provide a less biased evaluation than purely conceptual interpretations, enabling a better comparison of methodologies. We have chosen two quantitative metrics: Analytical eco-scale [121] and AGREE [122].

The Analytical eco-scale assumes the ideal green methodology (no solvent, minimal energy use, no toxicity, no waste) and attributes it 100 points. Then, for each deviation from the ideal (toxicity of solvents, energy use, waste generated), penalty points are attributed, reducing the overall score. Hence, the closer to 100 the score, the greener the methodology [121]. The AGREE metric closely follows the 12 principles of Green Analytical Chemistry, providing a quantifiable as well as visual way of measuring them. The principle is similar to the Analytical eco-scale, in that a maximum score of 1 represents a methodology fully compliant with the 12 principles, and deviations reduce the value. Each principle is measured separately and has the same weight on the final value (in the unaltered method). The authors [122] also developed a simple software package to assist in the calculation. Fig. 2 shows the result of the AGREE metric as per the author's software [122].

These two metrics were chosen for several reasons:

1. They are very well known and (along with GAPI) the most widely used green analytical chemistry metrics [119].
2. These metrics are applicable to all methods for pesticide analysis in soils described in this review and can be calculated with the data provided by the authors. Furthermore, additional data on solvents and reagents required for these calculations (such as GHS pictogram and hazard statements) are widely available in material safety data sheets. The United States National Fire Protection Association scores for example, which are required for the calculation of GAPI, can be hard to obtain for specialty chemicals such as the buffers often used in LC-MS mobile phases.
3. The result for each method is a single value which aids in comparing them directly (although it can also be too reductionistic) and significant variability in this value is obtained when comparing methods which are relatively similar.
4. The analytical eco-scale measures more precisely the hazard of every reagent used, whereas AGREE incorporates more green analytical chemistry principles (such as automation and the use of renewable chemicals). Therefore, these two metrics complement each other.

We have chosen to calculate the metrics unaltered (*i.e.* exactly as described by their authors) in order to maintain consistency and clarity. However, several values are the same for all methodologies and do not contribute to the comparison (the energy usage, for example, is given the same grade for all methods, as they all use either GC–MS or LC-MS).

The results are presented in Table 2. The lowest scoring analytical method (SLE-SPE-LC-MS/MS) involved several steps, including two sequential solid–liquid extractions (one with methanol and another with water), and an SPE stage for the aqueous extract [30]. It must be noted that if this method could be used to extract both polar and non-polar analytes, it might preclude the need for two separation methods in certain multi-residue analyses, making it the greener alternative. A more sophisticated chromatographic technique (such as LCxLC) could also permit the analysis of both polar and non-polar pesticides in a single run [123].

PLE extraction uses high amounts of diatomaceous earth (or other forms of silica), as well as disposable filters, which contribute to the overall waste. In addition, the equipment itself uses a considerable amount of energy. Still, it scored high due to most methods using relatively non-hazardous solvents and being a semiautomated technique, which increases sample throughput. Due to the high efficiency of PLE, a great number of analytes can be extracted from the same sample, an important goal in green analytical chemistry [32,116]. Furthermore, the avoidance of centrifugation reduces the number of vials used as well as time and energy spent.

The QuEChERS methods rated quite differently amongst each other. The lowest ranking method (QuEChERS-GC–MS/MS) used a small amount of hexane, which is highly hazardous [20]. The use of d-SPE did not seem to affect greatly the results, although it presents an extra step. The salts used in QuEChERS ($MgSO_4$, NaCl and citrate buffers) are all considered non-hazardous. There is a particular feature of QuEChERS which makes it potentially less environmentally conscious than the other techniques, especially considering its implementation: it produces a higher amount of plastic waste (in the form of conical tubes). This is because QuEChERS is usually performed with single use plastic centrifuge tubes which have the salts pre-weighed when bought. Although very time efficient and convenient, each sample will produce one or two (if d-SPE is used) conical tubes as waste. This type of waste is rarely considered in analytical laboratories, but it is very important [124]. Any method which employs a centrifuge will use conical plastic tubes. However, these should be washed and re-used as long as contamination and analyte carryover can be avoided.

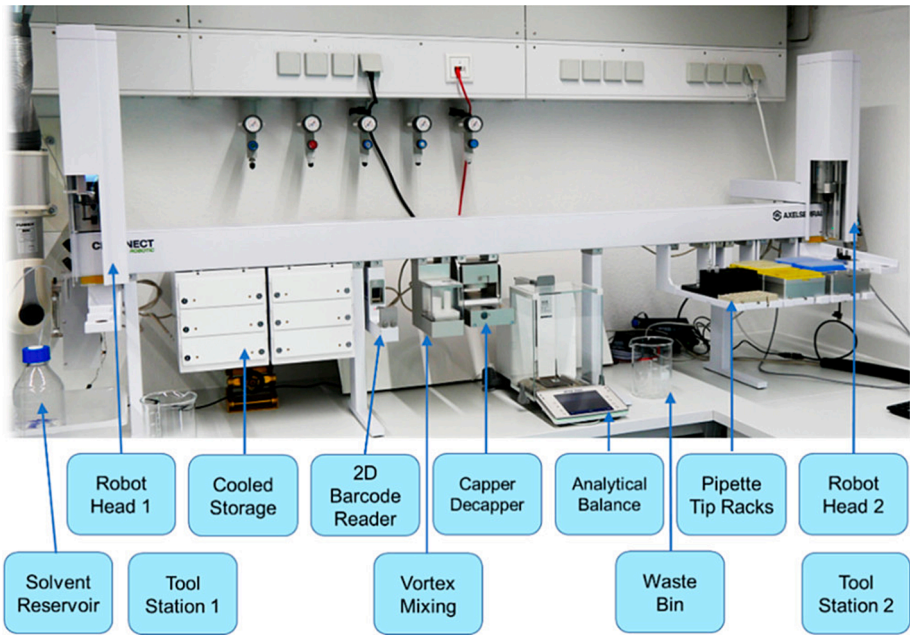

Fig. 3. Automatic multicompound working standard preparation robot (courtesy Axel Semrau GmbH). Reprinted with permission [125]. Copyright 2022 American Chemical Society.

Although GC–MS is inherently greener than LC-MS (due to less waste produced), sample preparation for GC can use highly toxic organic solvents, whereas LC is compatible with aqueous injection. Furthermore, the use of LC-MS is generally preferred to derivatization for GC–MS, in line with green analytical chemistry's principles 4, 6 and 8 (Fig. 2), especially if all target analytes can be chromatographed in a single LC-MS run alone.

In method "SLE-HS-SPME-GC–MS", the authors developed a miniaturized extraction (using only 0.5 g of soil), which ranked high in both the Analytical eco-scale and AGREE, due to the smaller volumes of solvents used, and the integration of a solvent-minimized clean-up and extraction (HS-SPME) [45]. When miniaturizing methods, there is a risk of compromising sample representativeness, especially in a highly variable material like soil, which even sieved by a 2 mm mesh will contain particles of varying sizes and densities. Sampling and sample homogenization are the critical steps to the correct application of such methods, and even when performed ideally poor repeatability and reproducibility can occur [45].

3.2. Cost and time

The cost of an analysis is seldom discussed. Instrument manufacturers are keen on guarding the prices of their products, analytical chemists only occasionally refer to costs and thus the subject of money is often buried under other considerations. This is surprising if one considers that the single most important factor for adopting an analytical procedure is availability of instrumentation or capacity (and inclination) to purchase and adopt it [112]. Instrument amortization and depreciation as well as consumable costs determine the price *per* sample analysed. From an environmental standpoint, a greater number of samples analysed (at a reduced cost) provides a far better monitoring and diagnosis.

Closely related to cost is time, which is an important consideration on two scores: less time *per* sample means more throughput (especially important with expensive instrumentation, increasing amortization rates) and less operator-time means reduced costs and possibility of human error, also increasing the analyst's throughput.

Our analysis in Table 2 provides a rough overview of the cost and time each technique entails. In terms of extraction, PLE equipment is more expensive, whilst most other methods employ cheaper shaker/vortex and centrifuges. QuEChERS will be cheaper in terms of consumables if no d-SPE is employed, but the increased strain on instruments, particularly if analysing soils with high organic matter content, will likely outweigh the benefit. The price of the technique can change greatly between buying pre-weighed kits and bulk reagents for weighing in the lab. SPE will always significantly increase cost and time expended. For chromatographic/mass spectrometric determination, LC instruments are more expensive than GC instruments with the same mass analysers, as is their running cost. Triple quadrupole instruments appear to be the ideal compromise due to their extremely good selectivity compared to single quadrupoles and midrange price. High resolution tandem mass spectrometers do not appear to provide significant metrological advantages compared to the triple quadrupole (QqQ), despite their much higher price and therefore seem to be more suited towards non-target analysis, where they excel [85,110].

4. Future Perspectives

Although analytical chemistry is a constantly evolving field, the bulk of "innovation" comprises more efficient or clever uses of existing technologies. The development of entirely new methods which break with the current paradigm is impossible to predict. Nevertheless, several recent advances from different areas of analytical chemistry could theoretically be applied to pesticide analysis in soil.

4.1. Automation

There is a constant movement towards greater automation of analytical tasks, especially in laboratories with large numbers of samples. Autosampler systems are now commonplace in nearly every instrument: their usefulness and accuracy can be attested by anyone who has had to inject a large number of samples manually. The upfront cost of automation is easily paid by less worker-hours and analytical errors. A fault from a properly validated equipment is often easier to diagnose than human error, because it's more predictable.

Nowadays, robotic systems for working standards preparation and certain forms of sample extraction and clean-up can be purchased [125] (Fig. 3). The claim that these systems are "greener" must be pondered

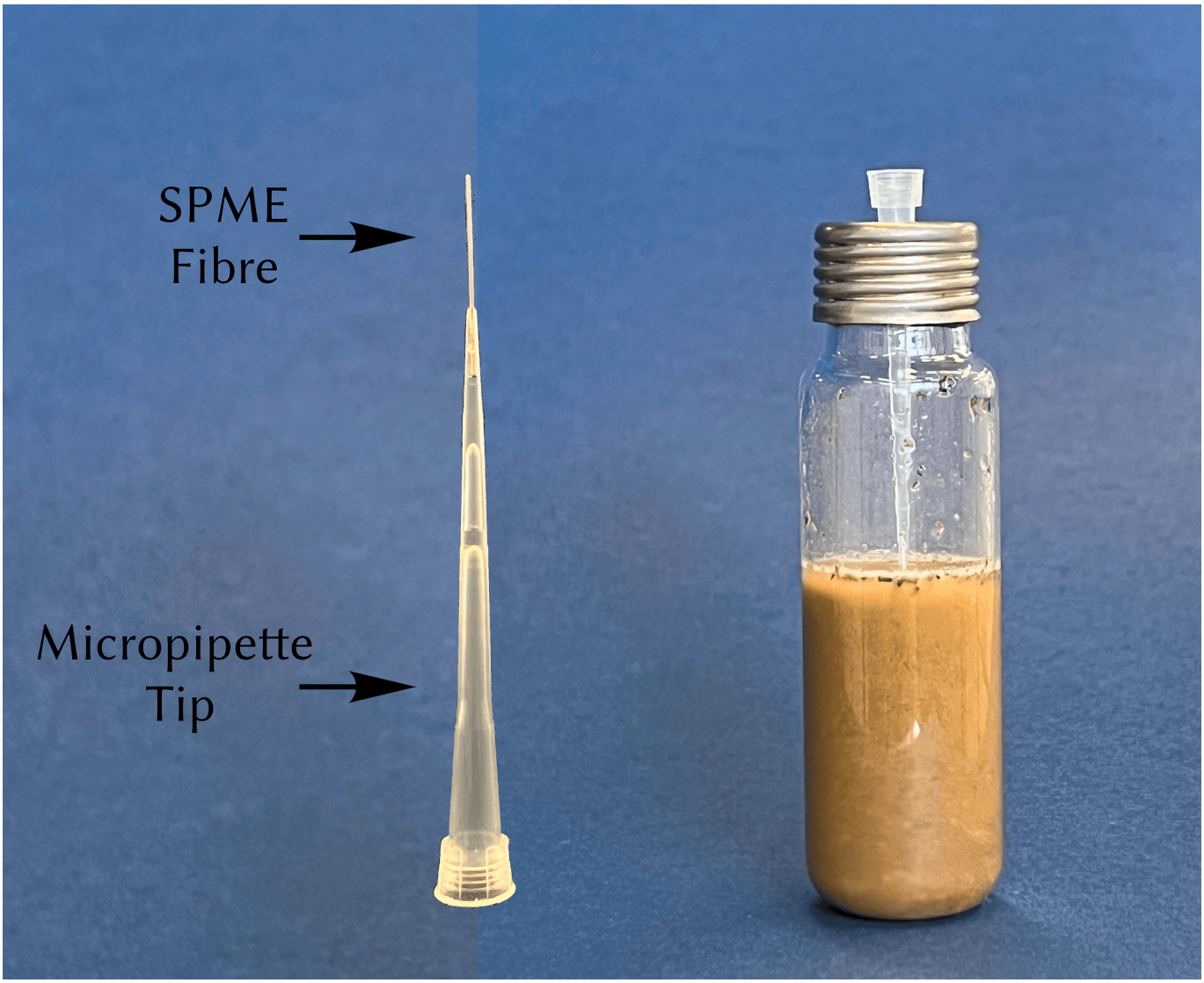

Fig. 4. SPME LC tips and a theoretical application for pesticide analysis in soil.

critically: the miniaturization of methods, reduced exposure of analysts to chemicals, increased sample throughput amongst other benefits are certainly in line with green analytical chemistry principles, but these need to be weighed against the increased energy and material use in the production and operation of such equipment, which can be substantial. Nevertheless, higher throughput and accuracy will undoubtedly result in their adoption by many laboratories.

4.2. Sample Preparation

In most methods of pesticide analysis, sample preparation involves the greatest production of waste, mostly in the form of chemicals (solvents, reagents) and disposable plastic [121,124]. Furthermore, it is often the longest and most labour-intensive step, especially since the introduction of instrument auto-samplers and automated chromatographic data processing. Therefore, sample preparation is the area with the greatest potential for development and application of greener methods.

New solid phase microextraction tips [126] (commercially called SPME LC tips) may overcome the limitation of traditional SPME for analysis of soil: small SPME fibres held to micropipette tips can be immersed in soil–water slurry and then desorbed onto an organic solvent for posterior analysis by GC–MS or LC-MS. A possible application is shown in Fig. 4. These can be used disposably because of their reduced cost (akin to SPE cartridges), and without high volumes of organic solvents.

Several studies have reported the use of switchable solvents as aids in extraction and pre-concentration of pesticides, mostly from aqueous samples [127]. These are compounds which under certain circumstances (by pH change or chemical reaction) switch from hydrophilic to hydrophobic, and vice-versa, allowing their co-dissolution in water and subsequent isolation as an organic phase. This type of switchable solvents seems to have limited application in soil analysis, but recyclable switchable solvents, which can undergo reversible chemical decomposition with temperature [128], present an interesting prospect for a greener extraction solvent, which might be theoretically decomposed and recycled, although purity and repeatability could be a problem.

4.3. Chromatography

Because both GC–MS and LC-MS are very widespread techniques with an enormous range of applications, there is abundant innovation. Although pesticide analysis in soil is not as economically significant as pharmaceutical or food analysis, new discoveries can be applied to this field with effective results.

Capillary columns with 0.25 mm i.d. are almost universally used in

all GC–MS instruments except those built specifically for fast GC. The use of narrower columns (0.18 and 0.15 mm) appears most logical for trace analysis [129], especially if a proper sample clean-up is conducted, as they give better chromatographic resolution with sharper peaks (consequently a faster separation) and require less carrier gas (also resulting in a slightly lower MS operating pressure). On the other hand, narrow-bore columns require higher head pressures and are much more susceptible to overload (particularly in splitless injections). Although 0.25 mm i.d. columns have been held as the ideal compromise for GC–MS, there is no reason why narrower columns should not be used in soil pesticide analysis, where analytes are usually present at trace levels. Indeed, this movement towards narrower columns has been seen in LC-MS, with the advent of UHPLC.

The use of helium as carrier gas in GC–MS presents a problem, since it is a limited resource with various other applications, most notably magnetic resonance imaging machines. The actual helium shortage problem has come into question [130], but even so the replacement of helium for hydrogen allows better resolution at high flow rates and it can be produced from ultra-pure water *in situ*. Unlike helium, hydrogen is reactive and can lead to reactions with the analytes. Furthermore, its lower viscosity results in less MS vacuum and consequently reduced sensitivity [131]. These effects have prevented hydrogen from replacing helium as GC–MS carrier gas, but in the future this change may be compulsory.

There is an interesting dynamic between GC and HPLC: Although GC–MS in inherently greener and mostly cheaper, due to less solvent and instrumentation demands, the rise of LC-MS instrumentation has allowed the analysis of polar and non-volatile analytes without recourse to expensive and hazardous derivatization steps. Furthermore, as LC-MS technology continues to develop (notably polarity switching and LCxLC, [123]) more classes of analytes can be determined in the same run, potentially eliminating altogether the need for GC in some analyses.

5. Conclusions

This work reviewed recently developed methods for pesticide analysis in soils and compared them in terms of cost, time and green analytical chemistry metrics. Our analysis shows that QuEChERS has revolutionized the field of sample preparation and continues to do so with new applications being published constantly. Still, other forms of extraction remain popular and indeed necessary for certain analyte classes such as the ionic pesticides glyphosate and glufosinate. In terms of instrumental determination, GC–MS and LC-MS remain the gold standard for nearly all pesticide residues, being somewhat complementary to one another, although LC-MS has asserted itself as the *de-facto* technique due to its versatility for nearly all pesticide chemical classes, with GC–MS being used increasingly only for semi-volatile pesticides which cannot be adequately quantified by LC-MS.

The accordance with Green Analytical Chemistry principles was found to be more influenced by the application than the technique itself. Any method can be made greener by clever selection of solvents, adequate miniaturization and hermitization of steps (for example). Although GC is inherently greener than LC, this comparison is meaningless for analytes which can only be chromatographed by the latter. Nevertheless, it is the choice of technique rather than its implementation which determines most of the cost and time expended in the analysis (which is also related to Green Analytical Chemistry).

It appears that pesticide analysis in soil is always downstream from food or water analysis, due to not being destined for direct human consumption. However, as an interface between mediums, soil is an extremely important and often overlooked support for life. Pesticide pollution in soils can contaminate natural systems and human food supply chains. New advances in sample preparation and analysis developed for food or water have yet to be tested in soil pesticide analysis, where they may yield simpler, greener and cheaper methods: an exciting prospect for the environmental analytical chemist.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109465.

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3.2 Method Selection

One of the main objectives of the present work was the development of a method which could analyse multiple currently used pesticides along with glyphosate using GC-MS/MS determination. In order to do this, several possible methods were theorized.

Since glyphosate is an ionic molecule at all pH's, it is more chemically similar to an amino acid than to other currently used pesticides, especially those which are analysed by gas chromatography. Because of its chemical nature, glyphosate is normally extracted with aqueous solutions and nearly always requires derivatization, followed by either GC or High-Performance Liquid Chromatography (HPLC). Several derivatization methods have been developed and used over the years [33]. If liquid chromatography is employed, fluorenylmethyloxycarbonyl chloride (Fmoc-Cl) can be used, which reacts with glyphosate's amine and adds a bulky Fmoc group [34], causing the resulting derivative to have sufficient retention on common reversed-phase HPLC columns, and allowing detection through fluorescence. For GC analysis, a complete protection of the carboxylic and phosphoric acid groups is required. Two methods are commonly employed: silylation with N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) in acetonitrile, or simultaneous acylation and esterification with trifluoroacetic anhydride (TFAA) and 2,2,2-trifluoroethanol (TFE) [33]. Both reactions are performed at high temperature, around 70 ^{o}C for silylation and 100 ^{o}C for acylation and esterification.

The very significant advantage of Fmoc-Cl over the other two methods described above is that it can be performed in the presence of water [34]. Both MTBSTFA and TFAA are extremely reactive towards water, and a dry extract is required before derivatization. Furthermore, handling of the reactants should be done in the absence of moisture (*e.g.* under a dry nitrogen stream), which further burdens the analytical procedure. This problem is exacerbated for MTBSTFA, which is not only significantly more expensive than TFAA per volume unit, but also has a much higher molecular mass and lower density. Consequently, since it has less molecules for the same volume, the presence of residual water can more easily consume the entire reagent. For this work, TFAA and TFE were chosen for the derivatization due to several reasons, as explained below in Section 4.4.

Given its chemical nature, a separate extraction would have to be employed for glyphosate, since it is not extractable by any of the commonly used methodologies described in the review above, and also to avoid submitting the other analytes to the derivatization reaction. The choice of extraction method was conditioned by the fact that the derivatization required a dry extract, and also by the wish to include the other two important ionic pesticides from the original analyte list (Section 2.3): 2,4-D and MCPA, as well as glyphosate's main degradation product, AMPA, which in the end was not feasible, as explained below in Section 4.4. For drying the extract and resuspending it after derivatization, a main requirement was the absence of non-volatile salts. Glyphosate extraction from soil has been most commonly done with a basic aqueous solution [35], which was also ideal for extracting 2,4-D and MCPA onto the aqueous medium, since

they both poses carboxylic acids. Nevertheless, the extraction is typically performed with hydroxide salts (*i.e.* KOH) [16, 35], which cannot be removed from the extract through drying or any simple chemical reaction. Thus, a previously developed extraction using ammonia solution [36] followed by its complete evaporation was much simpler than neutralizing the KOH solution with *e.g.* HCl and then filtering the precipitate before injection. A method by Hu *et al.* [36] employing the ammonia solution was selected as a starting point.

Having selected the extraction for the "ionizable" pesticides, an attempt was made to devise a method which could theoretically extract the other analytes (herein called "multiclass extraction") and be coupled to the aqueous extraction. Since a large number of currently used pesticides are known to degrade at high pH levels [37], the aqueous extraction with ammonia would have to happen after the multiclass extraction. QuEChERS was automatically ruled out, since it uses salts which mix with the soil, thereby "destroying" the original sample. The most promising idea was to do pressurized liquid extraction (PLE) with an organic solvent followed by another round of PLE with the aqueous solvent. This might have been feasible, but there were two issues: the first being that PLE instruments are not designed to work in this way, using two very different extractants with somewhat opposing chemical properties, and the second being that the equipment was not readily available. Another possibility was to perform some form of solid-liquid extraction (SLE) with an organic solvent first, such as ultrasound assisted extraction or simple SLE with shaking followed by the complete removal of the organic solvent before performing the aqueous extraction. However, these techniques, along with Soxhlet have fallen out of use largely due to their poor performance, as explained in the article above.

During the beginning stages of this work, some tests had been performed with the recent SPME LC-tips from Sigma-Aldrich (Steinheim, Germany), and these were theorized to be extremely well suited for coupling with the ammonia solution extraction, whilst possibly also being better in terms of green analytical chemistry principles, due to using an aqueous solvent for extracting low polarity analytes, something which none of the other common extraction techniques share. Thus, the idea was to develop a method for multiclass extraction using these SPME fibers, as described in the article above (Section 3.1). Interestingly, no published work employing direct-immersion SPME for pesticide extraction from a soil slurry was found, likely due to the resulting high cost of analysis, caused by the short lifespan of traditional SPME fibers under those conditions, which are much more expensive than the SPME LC-tips.

The proposed method consists of making an aqueous slurry with the soil sample, extracting with direct-immersion SPME, and then adding concentrated ammonia solution to the same slurry for the second extraction. The following chapter describes the development of that method.

4

A New Methodology for Pesticide Quantification in Soil

4.1 Introduction

After having decided on the development of a combined methodology employing DI-SPME from a soil slurry and then solid-liquid extraction with ammonia solution, the first step taken was to develop the DI-SPME technique as a standalone for multiclass analysis. This was done because a simpler and greener method only for multiclass pesticide analysis in soil could potentially be of great benefit, whereas the analysis of multiclass pesticides along with glyphosate might be a niche with only certain specific applications. Also, since no published work was found using these new SPME LC-Tips for this type of analysis (or indeed any type of DI-SPME for soil), the method had to be designed almost *ab initio*, which made it significantly more complicated than simply adapting a previously existing methodology, and thus a separate publication for its development was justified.

Section 4.2 presents the standalone DI-SPME extraction method which was developed and optimized for the analytes not requiring derivatization. Section 4.4 describes the attempted development of the combined methodology for multiclass extraction along with glyphosate, AMPA, 2,4-D and MCPA, which in the end proved only partly successful.

4.2 Article 3

Extraction of Pesticides From Soil Using Direct-Immersion SPME LC-Tips followed by GC-MS/MS: Evaluation and Proof-of-Concept

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Extraction of pesticides from soil using direct-immersion SPME LC-Tips followed by GC–MS/MS: Evaluation and proof-of-concept

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ABSTRACT

A new method was evaluated and developed for the analysis of pesticides in sandy-loam soil by direct-immersion solid phase microextraction (DI-SPME) followed by gas chromatography tandem-mass spectrometry (GC–MS/MS) determination. Ten pesticides were selected based on a literature survey of the compounds reported to be present in EU soils. The extraction was performed using SPME LC-Tips, a new SPME configuration with the coated fibers attached to a disposable and easy-to-handle micropipette tip, which was immersed into a soil slurry made by the addition of an aqueous solution to the soil sample. Ten experimental parameters were evaluated with a Plackett-Burman design, after which the extraction time and percentage of organic solvent in the aqueous extraction were optimized separately. The two fiber chemistries available (PDMS/DVB and C_{18}) were evaluated in parallel for the entire work. In the final method, slurry samples were made by adding an aqueous solution (6 % methanol v/v) to 2 g of soil. The fiber was conditioned and then inserted, for extraction, into the samples, stirred by a magnetic bar. Afterwards, the analytes were desorbed onto 100 μL of methanol. After the addition of analyte protectants (ethylglycerol, gulonolactone, and sorbitol) the extract was injected into the GC–MS/MS system. Isotopically labelled penconazole was used as internal standard. A calibration was performed by extracting spiked soil with analyte concentrations of 0.1–50 μg/kg. Coefficients of determination of the linear calibration were between 0.94–0.98 for the PDMS/DVB and 0.92–0.99 for the C_{18}. Limits of detection range between 0.01–10 μg/kg for the PDMS/DVB and 0.1–10 μg/kg for the C_{18}. Overall, the C_{18} analytically outperformed the PDMS/DVB but required a longer extraction time (120 min vs 75 min for the PDMS/DVB). This method allows automation and generates low residual toxic waste, having the potential to be introduced as a greener and simpler alternative to currently used sample preparation methodologies.

1. Introduction

Soil is crucial for sustaining life on land. Nevertheless, many soils around the world are contaminated with heavy metals and anthropogenic organic compounds [1,2]. The European Union (EU) is currently discussing a soil health law [3], in order to monitor soil quality and leverage its restoration if needed. Since EU soils have recently been found to contain very significant amounts of pesticide residues [2], a simple and effective method for their qualitative and quantitative analysis would be essential in drafting a monitoring and remediation plan.

The analysis of pesticides in soils is related to several of the sustainable development goals, namely: it promotes the safety and quality of agricultural production, food security and sustainable agriculture practices (Goal 2: Zero Hunger), it helps and supports the identification and mitigation of potential health risks associated with pesticide residues and thereby leverages human health and well-being (Goal 3: Good Health and Well-being), it contributes to prevent ecosystem pollution and the pollution of water bodies due to pesticide runoff and consequently supports the access to clean water (Goal 6: Clean Water and Sanitation) [4].

Several sample preparation methodologies have been developed for multiclass pesticide residue analysis in soil, but recently QuEChERS has become the most used and widespread [5,6]. Its low instrumental

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requirement (e.g. versus pressurized liquid extraction), simplicity, robustness, adaptability to in-house tailored methods and applicability to a wide range of analytes have made it a widely used extraction method, and it has been reported to outperform other techniques in metrological parameters and simplicity [6,7].

Nevertheless, QuEChERS is not a perfect technique: it generates significant amounts of waste *per* sample (albeit of relatively low toxicity). This is especially true because the conical centrifuge tubes are used disposably since their reuse is tedious and analytically not advised (due to the possibility of cross- and carryover contamination). Furthermore, like other multiresidue methods, it cannot be used to extract permanently ionic pesticides such asglyphosate, glufosinate ordiquat.

Solid phase microextraction (SPME) is a comparatively mature technique introduced in 1990 [8], which has found a remarkable array of applications. Headspace solid-phase microextraction (HS-SPME) is a solventless technique and has become the standard for volatile organic compound analysis in many areas of application [9], due to its high enrichment factor and simple operational and instrumental requirements when compared to classical alternative techniques such as static or dynamic headspace. For the analysis of low volatility compounds, direct-immersion SPME (DI-SPME) presents certain conveniences over traditional solid-phase extraction (SPE) in water analysis, being simpler to operate and greener, as it generates almost no waste and is potentially reusable [10,11].

SPME has been used in soil for the analysis of several pesticides by headspace sampling of a soil slurry made by adding water and sodium chloride to the sample (to promote salting-out and volatilization of non-polar compounds) [12,13]. Direct immersion into the soil slurry would result in reduced fiber lifetime and much increased analysis costs. Recently, tailor made SPME sorbent materials have been developed for the extraction of a wider range of pesticides [5]. Nevertheless, all HS-SPME methodologies require sufficient analyte volatilization into the headspace. Since most currently used pesticides are not sufficiently volatile, samples need to be heated, which reduces fiber adsorption and degrades thermo-labile analytes [5]. Several workarounds have been developed over the years, such as fiber cooling devices [14], but these techniques are operationally and technically much more complex than e.g. QuEChERS, which has prevented them from being used in routine analysis.

SPME extractions (direct immersion and headspace) often follow a complex equilibrium of the analytes between the fiber phase and sample matrix. Thus, due to being a non-exhaustive technique, quantitative methods employing SPME have traditionally been challenging. Matrix effects (changes in pH, competitive adsorption, *etc.*) influence the partition coefficients of the analytes and consequently influence the efficiency of their extraction [15]. The use of matrix-matched calibration as well as isotopically labelled surrogate standards (for mass spectrometric detection) is advised since standard addition is often too laborious and expensive [15].

Recently, a new configuration of SPME has become commercially available - the LC Tips (originally from Merck/Sigma-Aldrich and recently from Bruker under the commercial name “SPE-it tips”). In this configuration, the SPME fiber is attached to the end of a micropipette tip, which allows it to be used (typically) with 96-well microplates. These fibers were originally developed for biological sample analysis, but they permit the isolation of analytes from any aqueous solution. Unlike traditional SPME, after extraction, the analytes are desorbed onto an organic solvent (retro-extraction with acetonitrile or methanol) before being injected [16]. These new fibers are around thirty times cheaper *per* unit than traditional SPME assemblies (2023 prices from Sigma-Aldrich), which allows them to be used semi-disposably.

This work presents a novel application of DI-SPME, using the LC-Tips configuration, which attempts to overcome the limitations of traditional SPME when applied to the context of pesticide residue analysis in soil. Furthermore, it intends to provide a more sustainable alternative to currently used methodologies, in line with green analytical chemistry principles. For the extraction, a soil slurry is made with an aqueous solvent, and the extraction is performed under stir-bar agitation of the slurry. A Plackett-Burman experimental design was conducted to screen different variables in the extraction. Afterwards, the composition of the organic solvent and extraction time were optimized separately, and the final methodology was evaluated in terms of linearity, detection limits and repeatability.

In order to select the analytes for this study, we conducted a literature review of recent articles reporting on pesticide soil contamination within the EU and constructed a list of high priority analytes based on those most commonly found in EU soils, and those most used in Portugal from 2018 to 2022, determined by a monitoring campaign of used pesticide packaging [17]. The ten compounds selected for this study are presented in Table 1. Glyphosate and its metabolite aminomethyl-phosphonic acid (AMPA) were also singled out as especially important contaminants. However, due to their ionic nature (akin to amino acids), they are not expected to adsorb significantly to non-polar SPME fiber coatings and therefore were not considered for this work.

2. Experimental

2.1. Materials and chemicals

The ten pesticides (boscalid, diflufenican, epoxiconazole, indoxacarb, metalaxyl, metolachlor, metribuzin, penconazole, tebuconazole and terbuthylazine) were of analytical grade, obtained from Sigma-Aldrich (Steinheim, Germany). Penconazole-d7 was obtained from Toronto Research Chemicals (Toronto, Canada). The water used in the extraction was ultrapure, produced in a Milli-Q plus system from Millipore (Bedford, MA, USA). Methanol (MeOH) and acetonitrile (MeCN) were of HPLC grade, obtained from Honeywell (Charlotte, NC, USA). Dichloromethane was GC–MS grade, purchased from Carlo-Erba (Emmendingen, Germany). The remaining materials were all purchased from Sigma-Aldrich, namely: C_{18} and PDMS/DVB SPME LC-Tips, 3-Ethoxy-1,2-propanediol, gulonolactone and D-Sorbitol (analyte protectants) of purity 98, 95 % and 99 %, correspondingly, anhydrous sodium acetate and acetic acid of >99 % purity.

Stock solutions for the ten pesticides were prepared in acetonitrile at 250 μg/mL, and kept refrigerated at −20 ºC, for at most 1 month. Dilutions for injection and soil spiking were performed in methanol. A stock solution of the three analyte protectants was prepared in methanol at 2000 μg/mL each. Penconazole-d7 was dissolved in dichloromethane at 200 ng/mL.

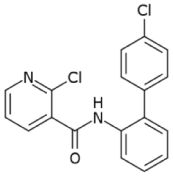
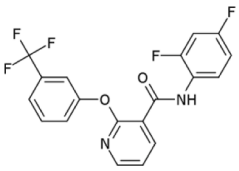
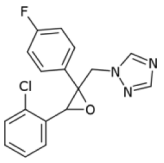
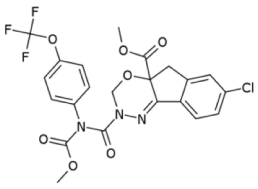
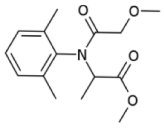
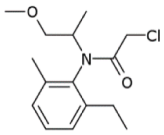
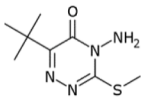
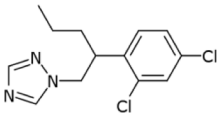
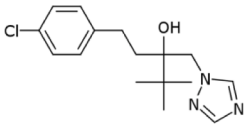
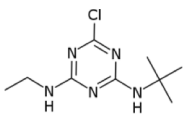
2.2. Soil sampling and spiking

The soil for the study was sampled from Idanha-a-Nova Municipality, in Eastern Portugal, from an area that was used in the past for tobacco culture (39,8454º N, 7,2544º W). Samples were collected at a depth of up to 25–30 cm. Once in the laboratory, they were sieved through a 2 mm mesh and allowed to air-dry at room temperature (~22º C), before being frozen at −20º C. Soil pH and pH_{KCl} ($n = 3$) were determined by mixing 4 g with 10 mL milli-Q water or 1 M of KCl, respectively, and shaken for 1 hour, prior to measurement.

The soil presents a sandy-loam texture (supplementary material, Table S1), 3.3 ± 0.3% organic matter ($n = 5$), pH of 7.73 ± 0.06, and pH_{KCl} of 6.94 ± 0.05. Soil metal content was also determined and can be found in supplementary material, Table S2.

For method development, the soil was spiked with the ten pesticides (Table 1) at 50 μg/kg. This was performed by adding an appropriate amount of solution to the soil (enough to disperse by capillarity, but not saturate), drying it under a nitrogen current to remove most of the solvent, and then allowing it to age for three days under aerobic conditions, after which it was kept at −20º C. A single spiked batch was used for all method development studies.

Table 1
Selection of compounds for this study, along with chemical structures and references which report their detection in EU soils. The references shown are those which report the compounds presence in soil. Logarithm of water-octanol partition coefficient (log K_{ow}) data from the Pesticide Properties Database [18].

Compound	Structure	Log K_{ow}	Action Type	Approval in EU**	CAS	References
boscalid		2.96	Fungicide	Aproved	188,425–85–6	[2,7]
diflufenican		4.2	Herbicide	Aproved	83,164–33–4	[7,17,19]
epoxiconazole		3.3	Fungicide	Not Approved	135,319–73–2	[2,7,19]
indoxacarb		4.65	Insecticide	Not Approved	173,584–44–6	[17]
metalaxyl		1.75	Fungicide	Aproved	57,837–19–1	[20]
metolachlor		3.4	Herbicide	Racemic mixture not approved, S-metolachlor (around 60 % enantiomeric excess) approved	51,218–45–2	[17,21]
metribuzin		1.7	Herbicide	Aproved	21,087–64–9	[17]
penconazol		3.72	Fungicide	Aproved	66,246–88–6	[17,22,23]
tebuconazole		3.7	Fungicide	Aproved	107,534–96–3	[7,17,19,20,23,24]
terbutylazine		3.4	Herbicide	Aproved	5915–41–3	[7,17,19–21,24]

** Data from the European Commission Website.

2.3. GC–MS/MS analysis

The analyses were performed by Gas Chromatography-Tandem Mass Spectrometry (GC–MS/MS) on a Bruker GC 456 and a Bruker Scion TQ (Triple Quadrupole) system equipped with a CTC CombiPAL automatic injector and a programmable temperature vaporizer (PTV) inlet (Bruker 1079). Data were acquired with Bruker MSWS 8.2 and analysed with Bruker MS Data Review 8.0. Chromatographic separation was achieved on a ZB-5MS Plus capillary column (20 m × 0.18 mm i.d., 0.18 μm df). The oven temperature program began at 50 ºC held for 3 min, raised at 20 ºC/min to 140 ºC, then 4 ºC/min to 250 ºC, and finally 20 ºC/min to 310 ºC held for 2 min. Helium of 99.9999 % purity was used as carrier gas at a constant flow rate of 0.7 mL/min. The injection volume was 5 μL, performed in PTV large volume mode, starting at 80 ºC with a split ratio of 1:120, held for 30 s, then splitless and a temperature increase of 200 ºC/min to 270 ºC. At 3 min, the split valve was opened at a ratio of 1:60, and after 3 more minutes reduced to 15 mL/min and held for the entire run. The mass spectrometer system was operated in multiple-reaction monitoring (MRM), with argon as collision gas at 2.4 mTorr. The transfer line was held at 300º C, and the ion source at 270º C. The solvent delay was set to 15 min. MRM transitions associated with the selected precursor and product ion pairs of the analytes, their relative ratios, and a GC–MS/MS chromatogram of the standards can be found in supplementary material, Table S3 and Figure S1, respectively. The quadrupoles were operated at unit resolution, and the ion ratios between quantifier and qualifier had to be within ±30 % the average of injected standards for positive identification [25].

2.4. SPME-Tips extraction method development

For the extraction, 2 g of soil sample were weighted into 16 mL with 22 mm diameter vials (Supelco, Steinheim, Germany), and then the aqueous solution (water with an organic modifier, MeOH or MeCN, in different concentrations) was added to create the soil slurry (Mili-Q water with 1–10 % (v/v) of MeOH or MeCN). The vial caps had pierced septa where 200 µL plastic micropipette tips were placed as holders for the SPME LC-Tip fiber (Fig. 1). Before each extraction, the fibers were conditioned by inserting them in a 2 mL glass vial (Chromacol, Thermo Fisher Scientific, MA, USA) with 100 % organic solvent (either MeOH or MeCN) for 30 min, followed by re-equilibration in another 2 mL glass vial with the aqueous solution (water with an organic modifier, MeOH or MeCN, in different concentrations) for another 10 min, always under agitation on a Bunsen AO 400 mechanical linear/orbital shaker at 250 RPM's. Afterwards, the SPME LC-Tip fibers were immediately inserted into the soil slurry (without drying), which was stirred using a magnetic bar. After extraction, the fibers were removed and retro-extracted to 100 μL of the same organic modifier as the one used in the aqueous solution (MeOH or MeCN), using 300 μL insert glass vials (Alwsci Technologies, Zhejiang PR, China), under agitation at 250 RPMs. Every step of the extraction was performed at room temperature (22 ºC).

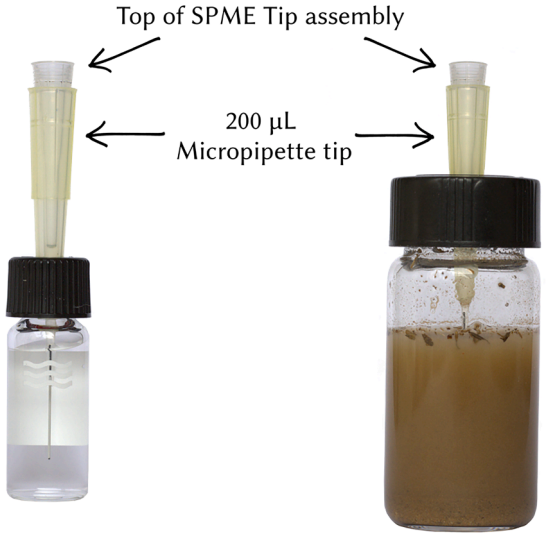


Fig. 1. Experimental setup for soil extraction using SPME-LC tips: (a) conditioning step, using 2 mL vials, (b) extraction step, employing magnetic stir-bar agitation. Retro-extraction is performed similarly to conditioning, except it uses vials with 300 μL inserts.

After initial experimentation, ten variables were determined for screening, to gauge their effect on method efficiency. A twelve experiment Plackett-Burman design was used for this purpose. The factors (variables) were: extraction time (30 or 60 min), use of a buffer (0.02 g of sodium acetate *per* mL of solvent and extraction aqueous solution with 1 % v/v acetic acid, values adapted from Lehotay et al. [26]), fiber coating chemistry (C_{18} versus PDMS/DVB), sonication of the slurry (prior to extraction, for 5 min, using Bandelin Sonorex Super RK 102 H, Berlin, Germany), salting-out (NaCl at 0.03 g/mL of aqueous solution, added to the dry soil), percentage of organic modifier (1 or 10 %), type of organic modifier (MeOH or MeCN), volume of solvent (10 or 12 mL, always for 2 g of soil), retro-extraction time (10 or 30 min) and concentration of analyte protectants [27] (250 or 500 µg/mL). Each experiment was performed in triplicate, and the SPME fibers were rotated randomly between experiments to prevent bias; twelve fibers were used, each three times. The main effect of each factor was calculated using the average of the three replicates of each experiment, and then tested for significance against a dummy factor (the eleventh variable in the design) using a one-tailed F-test at 0.05 significance level [28]. After the Plackett-Burman screening, the percentage of organic modifier and extraction time were further optimized for both fiber chemistries using the same spiked soil, whilst all other variables were fixed (Section 3.2).

2.5. Final extraction method

The final method was tested for both fiber chemistries in parallel. Samples were prepared by weighing 2 g of soil onto the extraction vial and adding 200 µL of internal standard solution (200 ng/mL in dichloromethane), which was allowed to air-dry for 30 min. Fibers were conditioned by being inserted in a 2 mL glass vial containing methanol for 30 min, followed by re-equilibration in another 2 mL glass vial containing water with 6 % methanol (v/v) for 10 min, under constant agitation at 250 RPMs. Afterwards, they were immediately exposed to the soil slurry, which was prepared by adding 10 mL of water with 6 % methanol (v/v) to the soil sample. The only operational difference between the fiber chemistries was the extraction time: 75 min for PDMS/DVB and 120 min for C_{18} (supplementary material, figures S2 and S3). After the extraction, the fibers were immediately inserted into a conical vial with 100 µL of methanol for 30 min at constant agitation (250 RPMs). From this methanolic extract, 60 µL were removed from the top (to eliminate any sediment deposited at the bottom, transported with the fiber) to another conical vial, and 20 µL of analyte protectant solution was added (to a final concentration of 500 µg/mL each), before analysis by GC–MS/MS.

2.6. Method validation

The calibration was performed for both C_{18} and PDMS/DVB fibers, by spiking the soil within the extraction vial with the ten pesticides at concentrations of 0.1–50 µg/kg (seven concentrations), and drying under a nitrogen stream, before following the final extraction method. For every concentration level, two different fibers were used, each two times, for a total of four extractions *per* concentration. Limits of detection (LoD) and quantification (LoQ) were determined visually for the qualifier MRM transition of each analyte (which had a worst signal-to-noise ratio than the respective quantifier) in the following way: the

lowest calibrated concentration with a signal-to-noise ratio above 3 (and clear peak identification) was assumed as the LoD, and above 10 was assumed as the LoQ. These limits were also calculated with a different method, from the calibration curve [29] (using the quantifier signals with higher signal-to-noise ratios), and the results can be found in the supplementary info, Table S4. Mandel's test was performed to judge the linearity of the calibration data [30]. Coefficients of determination for linear and quadratic regressions can be found in supplementary info, Table S8.

For estimating the mass of each analyte extracted, a calibration curve was performed by preparing six solutions in methanol with concentrations ranging from 0.1 to 40 ng/mL, and the same amount of analyte protectants as the extracts (500 μg/mL). These solutions were injected with the same method as the extracts. Least-squares fitting was performed in absolute units (no internal standard). The analyte mass injected into the GC–MS/MS was then calculated for the extracts of spiked soil at 10–30 μg/kg and transformed into a percentage of the total analyte mass in that spiked soil sample.

3. Results

3.1. Initial screening of variables

The first step was to evaluate the methodological operability of the extraction. SPME LC-tips were originally developed for biological samples on well trays, which allow process automation (*e.g.* robotic liquid handling systems), but this would not work for soil due to the larger sample amount and greater agitation required. The preliminary trials (data not shown) allowed us to determine that to produce a homogenous slurry (rather than soil deposition at the bottom), magnetic stir-bars were ideal, as opposed to shaking the entire vial. This also had the advantage of leaving the vial and fiber assembly static. Nevertheless, a very strong vortex is not advised, as it can leave the fiber exposed to air rather than the slurry, or damage it. The only disadvantages of using the stir-bars were (1) a more labor-intensive method and (2) increased strain on the materials, caused by the abrasive nature of soil particles, resulting in visible wear on both the vials and stir-bars. Still, observation of the fibers, which were located above the stirring bars, showed that they had good elasticity, and no visible mechanical damage was observed.

The first screening of variables was performed with a Plackett-Burman design (Section 2.4). These variables were chosen based on what were thought to be the influencing factors in the extraction. The results are presented in Table 2. The main effects were also calculated using the relative standard deviation of each experiment ($n = 3$), and tested for significance; However, only three significant values were present, at 0.05 significance level: diflufenican had a lower relative standard deviation (RSD) with no addition of salt, and both diflufenican and boscalid had lower RSD at 1 % organic modifier. Non-significant values can be found in the supplementary material, table S5.

The experimental results for some of the variables were similar across all analytes, namely acetate buffer, salting out and sonication, resulting in the exclusion of these steps from the extraction process. It was thought that increasing the ionic force of the slurry (salting-out) might potentiate the migration of analytes to the fiber. However, the results show the opposite, probably because the extraction from the soil to the aqueous solution was hampered. The buffer also increases the ionic force, and the neutral acetic acid molecules might compete for the fiber, especially the PDMS/DVB coating, further reducing extraction ability. It must be noted that, especially with alkaline soils, the use of a buffer may be preferable since several pesticides are known to degrade at high pH levels [26]. Furthermore, an unbuffered method will not be as robust to changes in soil chemistry.

A concentration of 500 μg/mL in the injected extract was used because it performed better overall (Supplementary material, table S5), even though most of these values were not statistically significant at 0.05 significance level. Nevertheless, a higher analyte protectant concentration should be beneficial for more polar and difficult analytes.

Fiber chemistry extraction performance varied among analytes, which is expected as they exhibit different polarities. Proper choice of fiber is one of the more important variables to consider for a specific pesticide class. Since this method aimed at analyzing several pesticide classes, further optimization was performed with both fiber chemistries.

According to the experimental data, at 30 min of extraction, no analyte had achieved the partition equilibrium point between the sample matrix and the extraction phase. Thus, the extraction time was one of the variables that needed further optimization. In terms of retro-extraction time, most analytes showed better signal and lower RSD at 30 min, but without statistical significance. A higher stirring during retro-extraction would likely improve desorption kinetics, but this was not necessary and could also create a vortex, causing the top portion of the fiber to not fully submerge into the solvent (as referred before).

The solvent volume was not a relevant variable for most analytes. Although a higher solvent volume increases the pesticide extraction from soil, it shifts the fiber adsorption-desorption equilibrium in the opposite way. A 10 mL volume was chosen for further studies because it was operationally simpler and produced slightly less waste.

3.2. Organic modifier and extraction time

The results of the Plackett-Burman screening point to a correlation between the modifier and its percentage in solution, with better results being achieved for 1 % methanol and 10 % acetonitrile. Methanol was selected primarily because the analyte protectants added before GC analysis have a low solubility in acetonitrile (especially the two sugars), but also because it showed better repeatability (Supplementary material, table S5).

The organic modifier may create different mechanisms within the extraction process: it favors extraction of the analytes from the soil

Table 2
Results of the Plackett-Burman screening. The values displayed are those which yield the highest signal for the average of three replicates. Non-significant main effects at 0.05 significance level are displayed by "-". Analytes are shown by elution order on GC–MS/MS (terbuthylazine first, indoxacarb last).

Analyte	Extrac. time (min)	Buffer	Fiber	Sonication	NaCl	%Organic modifier	Organic modifier	Solvent Volume (mL)	Retro-extrac. Time (min)	An. Protectant (mg/L)
terbutylazine	60	No	PDMS/DVB	–	–	1 %	MeOH	–	–	–
metribuzin	60	–	PDMS/DVB	No	No	1 %	MeOH	10	–	250
metalaxyl	–	–	–	–	–	1 %	–	–	–	–
metolachlor	60	–	–	–	–	–	–	–	–	–
penaconozole	60	No	–	–	No	10 %	–	–	–	250
tebuconazole	60	No	C18	–	No	–	–	–	–	–
diflufenican	60	–	–	–	–	–	–	–	–	–
epoxiconazole	60	No	C18	No	No	10 %	MeCN	–	–	500
boscalid	60	No	–	–	No	–	–	–	–	–
indoxacarb	60	–	–	–	–	10 %	MeCN	–	–	–

(increasing total extraction and equilibrium speed), it reduces the adsorption towards the fiber by increasing analytes solubility in the liquid phase, and it may compete with the analytes for the fiber. It remains unclear whether the organic modifier had a greater effect on the kinetics (speed) or thermodynamic equilibrium (overall "extraction").

The percentage of organic modifier (methanol) was tested with a 60 min extraction time and 30 min retro-extraction. The results are presented in Fig. 2 for both C_{18} and PDMS/DVB fibers.

The most polar compounds (metalaxyl and metribuzin) showed a significant decline in extraction efficiency for both fibers as the organic modifier concentration increased. This may be due to a reduced extraction from the soil or more likely because of reduced fiber absorption/adsorption. However, considering that most analytes have a similar behavior with both fiber coatings (C_{18} and PDMS/DVB), it can be inferred that the methanol concentration mostly modifies soil extraction step rather than the fiber adsorption-desorption equilibrium (although it is likely to have an effect here as well). This is also supported by the fact that, in general, less polar analytes' extractions were favored by higher methanol volumes. If fiber competition between the analytes and methanol was the most significant factor, this latter effect was not likely to be observed. Overall, the results show that no value will accommodate all chosen analytes due to their different chemical properties (*e.g.* polarity). A methanol concentration of 6 % (v/v) was chosen for both fibers.

The extraction time was evaluated for both fibers (supplementary material, figures S2 and S3). For the PDMS/DVB, most analytes reached the extraction maximum point at 75 min, followed by a small decrease. Thus, this extraction time was chosen. Concerning the C_{18} fiber, in the time range evaluated (30–120 min), no analyte was observed reaching the equilibrium point, except for diflufenican (which reached maximum extraction at 90 min). Thus, 120 min was selected in order not to extend the extraction time beyond two hours. This was done firstly to avoid an excessively long extraction (as the abrasion causes damage to the vials and stir-bar) and because some degradation-prone analytes should not be in aqueous solution for too long, especially considering the increase in temperature caused by the stir-bar, which was uncontrolled although experiments were performed at 22º C. At 120 min, the temperature was around 30 - 32 ºC, $n = 20$.

3.3. Carryover and clean-up

No instrumental carryover was detected in any blank run (MeOH injection) across the entire concentration range of the analyzed samples, even after the injection of the highest extracted concentrations. Fiber carryover was investigated firstly by retro-extracting the same fiber a second time (to a fresh 100 μL of MeOH) after the extraction of a soil with 50 μg/kg of each analyte. The second extract still had detectable amounts of nearly every analyte in both fiber chemistries, albeit low (under 10 % signal intensity compared to the 50 μg/kg extract). However, it was found that conditioning the fibers after extraction from soil (30 min in MeOH and 10 min in water with 6 % methanol) was sufficient to remove any detectable carryover. Consequently, in terms of carryover no opposition was found to re-using the SPME fibers, but since the conditioning solvent itself should not be reused (as it may concentrate carryover), it adds significantly to the overall solvent waste per sample.

A simple clean-up of the SPME fibers was tested: after extraction, the fibers were removed and inserted into 100 μL of Mili-Q water for 2 min, and agitated at 250 RPM, after which they were immediately retro-extracted accordingly to the methodology described above (Section 2.5). Although no analyte loss was observed (values within signal interval for that concentration), the method was insufficient to clean the fiber of attached matter (usually suspended organic matter from the soil which had clung to the fiber). Thus, after retro-extraction it was still necessary to let the extract settle and collect only 60 μL from the top, to ensure no solid matter was in the injected extract. Furthermore, the extract without clean-up was rather free of interferents, both in the MRM chromatogram and full-scan chromatogram when it was performed (supplementary material, Figure S4). Following this experimental observation, it was assumed that the fibers coatings would not be prone to carry any significant amount of matrix interferences (*e.g.* dissolved metals or soil colloids) and the clean-up step was considered unnecessary. The largest non-analyte peak observed in the MRM chromatogram was present in most samples and blanks (Fig. 3, number 10), possibly originating from the plastic micropipette tips used to measure volumes and hold the LC-tips. In terms of the analyte protectants, since the amount used in this work was quite large (mass equivalent to 2.5 mg/mL for a 1 μL injection), the use of GC-grade analyte protectant standards was desirable, but the authors were unable to find any commercially available.

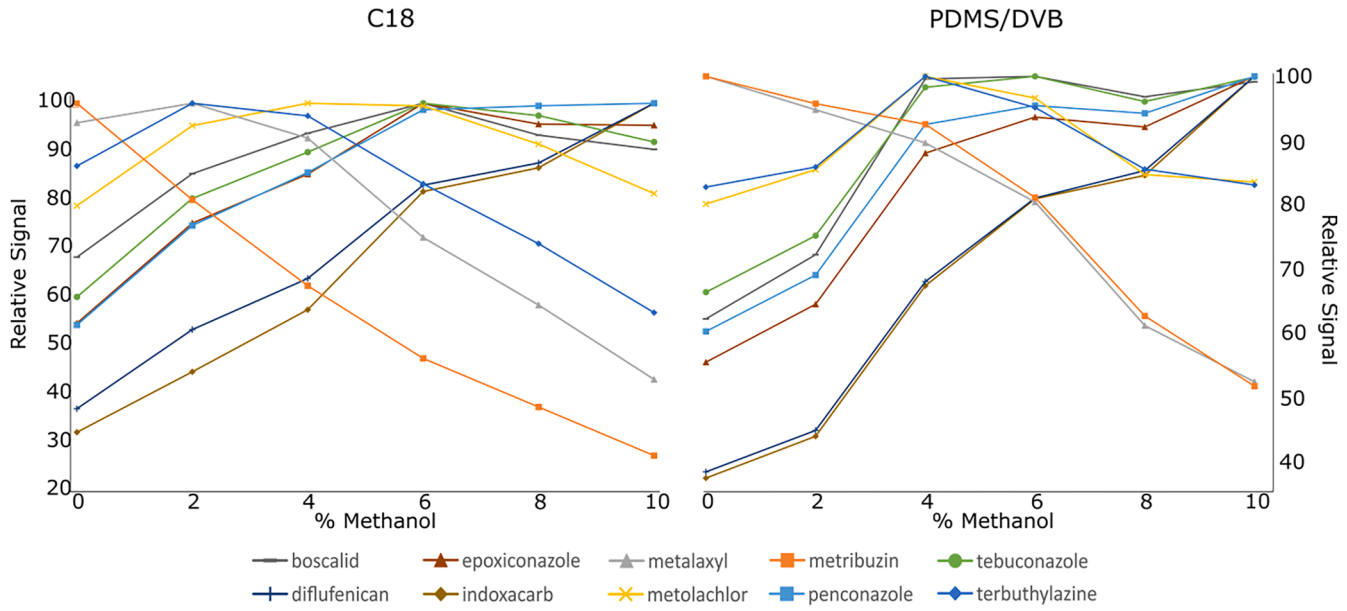


Fig. 2. Analytes extraction according to the percentage of methanol in the aqueous solvent. The highest absolute signal for each analyte was normalized to 100 %, and the remaining were scaled accordingly.

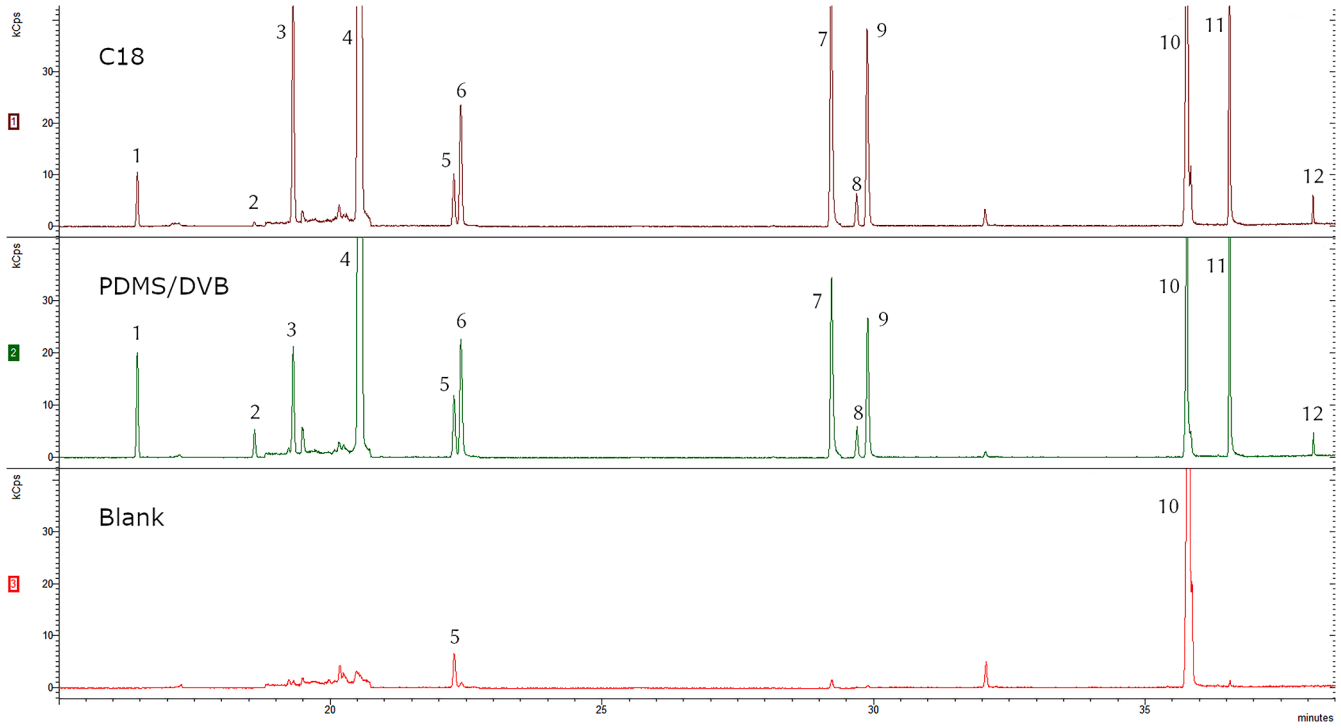


Fig. 3. Example MRM chromatograms (sum of quantifier and qualifier), displaying the analytes. Top and middle are soil extracts with 10 μg/kg spike for each analyte, using the final extraction method (Section 3.5). Bottom is an extracted blank using the PDMS/DVB fiber, from soil with internal standard only. 1 – terbuthylazine, 2 – metribuzin, 3 – metalaxyl, 4 – metolachlor, 5 – penconazole-d7 (Int. standard), 6 – penconazole, 7 – tebuconazole, 8 – diflufenican, 9 – epoxiconazole, 10 – unknown compound, 11 – boscalid, 12 – indoxacarb.

3.4. Calibration and method repeatability

The calibration results for both fibers are presented in Table 3. Every analyte in every sample had a quantifier/qualifier ion ratio within the acceptable range, except those that fell below the LoD. For the linearity study, Mandel's test was performed, which compares the residual's variance for the linear and quadratic fittings [30]. For the PDMS/DVB fiber, every calibration except that of metalaxyl was better modeled with a quadratic regression, whereas for the C_{18} fiber, this was only the case for tebuconazole and indoxacarb. Thus, it appears that C_{18} calibrations presented better linearity, whereas the PDMS/DVB, when calibrated at this concentration range, is better fitted by a quadratic equation. However, this phenomenon cannot be explained by fiber saturation, as the curves for every quadratic fitting tended upwards rather than towards a maximum signal. . For nearly every analyte, the C_{18} fiber showed better coefficients of determination for the linear fitting, and similar for the quadratic fitting (data in supplementary info Table S8).

In terms of detection and quantification limits, the C_{18} extraction performed equally or better except for metribuzin, penconazole and tebuconazole. These analytes (apart from penconazole), have significant polarity when compared to the remaining ones. Interestingly, even though terbuthylazine seemed to favor the PDMS/DVB fiber in terms of signal when tested during the Plackett-Burman screening (at 50 μg/kg), detection and quantification limits were lower with the C_{18} fiber. Overall, the results are congruent with the Plackett-Burman screening, in that the C_{18} fiber coating performed generally better. However, it must be noted that most analytes in this study are relatively non-polar within the spectrum of currently used pesticides, and that any further experimentation of more polar analytes that require LC-MS analysis, may achieve better results with the PDMS/DVB fiber. Also, when evaluating extraction time, a concentration of 50 μg/kg was used, and the fact that the longer extraction time used for the C_{18} fiber favors the extraction of lower analyte concentrations needs to be considered.

The addition of the analyte protectants before GC–MS/MS analysis resulted in a slight dilution of the extract, which directly influenced the limits of detection. However, for some analytes at low concentrations, the protecting effect greatly improved peak shape. Furthermore, since the extract was relatively clean, a larger-volume injection may have been considered, aiming to lower the LoD.

The repeatability of extraction replicates was quite poor. Relative standard deviations for six extractions were as high as 50 %, and generally in the 10–40 % range. The internal standard was very effective at mitigating the lack of precision, especially at concentration ranges close to its own (5–16 % RSD for 20 μg/kg). Regardless, calibration of a wider concentration range might result in significant heteroscedasticity; thus, the internal standard concentration must be adjusted to account for this factor. Because each calibration point has a significant number of preparation steps (weighed, spiked, dried, etc.), there is also room for random operator errors which might have compromised the values in

Table 3
Method performance parameters for the C18 and PDMS/DVB fibers using the final method as described in Section 2.5. R^2 values are of the linear regression. The asterisk denotes the calibrations in which the null hypothesis was rejected at 0.05 significance level for the Mandel test i.e. when the data is "better" fitted by a quadratic regression than a linear one.

Analyte	R^2		LoD		LoQ	
	PDMS/DVB	C18	PDMS/DVB	C18	PDMS/DVB	C18
terbutylazine	0.972*	0.988	10	1	20	10
metribuzin	0.965*	0.950	1	10	10	20
metalaxyl	0.952	0.970	1	0.1	10	1
metolachlor	0.976*	0.996	0.1	0.1	1	1
penconazole	0.975*	0.997	0.1	1	1	10
tebuconazole	0.963*	0.974*	0.01	0.1	0.1	1
diflufenican	0.948*	0.971	0.1	0.1	1	1
epoxiconazole	0.959*	0.986	1	0.1	10	1
boscalid	0.942*	0.987	1	0.1	10	1
indoxacarb	0.957*	0.915*	10	10	20	20

terms of repeatability, especially drying the spiked samples under nitrogen, where a sufficiently high flow could cause the finer soil particles to be blown off. Also, the SPME extraction itself is known to generate high replicate deviations, and thus its applicability in quantification has traditionally been difficult [15]. This phenomenon might be further aggravated by fiber variability, as this method uses different fibers (each used up to three times) rather than a single one for all extractions like traditional SPME assemblies. Consequently, it is unlikely that this type of DI-SPME will provide adequate repeatability without internal standard correction. Lack of repeatability could also be partly explained by the non-controlled temperature increase inside the extraction vial, as detailed above. The extraction temperature was not controlled because that would make the system more complicated, and thus less able to compete with relatively straightforward extraction methodologies such as QuEChERS. However, in the future this might be an interesting avenue for further optimization. Increasing the extraction temperature will reduce fiber adsorption but might increase the extraction from the soil onto the solvent, although this would not be a viable option for degradation-prone analytes. Finally, changing the sample size is likely to have an effect on both repeatability and detection limits. Although we found little statistically significant difference between an extraction of 10 or 12 mL from a 2 g soil sample, either miniaturizing the extraction (using for example 0.5 g of soil and 2.5–5 mL of solvent, possible due to the fiber's small size), or maintaining the solvent volume whilst increasing sample mass, could change these parameters. Unfortunately, miniaturization is known to have a negative effect on repeatability and sample representativeness, but for this method in particular it could result in a more homogenous extraction, if adequate stirring is provided.

The same fibers were used up to three times. The variability between replicates of different fibers (in absolute signal) was generally higher than the variability between subsequent extractions of the same fiber. Consequently, any elations as to fiber degradation/loss of efficiency were difficult, but no consistent proof of lower extraction efficiencies for second and third extractions was found. In terms of the calibration curve, due to internal standard correction, there is no significant difference in values from the first to third extractions of the same fiber. Furthermore, since they are relatively cheap, from a monetary perspective there is no need to reuse the fibers more than three times.

To roughly estimate the percentage of analyte being extracted onto the fibers, a calibration curve was constructed by injection of the analyte standards (described in Section 2.6). Every analyte had an R^2 above 0.99, and every instrumental LoQ was below 0.1 ng/mL except for indoxacarb, which was 1 ng/mL. The absolute signals from the soil extractions were translated into% of analyte extracted from the soil, considering the full mass of analyte present in the 2 gs of soil (full data in supplementary material, Tables S6 and S7). The C_{18} fiber performed better for every analyte except terbuthylazine and metribuzin, with an average extraction of 2.46 % (0.29 - 5.62 %) for 10–30 μg/kg concentrations, whereas the PDMS/DVB fiber had an average of 1.29 % (0.20 - 3.12 %). Since SPME is a non-exhaustive technique, these results show that, as expected, most of the analyte mass is not extracted onto the fiber. Furthermore, since this method utilizes a retro-extraction step prior to injection, it will never achieve as great a concentration factor as traditional SPME with direct fiber thermal desorption, since only a small percentage of the extract volume is injected.

3.5. Evaluation under green analytical chemistry

In order to evaluate the methodology, two different metrics were calculated: AGREE [31] and Analytical Eco-Scale [32]. The results were compared to twelve other methodologies for multiresidue analysis of pesticide residues in soil, whose values had been previously calculated and reported in a review [6]. These methods involve several types of extraction (pressurized liquid extraction, QuEChERS, solid-liquid extraction, microwave-assisted extraction) and gas or liquid chromatography coupled to various mass spectrometers (quadrupole, triple quadrupole, high-resolution tandem mass spectrometers). We have calculated these metrics exactly as described in that review [6]. Since the methods for C_{18} and PDMS/DVB differ only in the extraction time, which is not the bottleneck in terms of overall time (as several samples can be extracted concurrently), both methods yielded the same values.

For AGREE, a value of 0.46 was obtained (detailed in supplementary material, Figure S5). For the Analytical Eco-Scale, the result was 81. These values are tied to the highest ones obtained for the twelve methodologies previously evaluated (0.29–0.46 for AGREE and 58–81 for analytical eco-scale) [6]. Clearly, the method scored high in terms of green analytical chemistry principles but did not greatly outperform others. The greatest contribution for this good performance is the fact that water is used as the extraction solvent, although the small amount of methanol cannot eliminate dangers such as flammability and human toxicity, which is significant for this solvent. Another advantage over currently used methodologies is that it is operationally simple, involving a reduced number of steps and no drying under nitrogen or other complex procedures. Nevertheless, the method determines a relatively small number of analytes compared to other multiresidue methodologies, which reduces its performance. The "green" character of the fibers themselves is very difficult to judge, as there is no information about its manufacture, i.e. how much energy it uses, the generation of toxic effluents, etc. Using the fiber's low cost as a proxy might imply that the energy and waste cost is not too great, but this could obviously be misleading. In this respect, reusing the fibers is the best way to reduce ill effects from their manufacture, and as stated above, we have found them to be usable at least three times without any significant loss in performance parameters.

In the future, the greatest improvement in terms of green analytical chemistry performance would be attainable by the replacement of methanol with ethanol, both in conditioning and extraction. Besides (mostly) eliminating human toxicity, ethanol can be easily obtained from bio-based feedstocks. However, it is still flammable, and being a controlled substance might be difficult to obtain in some countries. For use in retro-extraction and injection, ethanol's high boiling point might pose a problem for traditional splitless injection, and the low solubility of the analyte protectants might also be a problem, which would be mitigated by co-dissolution with methanol. Nevertheless, ethanol could improve the retro-extraction of non-polar analytes over methanol and maybe allow the analysis of pesticides such as organochlorines with this technique.

4. Conclusion

In this study, a new methodology for the extraction of ten pesticides from soil was evaluated. The final method proved successful in terms of the preliminary validation performed and yielded acceptable detection limits. The main advantages of this methodology are the possibility of automation (to a large extent), low capital costs *per* sample and especially low generation of toxic wastes, thus providing a more sustainable alternative to currently used methodologies. Notwithstanding, it still suffered from relatively poor sample repeatability, which was compensated by the isotopically labelled internal standard. Of the two fiber chemistries commercially available, C_{18} performed best in most parameters, except for the more polar analytes (metalaxyl and metribuzin).

Given the inherent variability in the world's soils, an extrapolation of this method's effectiveness to other soil types (notably with finer textures, such as clay) cannot be inferred. Thus, in the future, it would be interesting to study and evaluate the applicability of this method for various soils, as well as expand it for the analysis of other pesticides. It is common knowledge, however, that no specific method will be able to cover all analytes under ideal conditions, but in-house and customized/tailored methods can be designed and evaluated. Therefore, if a more finely tuned method is required (*e.g.* to achieve lower LoDs), these may be validated for a specific class of pesticides in certain matrices and

conditions. According to the experimental data, the most important variables to evaluate are fiber chemistry, extraction time and composition of the extraction solvent.

CRediT authorship contribution statement

João Brinco: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Raquel Carvalho:** Methodology, Investigation, Formal analysis. **Marco Gomes da Silva:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Paula Guedes:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Alexandra B . Ribeiro:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Eduardo P . Mateus:** Writing – review & editing, Validation, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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4.3 Development of the Combined Extraction Methodology

After the previous work had been completed, a combined methodology was developed to integrate the other four analytes in the original list: glyphosate, AMPA, 2,4-D and MCPA, as described in Section 2.3. The first extraction, using DI-SPME was slightly altered and re-optimized. The main change was the substitution of methanol as organic modifier in the slurry for ethanol. The idea was to develop a greener method in terms of lower human/environmental toxicity and different choice of feedstock for the solvent: ethanol is arguably the easiest bio-based solvent to obtain (through fermentation). Being less volatile than methanol, it is better in terms of operator exposure, but also more problematic for GC analysis, especially when using solvent venting for large-volume injection. Furthermore, being slightly less polar than methanol, it was thought that it would be a better solvent for the relatively low-polarity analytes under study, including the glyphosate derivative. Methanol was not totally forgone, however, because ethanol alone could not dissolve the analyte protectants (sugars) at the required concentration. Thus, the two solvents had to be mixed to produce the analyte protectant solution which was added before injection.

Another change was the addition of other analytes to the study, notably some organochlorines (aldrin, lindane and pentacloroanisole) and a few other analytes which were found in the Valorfito samples from 2022-2024 after the article had been published, or which were of interest due to belonging to different chemical classes, namely buprofezin, chlorpyrifos, malathion, metazachlor, prosulfocarb, tefluthrin and tetraconazole. The idea was to test the method's efficacy for chemically different compounds.

4.4 Article 4

Simultaneous Determination of Glyphosate and 13 Multiclass Pesticides in Agricultural Soil by Direct-Immersion SPME followed by Solid-Liquid Extraction

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Article

Simultaneous Determination of Glyphosate and 13 Multiclass Pesticides in Agricultural Soil by Direct-Immersion SPME Followed by Solid–Liquid Extraction

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Abstract: A new method is presented for the simultaneous determination of 13 multiclass pesticides along with glyphosate. The multiclass pesticides were extracted by creating a soil slurry with 2% ethanol in water (*v*/*v*), and then, applying direct-immersion solid-phase microextraction (DI-SPME) with a new type of semi-disposable SPME fiber configuration called LC-Tips. The fibers were then retroextracted to ethanol, and aqueous ammonia was added to the slurry to extract glyphosate. Derivatization of the glyphosate extract was accomplished with a mixture of trifluoroacetic anhydride and trifluoroethanol, after which the reaction mixture was dried and resuspended with the SPME ethanol extract. To this, a mixture of analyte protectants was added, and it was analyzed by GC-MS/MS in multiple-reaction-monitoring mode. All analytes showed a coefficient of determination greater than 0.95 in the 0.1–100 µg/kg calibrated range, and the limits of detection were between 0.1 and 1 µg/kg, except for glyphosate, which was 0.01 µg/kg. The method shows relatively high replicate relative standard deviation (as much as 37% for five extractions at 20 µg/kg), but the isotopically labeled internal standard was effective at mitigating this effect for some analytes.

Keywords: multiclass pesticide analysis; glyphosate extraction; direct-immersion solid-phase microextraction

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1. Introduction

The use of pesticides in modern industrial agriculture is nearly ubiquitous, as they are essential in maintaining food security for the increasingly urban world population [1]. However, a large number of currently used pesticides have been found to be detrimental to ecosystem health and prosperity. Furthermore, several pesticides with adverse effects are known to persist in soils for a long time after use, such as some banned organochlorines [2]. Recent studies have pointed to the large amount of pesticides present in agricultural soils, including the very commonly used herbicide glyphosate [3–5]. Due to its permanently ionic nature, glyphosate (Figure 1) cannot be analyzed in multiresidue methods, and is often quantified separately [3]. To our knowledge, no method has ever been published which could determine permanently ionic pesticides and their degradation products (glyphosate, aminomethylphosphonic acid, glufosinate) along with other pesticide residues: This work presents a first attempt.

In general, methods for the analysis of pesticides in soil need to reach very low detection limits, due to the small quantities of these analytes often present in agricultural soils (commonly in the µg/kg range) [6], and also need to have a certain robustness in order to be able to extract the analytes from soils with different physical and chemical properties, the most important of which are soil texture, pH, and organic matter content [6].

In terms of GC-MS analysis, it is important to obtain an extract which is relatively free from matrix interferents, most notably those which are not detected by the technique, e.g., non-volatile compounds such as inorganic salts, which can compromise the instrument over many runs, causing among other things adsorption of the pesticides and eventual poor peak shape. Also, moderately volatile and/or polar matrix components can cause matrix-induced response enhancement, which results in the sample peaks being significantly larger and more symmetrical than those injected in pure solvent. Generally, matrix-matching calibration has been used to mitigate this problem, but the use of analyte protectants has also been proposed, in which moderately volatile compounds such as some sugars are added to the sample in order to "protect" the analytes from chemical adsorption, resulting in the same effect as matrix-induced response enhancement [7], both in the samples and calibration solutions.

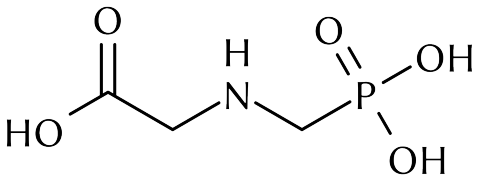

Figure 1. Glyphosate in its (theoretical) neutral form. At every pH the molecule either has a net charge or is a zwitterion.

When designing a method which could quantify glyphosate along with other commonly used pesticides, several avenues were considered. Since the extraction of glyphosate from soil is always performed with an aqueous solution [8], two serial extractions would always be needed, since low-polarity analytes will not readily extract onto aqueous phases. Furthermore, since glyphosate extraction is commonly performed with very basic solutions (either with KOH or aqueous NH_3), and some pesticides are known to degrade under basic pH [9], it was thought that the aqueous extraction would have to occur last. Thus, the idea was to modify a previously existent multiresidue method which can extract organochlorines, organophosphates, triazoles, among others, and then, perform the glyphosate extraction. The most important condition would be that the first extraction did not degrade or modify the sample to an extent that the glyphosate extraction would be impaired. Thus, QuEChERS was ruled out, since it requires the addition of salts, which would have made the aqueous extraction impossible.

The original conception was to perform ultrasound-assisted extraction (UAE) with an organic solvent, remove the entirety of the extract volume, and then, add the aqueous solution for glyphosate extraction. However, UAE has several drawbacks and does not inherently provide significant concentration factors [6]. Also, the removal of the entire extract solvent volume is operationally challenging, even after centrifugation, and could induce repeatability problems. Since a method for the multiresidue analysis of pesticides from soil using direct-immersion solid-phase microextraction (DI-SPME) was being developed [10], it was thought that it could be adapted to provide the extraction for this combined methodology.

The employment of direct-immersion SPME for soil analysis is made possible by a new type of semi-disposable fiber called SPME LC-Tips [11,12]. These fibers present one major advantage over traditional SPME, namely, their much reduced cost. Whereas commonly used SPME assemblies would quickly degrade when in contact with a stirred soil slurry (via abrasion), and thus, only be usable for a few samples, resulting in extremely high per-sample costs, these new fibers, although they can be used only one to three times, still provide a cost-effective analysis which rivals commonly used methodologies such as QuEChERS [13]. The major downside of this technique is that, unlike traditional SPME where the fiber can undergo direct desorption onto the GC inlet [14], SPME LC-Tips require solvent desorption prior to injection onto the GC or LC. This greatly reduces the

concentration factor of the extraction, because only a small percentage of the solvent extract is injected onto the chromatographic system, whereas for direct thermal desorption nearly the entire analyte mass on the fiber can be introduced onto the column. An attractive (but costly) possibility for direct desorption using LC-Tips is to use direct analysis in real time coupled with mass spectrometry (DART-MS), especially using a high-resolution mass analyzer to differentiate analytes with similar masses, where the pesticides trapped in the fiber are desorbed and ionized by a heated stream of excited and ionized helium or nitrogen, allowing their analysis with increased sensitivity and without the need for solvent retroextraction, chromatographic separation, or the increase in soil sample mass [15]. Nevertheless, for the proposed combined method, solvent desorption is ideal, as the SPME extract can easily be combined with the glyphosate extract.

2. Materials and Methods

2.1. Soil Sampling

The soil sample was collected from Idanha-a-Nova Municipality in Portugal (39′8454º N, 7′2544º W), at a depth of 25–30 cm. The land where the soil was sampled used to grow tobacco. Once in the laboratory, samples were sieved through a 2 mm mesh and allowed to air-dry at 22 ºC. The soil had a sandy-loam texture (25% coarse sand, 40.2% fine sand, 17.7% silt, and 17.1% clay), and 3.3% organic matter (st. deviation = 0.3, n = 5). Soil pH was 7.73 ± 0.06 (4 g with 10 mL of milli-Q water, shaken for 1 h, n = 3) and 6.94 ± 0.05 (4 g with 10 mL of 1 M KCl in milli-Q water, shaken for 1 h, n = 3).

2.2. Standards and Chemicals

Individual standards for 2-methyl-4-chlorophenoxyacetic acid (MCPA), buprofezin, 2,4-dichlorophenoxyacetic acid (2,4-D), aminomethylphosphonic acid (AMPA), boscalid, chlorpyrifos, diflufenican, epoxiconazole, glyphosate, malathion, metalaxyl, metazachlor, metolachlor, penconazole, prosulfocarb tebuconazole, tefluthrin, tetraconazole, and terbuthylazine were of analytical grade, obtained from Sigma-Aldrich (Steinheim, Germany). A multiresidue standard for organochlorine pesticides (at 100 µg/mL each) was obtained from Restek (Bellefonte, PA, USA). Penconazole-d7 was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Glyphosate 1,2-$^{13}C_2$-^{15}N was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Water used in the extraction and dilutions was ultrapure, produced in a Milli-Q plus system from Millipore (Bedford, MA, USA). Absolute ethanol (EtOH) and methanol were of HPLC grade, obtained from Honeywell (Charlotte, NC, USA). Dichloromethane was GC-MS grade, purchased from Carlo-Erba (Emmendingen, Germany). The remaining materials were all purchased from Sigma-Aldrich, namely, C_{18} SPME LC-Tips, trifluoroacetic anhydride and trifluoroethanol (≥99% purity), 25% aqueous ammonia solution (p. a. grade), 3-ethoxy-1,2-propanediol, gulonolactone, and D-sorbitol (analyte protectants) of purities 98%, 95%, and 99%, respectively. Stock solutions for the pesticides were prepared in methanol at 250 µg/mL, and kept refrigerated at −20 ºC, for at most one month. Dilutions for injection and soil spiking were performed in methanol. A stock solution of the three analyte protectants was prepared in a 50/50 (v/v) solution of ethanol–methanol at 1000 µg/mL each [7]. The internal standard solution (penconazole-d7 and glyphosate 1,2-$^{13}C_2$-^{15}N) was prepared with 80/20 (v/v) methanol–water at 800 ng/mL each.

2.3. GC-MS/MS Analysis

The analyses were performed by gas chromatography–tandem mass spectrometry (GC-MS/MS) on a Bruker GC 456 and a Bruker Scion TQ (Triple Quadrupole) system equipped with a CTC CombiPAL automatic injector and a programmable temperature vaporizer (PTV) inlet (Bruker 1079). Data were acquired with Bruker MSWS 8.2 and analyzed with Bruker MS Data Review 8.0. Chromatographic separation was achieved on a ZB-5MS Plus capillary column (20 m × 0.18 mm i.d., 0.18 µm df). The oven temperature program began at 40 ºC, where it was held for 3 min, increased at 20 ºC/min to 140 ºC, then 4 ºC/min to

250 ºC, and finally, 20 ºC/min to 300 ºC, where it was held for 1 min. Helium of 99.9999% purity was used as the carrier gas at a constant flow rate of 0.7 mL/min. The injection volume was 5 µL, performed in PTV large-volume mode, starting at 80 ºC with a split ratio of 1:120, held for 30 s, then splitless and with a temperature increase of 200 ºC/min to 270 ºC. At 3 min, the split valve was opened at a ratio of 1:60, and after 3 more minutes reduced to 15 mL/min and held for the entire run. The mass spectrometer system was operated in multiple-reaction monitoring (MRM), with argon as collision gas at 2.4 mTorr. The transfer line was held at 290 ºC, and the ion source at 270 ºC. The solvent delay was set to 7 min. MRM transitions associated with the selected precursor and product ion pairs of the analytes can be found in the Supplementary Material.

2.4. Final Method

A mass of 2 g of soil sample was weighed onto the extraction vial, to which was added 50 µL of internal standard solution, and it was allowed to air-dry for 1 h. C18 LC-Tip SPME fibers were conditioned by being inserted into a 2 mL glass vial containing 50/50 (v/v) ethanol–water for 20 min, followed by re-equilibration in another 2 mL glass vial containing water with 10% ethanol (v/v) for 10 min, under constant agitation at 300 rpm.

DI-SPME extraction: A volume of 10 mL of 2% ethanol in water (v/v) was added to the extraction vial, then the conditioned fiber was inserted into this solution, which was agitated for 3 h at 1000 rpm with a magnetic stir-bar. Then, the fiber was removed and immediately inserted into a 300 µL vial containing 120 µL of ethanol, and retroextracted for 30 min at 300 rpm, after which it was removed and the extract was stored in the fridge.

Glyphosate extraction: As soon as the fiber was removed from the extraction vial, 1.7 mL of 25% aqueous ammonia solution was added to it, and the soil was stirred at 1200 rpm for 1 h, at the same time that the fiber was being retroextracted. Afterwards, 1 mL of the aqueous solution from the extraction vial was added to a 2 mL Eppendorf tube, and 50 µL of dichloromethane was added, vortexed for 10 s, and centrifuged for 5 min at 4000 rpm. Then, 500 µL of the supernatant were transferred to a 1 mL reaction vial (Thermo Scienfitic, Waltham MA, USA), and completely dried under a nitrogen stream at 60 ºC. Then, 20 µL of 18% (m/m) aqueous hydrochloric acid was added to the reaction vial, and re-dried under nitrogen at 60 ºC. Afterwards, 200 µL of trifluoroacetic acid and 100 µL of trifluoroethanol were added to the reaction vials, and they were maintained at 100 ºC for 1 h. After being allowed to cool to room temperature, the vials were opened and gently dried under nitrogen.

Final extract: The ethanolic fiber extracts were removed from the fridge and allowed to return to room temperature, after which 80 µL of the extract was added to the dry reaction vial, which was then closed, vortexed for 10 s, and sonicated for 1 min. Finally, 20 µL of the analyte protectant solution was added to the reaction vial and the mixture was removed onto an autosampler vial and analyzed by GC-MS/MS. A graphical representation of the method can be seen in Figure 2.

2.5. Method Performance

Calibration curves were performed by spiking various 2 g samples with the appropriate amount of standard containing all the analytes to obtain seven different concentrations in the range of 0.1–100 µg per kg of soil, and then, drying the soil under a very gentle nitrogen stream, before following the full method described above. Each concentration was extracted in triplicate, except for 20 µg/kg, which was extracted five times, for the repeatability and recovery calculations. In order to determine recovery, a calibration was performed for glyphosate without internal standard, in the range of 0.1–10 ng, added directly to the derivatization vial, and then, dried under a gentle nitrogen stream.

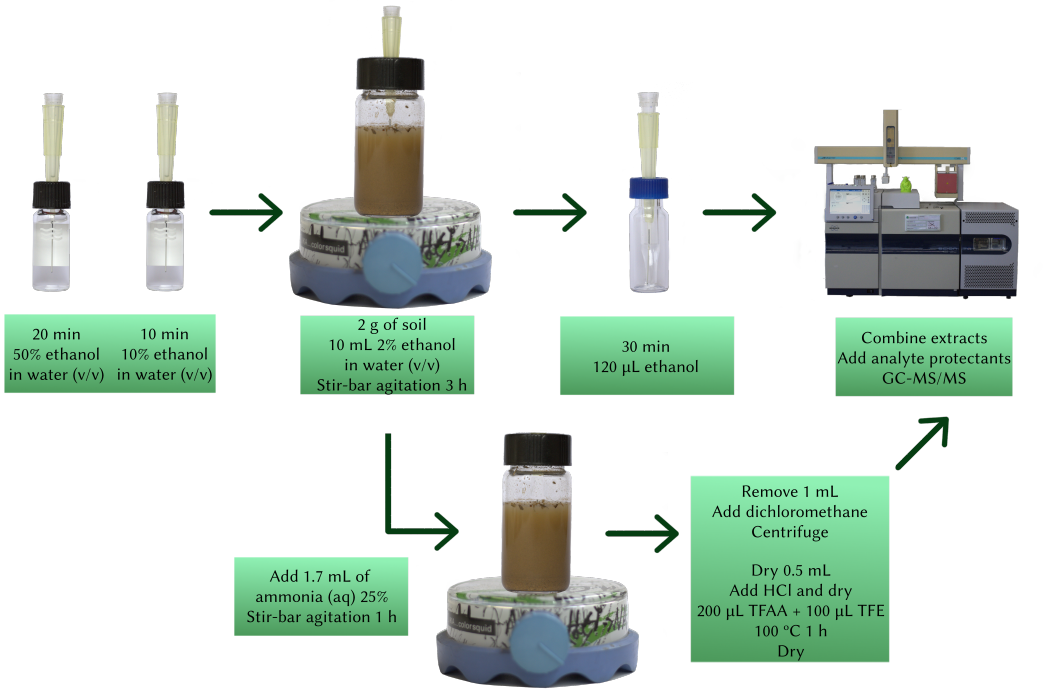

Figure 2. Schematic representation of the full method. SPME tip retroextraction happens at the same time as the extraction with ammonia solution.

3. Results and Discussion

3.1. Multiclass Pesticide Extraction by Immersion SPME

The pesticide selection chosen for the method development included several pesticide classes: Chloroacetamides (metazachlor and metolachor), organochlorines (aldrin, lindane and pentachloroanisole), organophosphates (malathion and chlorpyrifos), triazoles (tetraconazole, penconazole and tebuconazole), as well as one pyrethroid, thiocarbamate, and unclassified pesticides, namely, tefluthrin, prosulfocarb, and buprofezin. The aim was to gauge the applicability of the developed method towards different chemical properties.

The original multiclass extraction method which was adapted used 6% methanol in water (v/v) as a solvent for immersion SPME, followed by desorption of the fiber in methanol and subsequent injection [10]. In the present work, ethanol was used instead, mainly because it has a much smaller toxicity (negligible for humans considering normal laboratory exposure levels), can be produced more easily from renewable feedstocks, and is slightly less polar, which is relevant for the extraction of organochlorines, especially. Furthermore, most salts have lower solubility in ethanol than methanol [16], which is important if in the future this method is applied to soils with greater salt content, where the use of methanol for redissolution of the derivatized glyphosate extract might introduce salts into the chromatographic system. From the two fiber chemistries available, PDMS/DVB and C18, the latter was chosen because the method introduces the analysis of organochlorines, not contemplated in the previous work [10].

Initially, a three factor Box–Behnken design was used as an attempt to optimize the immersion SPME extraction It modeled the percentage of ethanol in the extraction solvent (2%, 6%, and 10%, v/v), the extraction time (60, 90, and 120 min), and the retroextraction volume (100, 120, and 140 µL). However, the results showed that for most analytes, the extraction time was by far the most relevant factor, as it seemed that even 120 min were insufficient to attain equilibrium. Thus, this factor was far outside the values at which a response surface might show a maximum. As a result, the retroextraction volume was fixed to 120 µL (as a compromise between obtaining a good concentration factor and enough working volume), and a two-factor, three-level full-factorial experimental design was constructed (nine experiments), where the percentages of ethanol in the extraction solvent

were 2, 6, and 10%, and the extraction times were 120, 150, and 180 min. Each experiment was repeated three times, for a total of 27 runs. The results (in terms of maximum average signal) are presented in Table 1.

Table 1. Results of the two-factor, three-level full-factorial design. Signals were normalized, with the largest signal for each analyte being 100 and the others scaled accordingly.

Percent Ethanol		**2%**			**6%**			**10%**		
Analyte	**Time (Min) Class**	**120**	**150**	**180**	**120**	**150**	**180**	**120**	**150**	**180**
Metazachlor	Chloroacetamide	100	86	79	33	27	33	15	13	15
Metolachlor	Chloroacetanilide	86	97	100	52	44	53	25	23	25
Pentachloroanisole	Organochlorine	58	82	83	74	70	95	75	85	100
Lindane	Organochlorine	100	97	92	77	63	72	54	48	40
Aldrin	Organochlorine	45	61	68	43	51	75	58	73	100
Malathion	Organophosphate	87	100	99	62	52	58	30	29	29
Chlorpyrifos	Organophosphate	37	57	75	65	57	91	65	76	100
Tefluthrin	Pyrethroid	42	65	67	65	52	89	67	72	100
Prosulfocarb	Thiocarbamate	66	84	100	77	74	92	61	71	84
tetraconazole	Triazole	69	89	100	76	64	81	47	51	54
Penconazole	Triazole	72	83	100	74	54	82	48	48	52
Tebuconazole	Triazole	59	82	100	70	57	83	39	43	47
Buprofezin	Unclassified	35	62	82	61	60	95	60	74	100

From the results, 180 min was selected as the extraction time. A long extraction time is not ideal, because the full method including glyphosate extraction and derivatization takes several hours, and at worst can be longer than a single 8 h shift, which would compromise its applicability in routine laboratories without the implementation of process automation. The results for just 180 min of extraction can be seen in Figure 3.

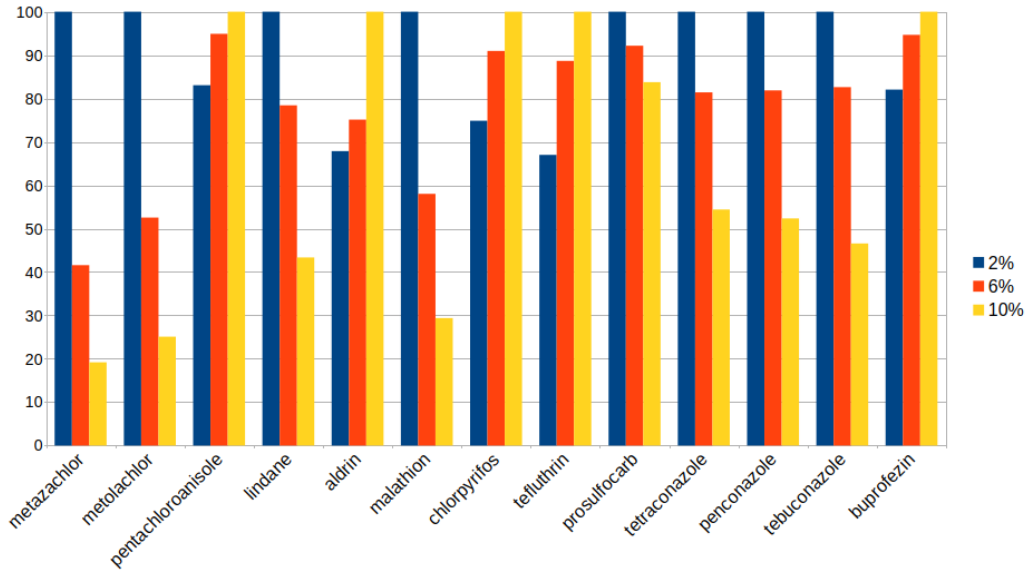

Figure 3. Relative signal values for 180 min extraction time under 2, 6, and 10% ethanol extractions.

From these results, 2% ethanol in water was chosen as the extraction solvent. Although the more nonpolar compounds were favored by the greater percentage of ethanol, most had the highest signals at 2%. Furthermore, a smaller amount of ethanol would be favorable for the subsequent glyphosate extraction, as the organic modifier would not help in the dissolution of glyphosate. Interestingly, no compound had a maximum at 6% ethanol, which seems to suggest that the ideal percentage for each pesticide lies either closer to 2% or 10%, or outside the evaluated range.

3.2. Glyphosate Extraction

The extraction of glyphosate from the soil matrix had to be carried out via some modifications to the extraction solvent for immersion SPME. After the three hours of extraction, the SPME fiber was removed from the soil slurry and retroextracted onto ethanol. Concurrently, the glyphosate extraction was performed. There were two possibilities explored: addition of potassium hydroxide (KOH) [3] or ammonia solution [17]. The ammonia was chosen because it could be almost entirely removed by drying the extract. KOH, on the other hand, could not be removed from the extract, and although it has a relatively low solubility in ethanol (as does KCl, which is formed after neutralization with hydrochloric acid), it is still sufficient to cause accumulation in the GC liner and eventual clogging and chemisorption. Thus, when an extraction with KOH was assayed (0.6 molar, added dry to the extraction slurry), an extra step of extraction after the derivatization was required. Furthermore, whether neutralization with hydrochloric acid was performed or not, the large amount of salts interfered with the derivatization step, especially at low concentrations of glyphosate.

The sample-to-extraction solvent ratio was slightly modified from a previous work [17], except only one extraction was performed instead of two. Using a larger sample size (e.g., 3 or 4 g) while retaining a similar extraction solvent volume could have been employed in an attempt to improve detection and quantification limits for the multiresidue pesticides extracted with DI-SPME, but would have been operationally more challenging (especially in terms of shaking), and might have compromised repeatability and extractability in terms of the soil–solvent equilibrium, even though it would ultimately result in a larger analyte mass being extracted onto the SPME fiber. Also, this could compromise the glyphosate recovery, unless a larger extraction solvent volume was added after DI-SPME.

After the extraction, a small amount of dichloromethane had to be added to the extract because it was found that it contained non-negligible amounts of the other analytes, and their presence in the aqueous solvent could compromise repeatability. The simple centrifugation of the extract both removed suspended soil particles, thus eliminating the need for filtration, and settled the dichloromethane layer. However, for finer soils (notably clay), it may be required to centrifuge at higher speeds for longer. Neutralization of the extract with hydrochloric acid before centrifugation (in order to precipitate some soil matrix components) was not feasible because of the formation of non-volatile salts which could not be removed in the drying step.

For derivatization of the glyphosate extract, two methods were tested: silylation with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) co-dissolved with acetonitrile [18,19], and simultaneous acylation and esterification with trifluoroacetic anhydride (TFAA) and trifluoroethanol (TFE) [17,20]. The silylation with MTBSTFA suffers from several drawbacks, including high reactivity of the reagent with water (more problematic than for TFAA), high cost per sample, and poor reaction yields for trace amounts of glyphosate [21], possibly caused by low solubility of the underivatized compound and adsorption to the glass vial. Furthermore, neither MTBSTFA nor the other reaction products are sufficiently volatile to allow drying of the mixture after derivatization, which compromises the subsequent combination with the SPME extract. Thus, derivatization with TFAA + TFE was chosen.

One of the drawbacks of this approach was that the glyphosate derivative was much more volatile than the other pesticides analyzed. This was even more pronounced for gylphosate's degradation product aminomethylphosphonic acid (AMPA), which was also tested in this method. When a starting GC oven temperature of 50 °C was used, AMPA's derivative showed poor peak shapes with significant variability between injections of the same extract. As a consequence, the initial oven temperature was reduced to 40 °C to permit better focusing of this analyte's band at the top of the column.

3.3. Combined Method and Performance

For the combined method, it was imperative to ensure proper dissolution of the derivative in the ethanol SPME extract. Sonication was employed in an attempt to ensure full dissolution, and thus, good repeatability. The full method was also tested for two phenoxy herbicides: 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D). It was possible to extract these compounds along with glyphosate due to the fact that at the extraction pH (around 12) the carboxylate is dominant, with almost no neutral carboxylic acid molecules present (calculated from the phenoxyacetic acid pKa). However, the repeatability was poor at every concentration (20–100 µg/kg), with coefficients of determination of 0.87 for MCPA and 0.88 for 2,4-D. This was likely caused by the extraction itself, or by the fact that a certain amount of the analytes might have been dissolved in the dichloromethane phase. Naturally, the isotopically labeled glyphosate was very poor at correcting extraction variability, but it may be possible to analyze these compounds with this methodology by using another internal standard which is chemically similar to them, although adding more isotopically labeled compounds to a method will significantly increase its cost per sample. For AMPA, there was also significant lack of repeatability (R^2 = 0.91), but this was probably also due to the poor chromatographic performance, since the volatile AMPA derivative is not compatible with the programmed temperature volatilization injection technique used.

Table 2 presents the method performance parameters. The extraction recovery for glyphosate was 92%, with a relative standard deviation of 12% (n = 3). Although for some compounds the internal standard is essential, for others (such as tebuconazole and tefluthrin) it did not increase repeatability. Glyphosate had a lower limit of detection, likely because of the greater concentration factor. Even though SPME is known for greatly concentrating samples, that effect is not present in this method, because instead of direct desorption onto the GC inlet (which transfers the whole extracted analyte mass onto the GC-MS), it employs solvent desorption onto 120 µL of ethanol, of which only 5 µL is injected. Nevertheless, in the future an increase in soil sample mass (e.g., to 4 g) might be an interesting avenue for exploration; it could lower limits of detection, although it could also increase RSDs. Another weakness of the method is that it does not take advantage of the inherent clean-up performed by SPME, since the SPME extract is then mixed with the glyphosate extract, whose only clean-up was the addition of dichloromethane. This results in a final sample with much more matrix interferents than the SPME extract alone, although these were not noticeable in the MRM chromatogram.

Table 2. Method performance parameters. Limit of detection (LoD) was the lowest calibration concentration with a signal-to-noise ratio greater than 3, and limit of quantitation (LoQ) was that which was greater than 10. Values for LoD and LoQ in µg/kg. %RSD with and without internal standard (IS) refers to 20 µg/kg concentration, extracted five times.

Analyte	LoD	LoQ	R^2	%RSD	%RSD with IS
Aldrin	1.0	20	0.989	28	13
Buprofezin	1.0	20	0.983	36	10
Chlorpyrifos	0.1	1.0	0.988	29	6.9
Lindane	1.0	20	0.981	21	22
Malathion	0.1	1.0	0.982	19	17
Metazachlor	1.0	20	0.954	17	18
Metolachlor	0.1	1.0	0.992	21	13
Penconazole	0.1	1.0	0.994	26	3.0
Pentachloroanisole	1.0	20	0.990	18	15
Prosulfocarb	1.0	20	0.979	27	2.5
Tebuconazole	1.0	20	0.965	35	37
Tefluthrin	0.1	1.0	0.986	35	37
Tetraconazole	1.0	20	0.990	26	5.6
Glyphosate	0.01	1.0	0.991	26	5.5

The method was also tested for other currently used pesticides, namely, boscalid, diflufenican, epoxiconazole, metalaxyl, and terbutylazine, but was found lacking in terms of precision (R^2 = 0.899–0.94; RSD as high as 40% for 20 µg/kg extractions; n = 5). In the future, an isotopically labeled internal standard which better mimics the chemical properties of these compounds should be used to mitigate such problems. Further optimization of the method in order to achieve better concentration factors from the immersion-SPME extraction would be desirable. In terms of the GC-MS/MS determination, it was possible to isolate every analytes' signal, and thus, a combined method involving more compounds could be viable. A chromatogram from a spiked sample can be seen in Figure 4.

The limit of quantification obtained for glyphosate was significantly lower than other published methods (e.g., Ref. [3], LoQ of 50 µg/kg), likely due to the low extraction solvent volume used and high concentration factor generated by drying the aqueous extract, which nonetheless is a difficult and time-consuming process, often avoided. It is possible that a different soil, particularly of finer texture, might require a larger extraction solvent volume to obtain an adequate recovery for glyphosate, which would compromise the detection limit, unless an even larger concentration is performed. In terms of the other analytes, detection and quantification limits are within normal ranges for most multiresidue methods, but not significantly better [6].

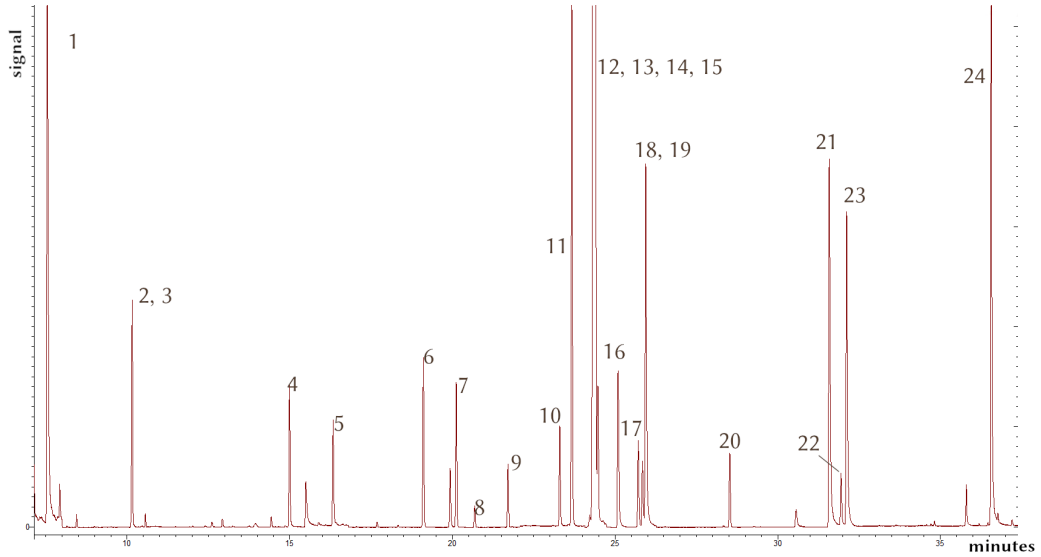

Figure 4. Sample GC-MS/MS (MRM) chromatogram from an extract of spiked soil at 60 µg/kg. 1—aminomethylphosphonic acid; 2, 3—glyphosate and isotopically labeled glyphosate (co-eluting); 4—MCPA; 5—2,4-D; 6—pentachloroanisole; 7—lindane; 8—terbutylazine; 9—tefluthrin; 10—metalaxyl; 11—prosulfocarb; 12, 13, 14, 15—aldrin, metolachlor, malathion, and chlorpyrifos; 16—tetraconazole; 17—metazachlor; 18, 19—isotopically labeled penconazole and penconazole; 20—buprofezin; 21—tebuconazole; 22—diflufenican; 23—epoxiconazole; 24—boscalid.

4. Conclusions

In this work, a new method for the simultaneous determination of glyphosate and 13 pesticides of different chemical classes was developed. The method shows acceptable performance parameters, and is relatively straightforward in terms of operation. In the future, different ratios of sample to extraction solvent will be tested and optimized in order to achieve a better concentration factor for the SPME extraction. In soils with greater matrix interferents, it may be necessary to employ some form of clean-up of the glyphosate extract prior to derivatization. The use of SPME LC-Tips permitted the development of this combined method in a way that would have been difficult otherwise (by performing serial extractions) because the extraction of multiclass pesticides is itself performed in an aqueous solvent, whereas it normally uses an organic solvent. This new SPME configuration appears to have interesting properties and opens several avenues for further exploration, such as

direct screening of soils without prior sample preparation by the insertion of the fibers onto wet soil, and then, transporting them to the laboratory.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14198584/s1, Table S1: Multiple-reaction monitoring transitions for MS/MS experiments.

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4.5 Conclusions on the Developed Methodologies

In the first article (Section 4.2) a green analytical chemistry evaluation was performed. As explained, the values are excellent, but not strictly higher than all other methodologies. It is possible that the combined methodology from the second article (Section 4.4) is better than having to perform two different methods for analysing multiclass analytes and glyphosate, but it is difficult to calculate these metrics for two separate methods as one. In AGREE, for example, there is no benefit to performing a single chromatographic run rather than the two which would be required for analysing the multiclass pesticides and glyphosate separately. Then again, it is possible that the combined methodology developed would not be significantly better in terms of green analytical chemistry due to the use of a small amount of dichloromethane and the extremely long time required to perform a single extraction, nearly an entire working day of 8 hours (offset by the fact that many extractions can be easily performed in parallel). It is also pointless to compare the combined methodology with any other single method since it was developed specifically to replace the use of two different extractions and chromatographic runs.

Even more important than accordance with green analytical chemistry principles is metrological performance. The first method employing only DI-SPME had an adequate performance, especially considering that the calibration was performed by spiking the soil before extraction rather than a blank extract. This, along with the fact that it is a new form of extraction with very little study makes it a very interesting candidate for further exploration, and likely to be useful if some of its flaws can be corrected. Probably, testing different analyte mass to extraction solvent volume combinations can eventually yield a better performing method. Perhaps an increase in analyte mass or a decrease of extraction solvent could help improve repeatability and detection limits. This was not tested further in the original work because of operational problems (*e.g.* ideal vial size, inefficient sample shaking, etc).

The combined method, however, has several problems unlikely to be resolved by further optimization. The fact that no work has ever been reported (to my knowledge) attempting to analyse glyphosate (or similar analytes) along with "multiclass" pesticides should give an indication of how difficult/unfeasible it is, especially since glyphosate is well known as an important pesticide and environmental contaminant [16]. Nevertheless, although the combined methodology worked reasonably well for glyphosate, its degradation product AMPA could not be adequately chromatographed. The very high volatility and low molecular mass of the AMPA derivative resulted in poor chromatographic repeatability and the presence of some co-elutants, especially detrimental at low concentrations. Derivatization with MTBSTFA produced a much heavier derivative with cleaner Multiple-Reaction Monitoring mass spectrometry (MRM) transitions, but the reagent mixture was not compatible with the combined methodology, because it could not be dried. Several of the "multiclass" analytes also presented poor repeatability, likely due to errors stemming from the very high number of analytical steps, or because the

dichloromethane added was not enough to consistently remove these compounds from the aqueous extract.

The next Chapter is centered around the development of a direct extraction of pesticides from soil without requiring off-line handling of the sample, using the SPME LC-Tips. The work also employs the methodologies shown in this Chapter. However, due to the analytical problems of the combined methodology, the method was divided into two, one using DI-SPME and another employing the aqueous ammonia extraction, but nevertheless maintaining the other parameters. The improved performance of the two separate methods versus the combined one further indicates that the major problem was the coupling of the two extractions, namely the necessity to remove the "multiclass" analytes from the aqueous extract and the several drying/resuspension/dissolution steps involved. Regardless, AMPA remained a difficult analyte since its main problems are not directly related to the extraction, but rather with chromatographic performance. Still, quantification was possible, within these limitations.

5

On-the-Fly Soil Monitoring with SPME extraction

5.1 Introduction

The following Chapter presents an attempt to develop a method for on-line real-time monitoring of soil pesticide contamination, and an application to a process of electrokinetic remediation. The method uses the same SPME-Tips introduced previously, but uses them for a much more streamlined analysis, by directly inserting them in the undisturbed soil, for pesticide extraction.

To my knowledge, there is currently no method described for accurate multiresidue analysis of pesticide residues (or other organic contaminants) in soil which does not presuppose sampling and sample transport. Traditional methodologies involving extractions such as QuEChERS followed by chromatographic separation always require "invasive" sampling (in the sense that the sampled medium is disturbed) and extensive sample treatment. The proposed method uses SPME fibers for direct, non-invasive pesticide extraction from soil, which can then be immediately retro-extracted and stored/transported. Although this might not present a great advantage for field sampling, since transport to a laboratory for chromatographic/mass spectrometric determination is still required, it is especially interesting for lab-scale experiments where removing a soil sample would destroy the experiment itself, as is the case with laboratory trials of electrokinetic remediation. Still, even in the field the method might have some use if paired with a mobile chromatography or mass spectrometry system, although these are quite rare and somewhat limited [38].

Electrokinetic (EK) remediation is a set of techniques which use a direct current electric field to mobilize and remove contaminants. It has been applied to soil for the remediation of several organic and inorganic contaminants [39–43], including pesticide contaminated soils [44], and can work alongside bioremediation in order to enhance it (a process called EK-Bio [45]).

This work consisted firstly in developing and optimizing the herein called "On-the-Fly SPME" extraction method for selected pesticides, and then judging its fitness-for-purpose

through a validation process. Finally, the method was tested for monitoring a simple EK remediation experiment based on a published design [46], targeting those same pesticides. At the end of this EK experiment, the pesticides were quantified using the methodologies described in Section 4.4, albeit slightly altered, to determine the EK degradation efficiency and compare with the On-the-Fly SPME monitoring results. Glyphosate, AMPA, MCPA and 2,4-D were also targeted in the EK remediation experiment even though they cannot be monitored by On-the-Fly SPME, in order to test this remediation design's effectiveness for such compounds and also to test the quantification method described in Section 4.4.

5.2 Experimental

5.2.1 Materials

The main soil used for the work, here called soil A, had a sandy-loam texture and has been described elsewhere (See *Soil sampling and Spiking* in Section 4.2). For validating the On-the-Fly SPME extraction, a second soil was also used, called soil B. It originated from an organic tomato farm in Santarém, Portugal, and presented a clay texture (61% clay, 29% silt and 10% sand) [46]. Both soils were allowed to air dry at room temperature before being used. C18 SPME LC-Tips were purchased from Sigma-Aldrich (now sold by Bruker as SPME DIP-it Tips). Water used for extractions and soil wetting was ultrapure, produced in a Mili-Q plus system from Millipore (Bedford, MA, USA). Absolute ethanol and methanol were of HPLC grade, obtained from Honeywell (Charlotte, NC, USA). Pesticide standards, namely 2,4-D, AMPA, MCPA, boscalid, buprofezin chlorpyrifos, diflufenican, epoxiconazole, glyphosate, metalaxyl, metazachlor, metolachlor, metribuzin, penconazole, prosulfocarb tebuconazole, tetraconazole and terbuthylazine were of analytical grade, obtained from Sigma-Aldrich (Steinheim, Germany). Aldrin, lindane and pentachloroanisole came from a multiresidue standard for organochlorine pesticides (at 100 µg/mL each), obtained from Restek (Bellefonte, PA, USA). Trifluoroacetic anhydride, trifluoroethanol, 25% aqueous ammonia solution (p. a. grade), 3-ethoxy-1,2-propanediol, gluconolactone and D-sorbitol (analyte protectants) [47] were all purchased from Sigma-Aldrich. Compounds used as internal standards were penconazole-d7, obtained from Toronto Research Chemicals (Toronto, Canada) and glyphosate 1,2-$^{13}C_2$-^{15}N, from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions for the pesticides were prepared in methanol at 250 µg/mL and kept refrigerated at -20 ^{o}C for at most one month.

5.2.2 On-the-Fly SPME

Experiments for developing and validating On-the-Fly SPME sampling were performed by first conditioning the fibers, which were inserted into a 300 µL glass autosampler vial (Alwsci, Zhejiang P.R., China) containing 50/50 (v/v) ethanol-water, shaken at 300 rpm for 20 min, followed by another 300 µL vial with 2% ethanol in water (v/v) and shaken for at least 10 min (Section 4.4). The experiments were conducted using 15 mL glass vials

with 21 mm outer diameter (Sigma-Aldrich), to which 5 g of soil were weighed, and then an appropriate volume of a stock solution containing all pesticides (1 µg/mL each in methanol) was added. It was then dried under a very gentle nitrogen stream and shaken to ensure even distribution. Afterwards, water was added to the soil and allowed to settle. The conditioned SPME fibers were then carefully inserted so that the whole coated surface was in direct contact with the soil and were maintained static for the duration of the extraction time (2-4 h). Then, each fiber was removed and immediately inserted into a 300 µL glass vial with 120 µL of ethanol and shaken for 30 min at 300 rpm for retro-extraction. The fiber was then removed, and the extract was stored in the fridge until the soil particles had settled (longer times were required for finer soil textures, overnight for soil B which had high clay content). Finally, after allowing the extract to reach room temperature, 60 µL were removed from the top into an autosampler vial. To this was added 20 µL of a 50/50 ethanol-methanol (v/v) solution containing the analyte protectants (1000 µg/mL each) and penconazole-d7 (80 ng/mL), which was then injected into the GC-MS/MS. For these experiments, the internal standard was only used to check system performance, not for quantitative purposes. GC-MS/MS conditions were the same as in Section 4.4, except that the GC oven temperature was slightly altered, instead starting at 40 ºC held for 3 min, then 20 ºC/min to 160 ºC, then 5 ºC/min to 230 ºC and finally 10 ºC/min to 300 ºC, held for 3 min.

5.2.3 Electrokinetic Experiment

The electrokinetic experiment was based on a previously published design [46]. The experimental microcosm was a rectangular plastic container (95 x 47 x 49 mm), into which 115 g of the A soil were weighed. These containers were lined with aluminum foil on all sides to reduce soil exposure to light. Two mixed-metal oxide electrodes (90 x 20 x 1 mm) were inserted into the soil, on opposing sides of the container, 5 mm from the edge and around 2 mm from the bottom (Figure 5.1). These were connected to a power supply for direct current generation (Hewlett Packard E3612A, Palo Alto, USA). Before starting the experiment, a spiking solution was added to every microcosm, which consisted of 40 mL Mili-Q water/methanol (40:1, v/v) with 11.5 µg of every analyte except the organochlorines (aldrin, lindane and pentachloroanisole), for a final concentration of 100 µg/kg each in the soil. The biotic control experiments were prepared in the same way, but without the electrodes. Two EK and two biotic control experiments were performed concurrently. In the EK experiments, current was set to 20 milliampere (mA) with an ON time of 14 h followed by an OFF time (no current) of 10 h [46]. The experiments ran for eight days, with daily moisture control (by weight) and irrigation with Mili-Q water to match the initial weight (after spiking). Soil temperature was measured daily.

During the experiments, On-the-Fly SPME monitoring was applied by performing two extractions, one at day 1 (D1) and another at day 5 (D5). For this, three previously conditioned C18 SPME LC-Tip fibers were inserted into each microcosm 1 h after wetting,

one at 1-2 cm from the cathode, one at the middle and one at 1-2 cm from the anode (Figure 5.1). These were left to extract for 3 h and then retro-extracted and analyzed as described above (Section 5.2.2). For each compound, all the D1 signals of the two EK experiments were averaged assuming the concentrations would be the same in all sections at the beginning. For D5, the signals of each section of the two EK experiments were averaged (D5-Cathode, D5-Center, D5-Anode, n = 2). For the biotic control samples, no significant difference was observed between the D1 and D5 averages.

Figure 5.1: SPME LC-Tips inserted onto the soil during the EK experiment for On-the-Fly analysis.

After the experiment, the electrodes were removed and all microcosms (including biotic controls) were sectioned into two equally sized parts along the shorter width and removed separately. Each section's moisture was determined by mass difference after drying overnight at 105 °C. pH was determined by making a slurry with Mili-Q water at a ratio of 1:2.5 (soil/water, w:v), stirring for 1 h and measuring with an Hanna edge pH meter (Woonsocket RI, USA). The EK experiment sections were named Cathode and Anode. Biotic control sections were analyzed separately and then averaged. After extraction and determination by GC-MS/MS (described below) pesticide concentrations were transformed to µg per kg dry weight according to moisture content of each sample. Each section was extracted and analyzed in duplicate.

5.2.4 Pesticide Quantification

At the end of the EK experiment, quantification of target analytes was achieved using two separate methods adapted from the work presented in Section 4.4, one for the SPME extractables and the other employing derivatization.

The determination of all analytes except glyphosate, AMPA, 2,4-D and MCPA was performed using direct-immersion SPME LC-tips extraction, as follows: to a vial containing 2 g of soil were added 50 µL of the internal standard solution (as per Section 4.4). After allowing to air-dry, 10 mL of 2% ethanol in Mili-Q water (v/v) were added. The previously conditioned fiber was inserted into the slurry, which was agitated for 3 h using a magnetic stir-bar at 1000 rpm. Afterwards, the fiber was removed and retro-extracted to 120 µL of ethanol by shaking for 30 min at 300 rpm. The extract solution was allowed to settle before removing 60 µL onto an autosampler vial, adding 20 µL of analyte protectant solution (1000 µg/mL each in 50/50, v/v ethanol-methanol) and injecting into the GC-MS/MS system. The remaining analytes (glyphosate, AMPA, 2,4-D and MCPA) were analyzed by the following method: to a vial containing 1 g of soil was added 50 µL of the internal standard solution (glyphosate 1,2-$^{13}C_2$-^{15}N at 400 ng/mL in 80/20, v/v methanol-water). Then, 6 mL of aqueous NH_3 at 2 M were added, and stirred for 1 h with a magnetic stir-bar at 1000 rpm. Afterwards, 1 mL of this slurry was removed onto an Eppendorf tube and centrifuged for 5 min at 4000 rpm. 500 µL of the supernatant were transferred to a 1 mL reaction vial, and the derivatization process was followed as in Section 4.4. Finally, 80 µL of acetone were added to the dry reaction vial, vortexed 10 seconds and sonicated for 1 min; 60 µL were removed to an autosampler vial, 20 µL of the analyte protectant solution were added, and it was injected into the chromatographic system.

The analysis by GC-MS/MS followed the parameters described in Section 4.4, except that the temperatures were slightly altered from the original method. For the SPME extract analysis, the oven temperature was altered as follows: 40 °C held for 3 min, then 20 °C/min to 160 °C, then 5 °C/min to 230 °C and finally 10 °C/min to 300 °C and held 3 min. For the derivatized extract, the injector temperature started at 60 °C held for 0.5 min and then increased at 200 °C/min to 250 °C, which was held for the remainder of the chromatographic run. The chromatographic run started at 40 °C held for 3 min, then an increase to 100 °C at 20 °C/min, followed by 4 °C/min to 120 °C, then 10 °C/min to 180 °C and finally 20 °C/min to 300 °C and held 1 min.

5.3 Results

5.3.1 On-the-Fly SPME Method Development

After initial experimentation using the fibers for direct extraction from spiked soil (2 mL water for 5 g of soil, 3 h extraction), it was found that this method, followed by GC-MS/MS provided adequate sensitivity, with signal-to-noise ratios (S/N) larger than 30 for all evaluated analytes at 50 µg/kg. Repeatability was poor when compared to

standard "off-line" methods such as QuEChERS, but adequate for this method's intended purpose, with relative standard deviations between 32-54% for 50 µg/kg and 22-40% for 100 µg/kg (n = 4). Since there is no way to add a surrogate standard (e.g. penconazole-d7) in real On-the-Fly monitoring conditions, and GC-MS/MS repeatability was very good (under 5% RSD for five injections of the same extract at S/N larger than 100), internal standardization was not found to increase precision. However, for longer monitoring experiments, where samples are analyzed several days (perhaps weeks) apart, an internal standard added before injection would be more important, in order to correct system variation over that time period and allow efficient comparison of samples.

Assuming that both fiber conditioning and retro-extraction were well studied and established from previous works (Chapter 4), the most important variables to optimize were the amount of water in the soil and the extraction time. Given that this is a static extraction (there is no movement of the fiber or soil for the extent that they are in contact), it is physically and chemically distinct from the previously developed methods in important ways. The water content was assumed to be the most important factor for extraction efficiency and repeatability. Unlike previous methods, no organic modifier would be used (in order not to disturb the soil and maintain feasibility of an eventual up-scaling to field conditions). Since the studied analytes show little volatility, the air volume in the soil would not only hinder extraction but also possibly increase its variability. Furthermore, direct mass transfer from the soil particles to the fibers is also likely low, especially in coarser soils (notably sandy textures), mostly because of low contact area. For these reasons, water was added in all experiments, as a way to increase mass transfer to the fiber and homogenize the soil water content itself. A two factor, three level full-factorial experiment was developed, testing the variables of water content (1, 2 and 3 mL) and extraction time (2, 3 and 4 h). The center point for water content was 2 mL, which showed an even dispersion through capillarity for the 5 g of soil. 1 mL was slightly "undersaturated", with dry areas initially visible and 3 mL showed evident "pooling" of undispersed water above the soil. These values would naturally differ for other soils, but no great difference was observed when adding the same volume to the B soil, with clay texture. The extraction time was modeled after the previous works (Chapter 4), with practicability being an important factor: too long of an extraction time would not only be unfeasible in some situations, but would also increase the difficulty of maintaining a stable water content in the soil. The results for selected analytes are presented in Figure 5.2.

Clearly, extraction efficiency was very dependent on soil water content, more so than extraction time. This is expected, as soil moisture is difficult to be accurately measured and maintained, especially between different samplings. Interestingly, when comparing between different water volumes for the same extraction time, the most distinctive signals were obtained for 1 mL, whether they are significantly higher than the others, as was the case with the organochlorines aldrin and pentachloroanisole, or whether they are lower, for nearly every other analyte, especially at 2 h of extraction. The relative signal homogeneity

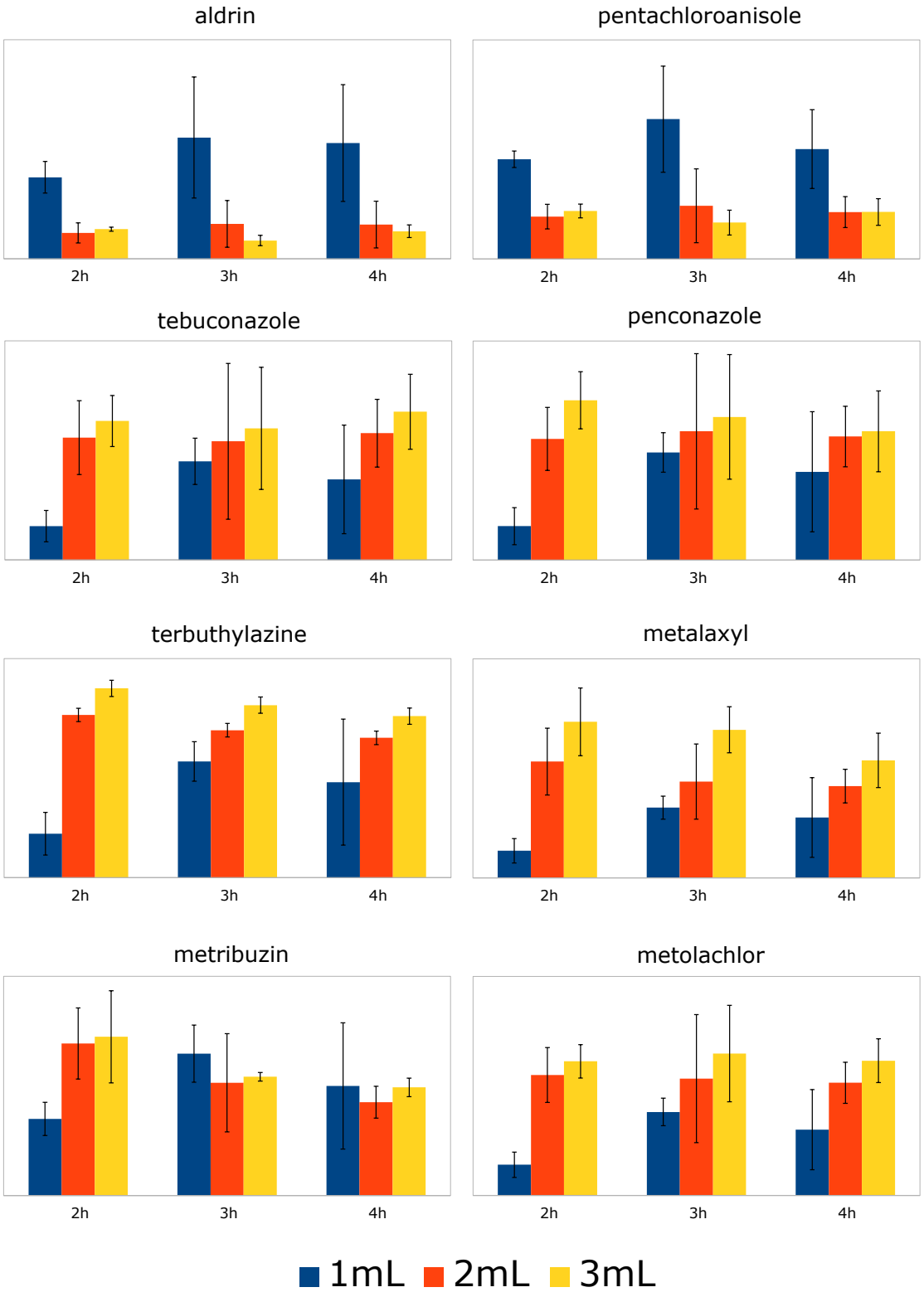

Figure 5.2: On-the-Fly SPME extraction optimization results for selected analytes (n = 3) as a function of time and water content. Vertical axis represent absolute signal, with each analyte's graph scaled differently for legibility.

for different extraction times might mean that even with a water-saturated soil, "long-range" mass transfer within the medium is not a quick process considering the extraction timeframe, and the extraction is localized to a relatively small area around the fiber. If this is the case, then the analyte mass extracted onto the fiber should be independent of the sample size, and only dependent on pesticide concentration. Otherwise, a small soil sample could result in lower analyte mass extracted onto a single fiber when compared to a larger sample, since there is more mass in the larger sample.

In terms of repeatability, it is difficult to draw conclusions from the results. Clearly, standard deviations are too high for accurate quantification, even in an environment with precisely controlled parameters (and would likely be higher under real conditions). However, for the intended purpose of non-destructive, On-the-Fly multiresidue sampling, the method has (to my knowledge) no competitors.

From the results, it appears that the soil should be at or over saturation in terms of water, as even for the organochlorines evaluated the difference between 2 and 3 mL of added water was minimal compared to 1 mL, suggesting that over the saturation mark results will be more precise despite differences in water content. Thus, for further testing the method, 2 mL of water was used for the same sample size (5 g). The extraction time was fixed at 3 h, both because at 2 h there was a greater disparity between different water contents, and because there was no appreciable benefit in 4 h of extraction.

5.3.2 On-the-Fly SPME Validation

In order to test the method's applicability for quick non-destructive qualitative analysis of pesticides in soil, an experiment was conducted, using the two soils of different textures and compositions, described in Section 5.2.1. The soils were spiked at 25, 50 and 100 µg/kg, and the experiments were conducted as described in Section 5.2.2, using 2 mL of added water for both soils, and 3 h of extraction time.

For the blanks of both soils, chromatograms were extremely clean, with little interferents in the MRM signals for every analyte. At 25 µg/kg, it was possible to distinguish a peak of S/N above 10 for all evaluated analytes in all injections, whereas in the blanks no peak was distinguishable from the noise at the compound's retention times, thus placing the detection limit for the method below this value, significantly so for some analytes. Although it was possible to visually distinguish blanks from spiked samples, the objective of the study was to determine if the mean analyte signals from the different concentrations (25, 50 and 100 µg/kg) were statistically significant, i.e. if they were reliably distinguishable. For this, the replicates of each concentration (n = 4) were tested against the other concentration's replicates (25 vs 50, 50 vs 100 and 25 vs 100 µg/kg). Firstly, a two-tailed F-test was performed for each pair to ascertain if variance differences were statistically significant at $p = 0.05$. Then, a one-sided t-test was performed at $p = 0.05$, modified according to whether variances were different or not for each pair. Results are presented in Table 5.1.

Table 5.1: Results for On-the-Fly SPME analysis of different pesticide concentrations. Pairs whose mean difference is statistically significant for a one-sided t-test at $p = 0.05$ are marked "Yes". All values are in µg/kg.

	Soil A			**Soil B**		
	25 vs 50	**50 vs 100**	**25 vs 100**	**25 vs 50**	**50 vs 100**	**25 vs 100**
aldrin	Yes	-	-	-	-	-
boscalid	-	-	Yes	-	Yes	Yes
buprofezin	-	-	-	-	Yes	-
chlorpyrifos	Yes	-	-	-	-	-
diflufenican	-	-	-	-	Yes	-
epoxiconazole	-	-	-	-	Yes	Yes
lindane	Yes	-	Yes	-	Yes	Yes
metalaxyl	-	-	-	-	-	-
metazachlor	-	-	Yes	Yes	-	-
metolachlor	-	-	Yes	Yes	Yes	Yes
metribuzin	-	-	-	-	-	-
penconazole	-	-	Yes	-	Yes	Yes
pentachloroanisole	Yes	-	-	-	-	-
prosulfocarb	Yes	-	-	-	Yes	Yes
tebuconazole	-	-	-	-	Yes	Yes
terbuthylazine	-	-	Yes	Yes	-	Yes
tetraconazole	-	-	Yes	-	Yes	Yes

The results show that the method cannot be reliably used for semi-quantitative purposes, at least for this relatively narrow concentration range. However, as stated previously, it could reliably distinguish between the blank and 25 µg/kg for every analyte. Thus, it may be employed for detection of pesticides in soil at concentrations above a specific confidence threshold (the method's detection limit). Furthermore, it is likely that an increase in the number of samples taken (*e.g* n = 10) would increase its reliability. However, this would also make the method much more cumbersome and expensive. It is noteworthy that a single soil sample, extracted with four fibers, obviously requires at least four GC-MS/MS runs, if no instrumental replicates are made. This for a method which in inherently much less precise than one like QuEChERS, probably means that its applicability will always be restricted to situations where "destructive" sampling wants to be avoided. The exception would be the use of Direct Analysis in Real Time ionization source (DART) [48], which can be used to desorb the SPME fibers directly, resulting in a theoretically much simpler method.

The applicability to field soil is somewhat more complex. On the one hand, it is much more difficult to maintain an adequate water content, since percolation will always happen, and saturation is more difficult to maintain, although for a 3 h extraction this is unlikely to present a problem except in extremely well-draining soils. Furthermore, given the large size of a common sampling area compared to the fibers themselves, and that there is no homogenization such as mixing subsamples, representativity can be a problem.

Sampling depth must be carefully studied, and likely several extractions will have to be made, in order to obtain a relevant average measurement for a particular site.

5.3.3 Electrokinetic Remediation Experiment

As stated above, the electrokinetic experiment (EK) was designed based on a previous work [46], where operational parameters had been studied and optimized, albeit for a different soil and contaminants. The experiment was used to test the possibility of using On-the-Fly SPME for monitoring.

Soil temperature was stable in both biotic and EK experiments (21 ± 1 ºC), with no discernible increase due to joule heating in the EK microcosms. After eight days the experiments were ended and each microcosm sectioned and analyzed. In terms of pH, a large deviation from the initial value (circa 7.7) was observed for the EK treatment, with the anode section having a value of 2.7 ± 0.2 and the cathode 10.4 ± 0.8 (n = 4). These changes are expected, since water electrolysis will generate H^+ (H_3O^+, aq.) at the anode, and OH^- at the cathode. However, these values are much larger than previously reported for other soils under similar treatment [46], especially the significant acidification at the anode section. This may be indicative of this soil's poor buffering capacity but may also have been caused by using a smaller soil mass compared to the previously reported experiments whilst retaining the same current (20 mA), as well as using a slightly larger current ON time (14 h vs 12 h). Nevertheless, it highlights the need for significant change in operational parameters before attempting to use this EK methodology on a soil of this nature, as these changes would significantly damage its health and change ion solubility, although more extreme pH values can also catalyze certain reactions which favor pesticide degradation.

Quantification of target pesticides was carried as explained in Section 5.2.4. Figure 5.3 shows the relative degradation attributable to the EK process, obtained by subtracting the average biotic degradation percentage to the EK degradation values.

2,4-D and MCPA are the only evaluated contaminants with carboxylic acid functional groups (along with glyphosate), which at the initial soil pH (around 7.7) will be mostly in the form of carboxylate, with a net negative charge. Thus, significant electromigration towards the anode is expected, and indeed a large removal percentage was seen in the cathode for both contaminants. However, it is not possible, given these results, to infer as to actual degradation, since the concentration in the anode was far larger than the average control. Glyphosate, on the other hand, has several ionizable groups, and can be in a net positive, negative or zwitterionic state. However, no differences in concentration between the cathode and anode can be discerned, hinting to the fact that electromigration was not a relevant phenomenon for this contaminant. The same is true for AMPA. Unfortunately, none of these four compounds could be analyzed through On-the-Fly SPME.

As for the remaining contaminants, the results for On-the-Fly monitoring can be seen in Figure 5.4. These were obtained by calculating the estimated day 5 (D5) "removal" by

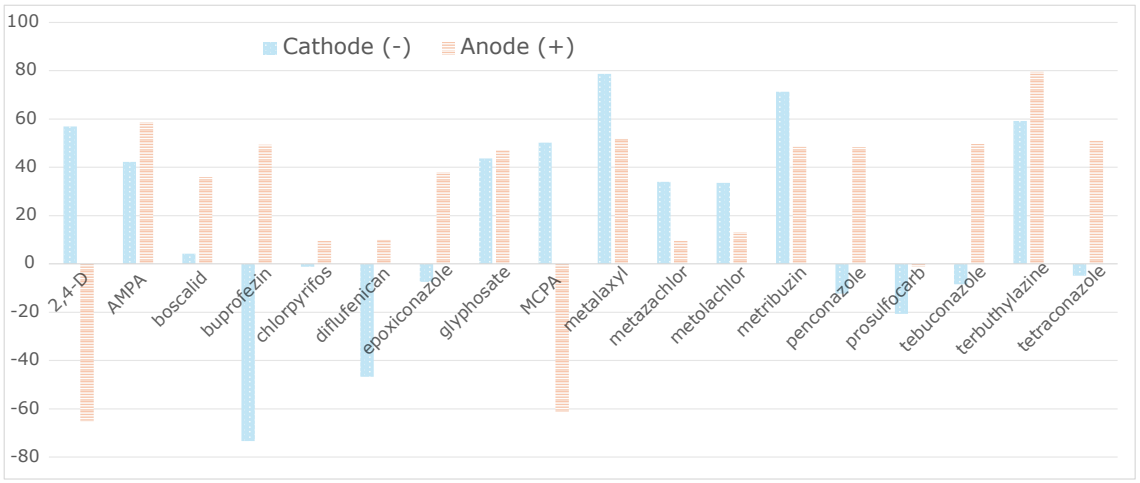

Figure 5.3: Relative degradation due to EK treatment. Vertical axis denotes percentage degradation above biotic control. Large values denote a larger degradation in comparison to biotic control (*i.e.* lower final concentrations) whereas negative values denote an enrichment of the contaminants in relation to control.

subtracting the D5 average signal of each experimental section from the initial (D1) average signal, and then plotting this "removal" as a percentage of the D1 average. Thus, positive percentages represent D5 signals lower than the initial, whereas negative percentages denote higher signals on D5. Unlike in Figure 5.3 above, these results were not normalized with the biotic control, since there were no statistically significant differences between D1 and D5 averages for biotic control samples using On-the-Fly SPME. Therefore, they refer only to differences between D1 and D5 for each EK experimental section (Cathode, Center and Anode).

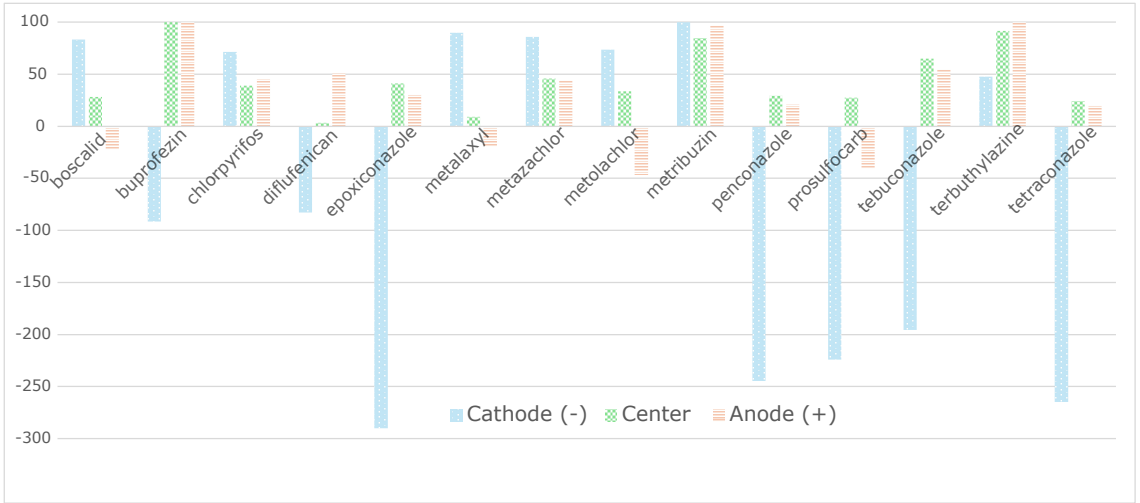

Figure 5.4: Percent degradation between D1 and D5, according to On-the-Fly SPME. Positive percentages denote lower values on D5, whereas negative percentages denote higher values on D5 (enrichment).

The results are mostly congruent with the quantifications performed at the end of the experiment, even though there is a 3-day difference between the samplings (day 5 vs day 8). Most compounds show the same pattern, with evident enrichment in the cathode for buprofezin, diflufenican, epoxiconazole, penconazole, prosulfocarb, tebuconazole and tetraconazole. However, it is clear that On-the-Fly SPME cannot be reliably used for quantification. For example, the compound with largest signal increase at D5-Cathode (epoxiconazole) shows only a moderate increase versus control in Figure 5.3. Furthermore, as the EK experiment progresses, soil parameters such as pH, mineral concentration, etc. are expected to change. In this experiment, pH was found to significantly change due to the electric current application, which can also influence SPME extraction. Nevertheless, since several compounds still exhibited lower signals at D5-Cathode (boscalid, chlorpyrifos, metalaxyl, metazachlor, metolachlor, metribuzin and terbuthylazine), in line with the quantitative results at the end of the experiment (Figure 5.3), it is reasonable to propose this On-the-Fly SPME methodology as a simple, relatively inexpensive, qualitative method for rough monitoring of the EK process. However, the most interesting and potentially useful application would be direct field sampling for determination of contamination from pesticides and eventually other organic contaminants such as polycyclic aromatic hydrocarbons, but this would have to be paired with some form of portable mass spectrometer [49], ideally with a DART source, which seems unlikely to appear in the near future.

6

Free Software for Chromatographic Data Analysis

6.1 Analytical Chemistry and Free Software

Modern analytical chemistry is dominated by computers. Today one would be hard pressed to find a single analytical process which does not employ microprocessors (even traditional titration is being automated). And yet, analytical chemists are strangely disempowered with regard to the apparatus they use daily, which comprises mostly proprietary hardware and software. It would be no exaggeration to say that today's advances in analytical chemistry are held by a handful of corporations.

There is a sense in which this corporate control is beneficial: it helps maintain a high level of quality and reproducibility in the results obtained; Furthermore, the widespread availability of these instruments as well as their relatively low cost (compared to "lab-made" prototypes) are also benefits. It is only natural that companies want to protect their intellectual property in order to maximize profits, which also pays for the development of those same instruments. However, it can also be a source of danger and ethical dilemmas.

In the realm of hardware (*i.e.* physical components), several issues can be easily detected. One of these is the monopoly of replacement parts for a particular instrument, opening the possibility for profiteering. This problem is endemic to most technological sectors, but not all. Another issue, and perhaps one more important in the context of scientific development itself, is the fact that new advances are dependent on the use of proprietary systems, which not only breaks with the tradition of knowledge sharing, but crucially makes the entire modern scientific edifice fragile. An example is apt: several developments have been made in the realm of proteomics using high resolution mass spectrometry [50]. Although more than one company makes these instruments, the technology behind them is proprietary. Thus, the continued use of such technologies, for *e.g.* medical diagnostics, is entirely dependent on companies whose main driver is profit. Again, it is only natural that these companies would want to protect their property and capitalize on the substantial capital invested in developing these systems, but the

issue here is the dependence which is created. In this respect, competition/antitrust laws are important for controlling the market, but ultimately the consumer (in this case the analytical chemist) is utterly dependent on the technological companies.

This chapter deals with another issue, however: that of software, which is commonly perceived as belonging to a totally different category from physical materials. Indeed, software has certain distinguishing characteristics, and also a certain ambiguity which makes it difficult to say whether it is a good or a service (individual pieces of software can be either, depending on how they are marketed and used). Nevertheless, software is essential in modern-day analytical chemistry, and indeed all scientific activities.

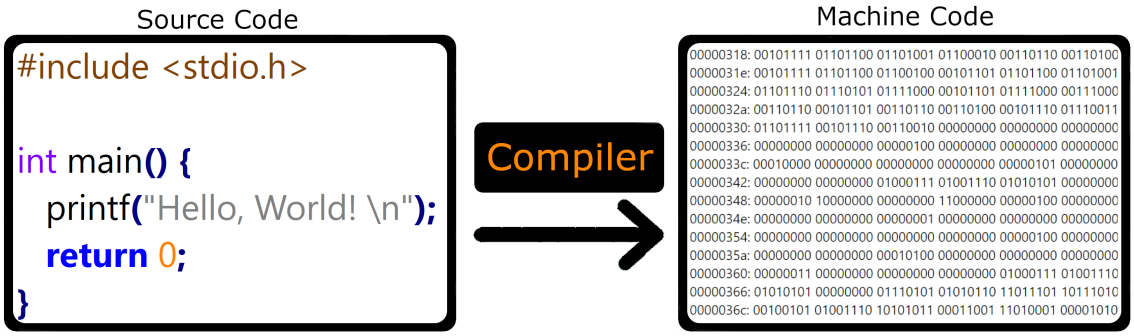

Figure 6.1: A very simplified view of the compilation process.

One important issue with software is that its workings can be hidden from the end-user. Essentially, after it is written in human-readable languages such as C, Fortran *etc.*, proprietary software is compiled to machine code (Figure 6.1), and distributed as an executable which is remarkably difficult (although not impossible) to reverse engineer in order to see the original code. As a consequence, end users do not have access to modifiable versions of the program, nor do they know exactly what is being executed (apart from what they see). Even when code is not compiled, there are various strategies known as obfuscation which make it harder for humans to read the code [51]. This is an old and well discussed issue, which led to the development of the "Free Software Movement" [52], an ideological approach to software development and licensing which is asserted by four essential freedoms [53]:

- The freedom to run the program for any purpose.
- The freedom to study how the program works and adapt it to one's own needs. [Access to the source code is a precondition for this].
- The freedom to redistribute copies [of the program].
- The freedom to make improvements to the program and release them to the public.

Here "free" stands for "freedom", not for "no price/gratis". Free software can be commercial, although it is inherently more difficult to profit from, since the program is

essentially exposed to legal or illegal redistribution. Nowadays, a large proportion of the software used is distributed under "free software" licenses such as the GNU General Public License (GPL) or the more permissible MIT license. Nevertheless, most software used by analytical chemists is proprietary and commercial, which prohibits users from modifying or even seeing its workings. Although this helps to maintain a high level of quality and reproducibility in the results obtained from such software, it also stunts progress and freedom.

Recently, some free (as in freedom, as opposed to proprietary) software packages have been developed for the treatment of chromatographic and mass spectrometric data. Most notably OpenChrom [54], which is still widely used, as well as other projects which are no longer in active development, such as mMass [55]. Also, some small libraries have emerged for data treatment, such as PythoMS [56], which provides several scripts for mass spectrometric related operations, from the command line. Nevertheless, direct interaction with most analytical instruments is precluded since manufacturers design closed hardware which can only be interfaced through their optimized, proprietary software.

The remainder of this chapter describes a new software package developed for chromatographic data processing. The intent of developing this small piece of software was firstly to assist the other tasks of the present work, such as creating and analysing experimental designs or easily calculating HPLC solvent usage for green analytical chemistry calculations. The other objective is to function as an abstraction layer for non-programming oriented people to have access to a relatively easy to use, free software. It also aims to give analytical chemists familiar with programming a starting place to further develop programs for their own professional use.

6.2 Chromapy

Chromapy (Figure 6.2) is derived from *chroma* (color, chromatography) and *py*, from the main language used in its development, Python. This language was used because of the rich set of previously developed, free software libraries, which enable the analysis of complex datasets such as matrices imported from excel or `.csv` files. Numpy and Pandas [57], in particular, are the python libraries most used for this type of analysis, and can handle data similarly to excel. This type of analysis would be incredibly difficult to implement *ab initio* in a language like Fortran or C. Other libraries such as Scikit-learn [58] have been used for most of the computations involved, such as calculating principal components for multivariate analysis. Thus, it is important to note that this package is almost totally based on these previously validated libraries: almost none of the code which does the true computation was written by me, apart from a few exceptions. Chromapy itself simply allows a much simplified use of these tools. It provides a simple way to import the data, evaluates it, and performs the calculations with sensible defaults (which can be changed), allowing the operator to forgo any knowledge of programming. The

idea behind distributing this code is to allow people who would never have been able to access these libraries through python itself to have an easier experience.

The chromapy package along with a detailed overview and instructions is hosted on Github: `https://github.com/JBrinco/Chromapy`. This chapter describes some of the functionality, how it was used throughout this work and why, but does not go into technical details regarding the software itself.

Figure 6.2: The Chromapy software logo.

6.2.1 Package Structure

For convenience, the package was divided into four modules which perform different functions, namely:

- **Multivariate Analysis** implements PCA and PLS with sensible graphical biplot outputs. Also implements several normalization algorithms commonly used for chromatographic data.
- **Design of Experiments** generates design matrices for full-factorial, Plackett-Burman and Box-Behnken designs. Calculates main effects for two-level designs and response surfaces for Box-Behnken.
- **Quantification Assistant** automates data processing for quantitative experiments. Fits a linear regression model, calculates final values based on recovery and generates some method performance parameters (estimates of detection and quantification limit) using the calibration curve.
- **Chromatography Calculators** performs very simple computations relevant for chromatography.

Each of these modules is a python file with a series of functions which can be accessed through python itself. For example, the function `full_factorial()` in the Design of Experiments module takes a set of correctly formatted `data` (usually a .csv file) and generates a full factorial design with however many factors and levels the data allows. For ease of use, a script was created for each of these four modules. These scripts can be accessed from the command line without any knowledge of programming, and automate a lot of the interaction with the module. Taking the function `full_factorial()` as an example, instead of having to import the data, call the function, store the output in a

variable and then exporting it to another .csv file, one can simply write in a terminal emulator: `'python DOE.py -F MyData.csv'`, where `DOE.py` is the design of experiments script, `-F` is the option that asks for a full-factorial design and `MyData.csv` contains the input data for the design. Equally, `'python PLS.py DataForPLS.csv'` calls on the `PLS.py` script to perform a partial least squares regression with the data in `DataForPLS.csv`. Although shown here in their simplest form, these scripts have several options to change the behaviour and output. Thus, someone with python knowledge can write their own scripts and routines by accessing the functions themselves, whereas people who do not know programming need only have a little patience in setting up the environment so that they can use the software in a simpler way through the command line. Naturally a graphical user interface (GUI) would further help in this respect, and might be developed in the future.

6.2.2 Multivariate Analysis Module

This is the only module which was not directly used in other parts of the present work. It provides calculations for PCA and partial least squares (PLS). Principal component analysis in particular is extremely used in chromatography. It is also sometimes misused, especially when the values of the variable under study are already known (*e.g.* studying the effect of different types of wine treatment on the volatile composition) [59]. In this case, where both the volatile composition and the types of treatment are known, an unsupervised method such as PCA (which does not take into account the "response variable") can be used for exploration of the data, but not in isolation. If the main variance in the volatile composition is indeed caused by treatment type, then PCA will be able to classify the samples "correctly", thereby indicating that different treatments do result in distinguishable volatile profiles, and be able to draw conclusions. However, if the samples are clustered in a different way, it does not necessarily mean that the different treatments have no effect on volatile composition, simply that it is not the main source of that dataset's variance. Often, PCA works well because the experiments are controlled in a way that only the studied variable changes, and thus the main variance is likely to originate from this variable. This is only an example of how easy it is to use statistical methods in wrong ways, something which can be illustrated with examples from this thesis.

Figure 6.3 shows an example of a PCA biplot output from Chromapy, adapted from a published work [60]. The data is concerned with an experiment which studied the effect of a new polymer for white whine protein stabilization versus the traditional method, which is bentonite. Here, the samples were analysed by headspace-SPME followed by GC-TOFMS and 71 volatile compounds were identified. Their relative intensities (peak areas) were used to produce this PCA biplot. The different colors of the loadings (numbers 1-71) reflect the main "chemical class" of that compound in terms of functional group. Red numbers, for example, represent esters, an important chemical class in wine's aromatic profile. By default, chromapy will print all loadings in black, unless some classification

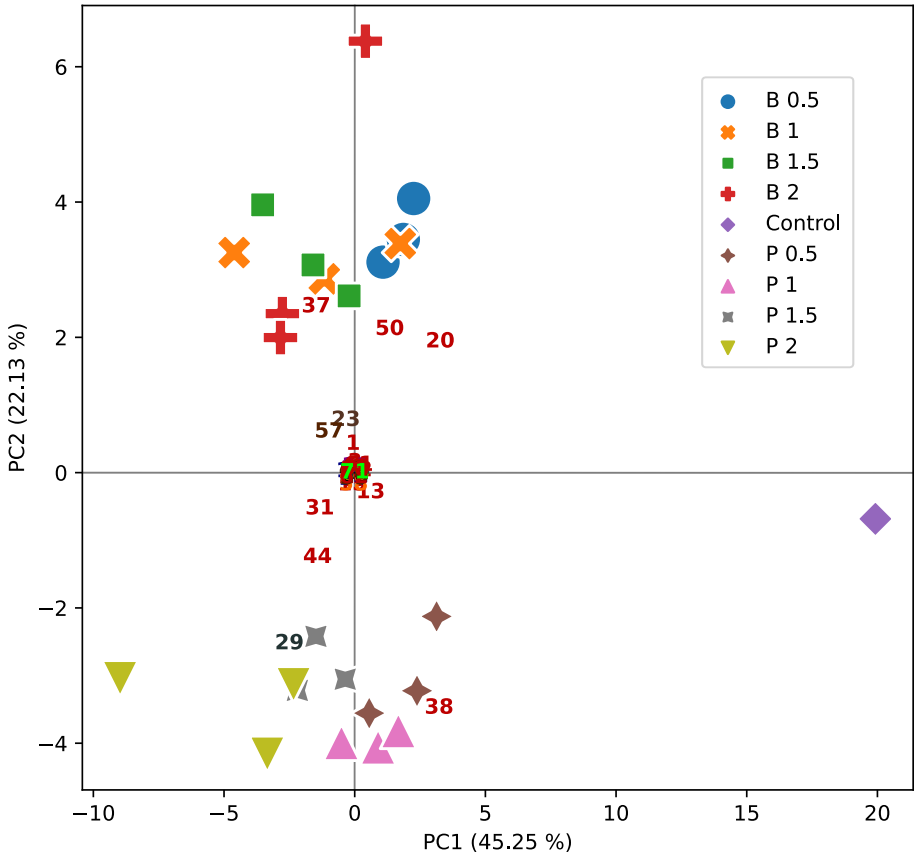

Figure 6.3: A PCA example made with Chromapy. Adapted from [60]. Numbers refer to loadings (the volatile compounds). B and P specifies samples treated with bentonite or the polymer, respectively, at 0.5-2 g/L. Control was untreated. Loadings were scaled by a factor of 6 for legibility.

is given. It is important to note that the loadings were scaled in this figure (an option in chromapy), otherwise they would all have crowded in the center, since the individual contributions to the overall principal components are small when compared to the sample's values in the 2D space. Nevertheless, Chromapy also outputs the actual contributions of each loading to the principal components, and can also output the loadings in a separate graph. Calculation of an unknown sample (for example in order to ascertain if it was treated with bentonite or the polymer) can be performed with the principal components using a technique called principal component regression, but it is not currently supported by Chromapy.

The clusterings in Figure 6.3 clearly show a separation between the bentonite and polymer treatment, showing that this is the main cause of variance in the dataset. Nevertheless, in this case PCA should not have been applied alone (as explained above), but in conjunction with another technique such as linear discriminant analysis or partial least squares (PLS) [60]. Figure 6.4 shows an example PLS biplot straight from the software, with the only applied option being a scaling of the loadings by a factor of 10. In this case, the data comes from the same dataset, but PLS was applied to classify the three

different wine varieties present: viozinho, moscatel and encruzado. The response variables are labeled as "R1", "R2" and "R3", where R1 was set to 1 for all viozinho samples and to 0 for the others, R2 was set to 1 for moscatel samples only, and R3 was set to 1 for encruzado samples. This is a common way to perform PLS when the classification is non-numerical (*i.e.* wine varieties rather than numerical values). PLS has many uses, one of which might be to determine the variety of an unknown sample by analysing its volatile profile and applying the trained model to that sample, although in this particular case that could be achieved simply by a taste test. The command to obtain this PLS biplot was `python3 PLS.py wine_data.csv -r 3 -s 10`, where `wine_data.csv` contains the data, `-r 3` specifies tree response variables and `-s 10` scales the loadings by a factor of 10 for legibility.

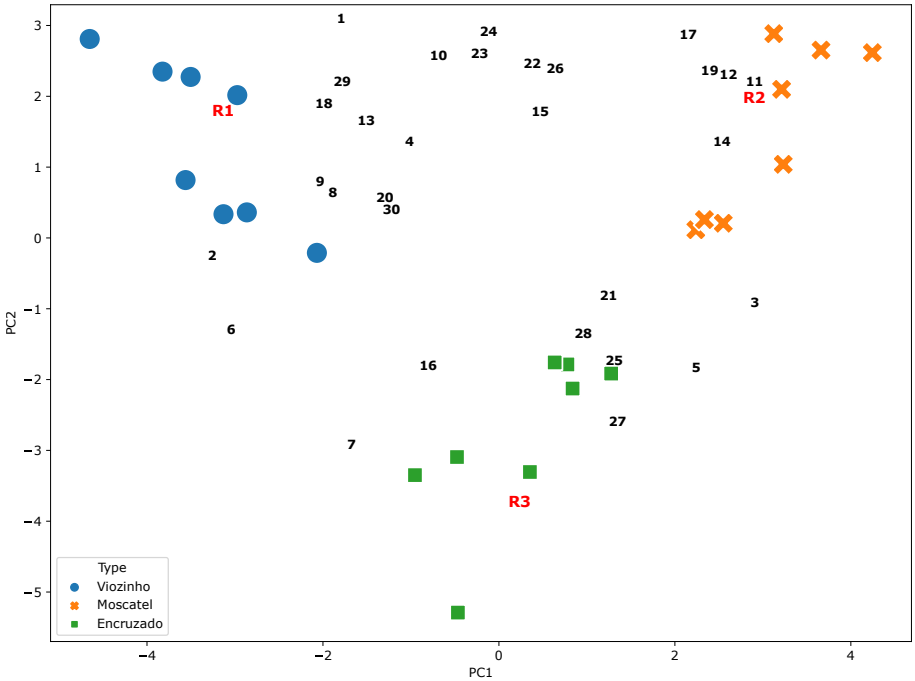

Figure 6.4: A PLS example made with Chromapy. Numbers refer to loadings (the volatile compounds). R1, R2 and R3 are the response variables associated with each classification: viozinho (R1), moscatel (R2) and encruzado (R3). Loadings were scaled by a factor of 10 for legibility.

All the computations in this module are performed by calling a Python library called Scikit-learn [58], which implements the algorithms for normalization as well as PCA and PLS calculations. It also supports several other forms of classification, regression and clustering useful to chromatography, which can be added in the future, if needed.

6.2.3 Design of Experiments Module

The module which implements experimental designs and their analyses is by far the largest and most complex of the whole package. It was extensively used throughout this work, mainly for optimization of the analytical methodologies developed. The code

does not do any checks to the data or the results other than running the computations themselves, which means that the operator must take care to ensure the data is reliable and the results significant. One example of this is the interpretation of screening designs, such as the Plackett-Burman design used to screen ten variables in the newly developed method of direct-immersion SPME of soil, in Section 4.2. This was an unsaturated design (10 variables of a maximum 11 were used), in which the eleventh variable was used as a "dummy" with which to calculate the statistical significance of the results against. Nevertheless, the original conception of the experiment was to simply use the main effects (*i.e.* the effect of each variable on the detected signal) to draw conclusions. It was only after the experiment was concluded that the need for a statistical test was ascertained, since the calculation of a main effect will always yield a result, even if it is not statistically significant. Ideally, an unsaturated design with at least two dummy variables instead of only one should have been used, which would have provided a more robust analysis, but at the time this was not considered. This example is meant to illustrate the danger in using such methods without a good understanding: Chromapy will not advise the user to perform an unsaturated design in order to reach more robust conclusions, nor will it set a threshold of significance for main effects. These issues are more pronounced in methods with a relatively high replicate variance (poor repeatability), especially since any form of internal standardization is not useful in an optimization stage, because operating variables are constantly being changed. Although some more assistance might be implemented in the future, at the moment it falls on the operator to have a good working knowledge of the techniques used.

The full-factorial design used to optimize the DI-SPME extraction method in Section 4.4 was also made with Chromapy, although it could have been done by hand. The calculation of main effects with Chromapy cannot be made for designs with more than two levels (*i.e.* two values per variable, a low and a high one). Thus, analysing the results from this three-factor design is a little more complex, but offers more than a simple screening of significance, as can be seen in Section 4.4 (see Table 1 of the published article). The general workflow adopted in this work was to first do a screening with a Plackett-Burman design to ascertain which variables most influenced the desired outcome (signal intensity and relative standard deviation of replicates), and then perform a stepwise optimization of each important variable in turn, or optimize them together in cases where they appear to be strongly correlated.

Chromapy also supports response-surface modeling, a more complex method which involves fitting a response surface (a polynomial mathematical model) to the data, thereby producing a surface representing the theoretical value of a response variable as a function of two or more modeled variables. The computations involved were implemented by `rsm` [61], a package for the R programming language. Thus, for response surface modeling, an R installation is required. No adequate package was found for the Python programming language, and whence the use of R.

A response-surface model was attempted for optimizing the DI-SPME extraction with

ethanol (Section 4.4), using a Box-Behnken design, the only one currently supported by Chromapy. In this case, the response variable was the analyte signal, and the modeled variables were the percentage of ethanol in the extraction slurry (2, 6 and 10%, v/v), the extraction time (60, 90 and 120 min) and the retroextraction volume (100, 120 and 140 µL).

Figure 6.5 shows two examples of response surfaces obtained with Chromapy for that dataset, which were not published in that work [19], since in these cases a maximum is not observable, and the experimental space seemed to lie outside the optimum. The coloring of the surface is redundant with the vertical axis, which in this case is the analyte's signal, and is done so for legibility, considering it is difficult to see an abstracted 3D object on a 2D surface. As three-dimensions is the maximum that can be simulated on a two-dimensional surface, the program "slices" (*i.e.* fixes) one of the variables and shows the others, as described below the graphs in Figure 6.5.The program will output several of these "sliced" graphs, in order to get an idea of each variable's contribution and effect on the response variable, of which Figure 6.5 shows only one. Naturally this behaviour can be configured. An experiment with even more variables would obviously be significantly more challenging to analyse graphically.

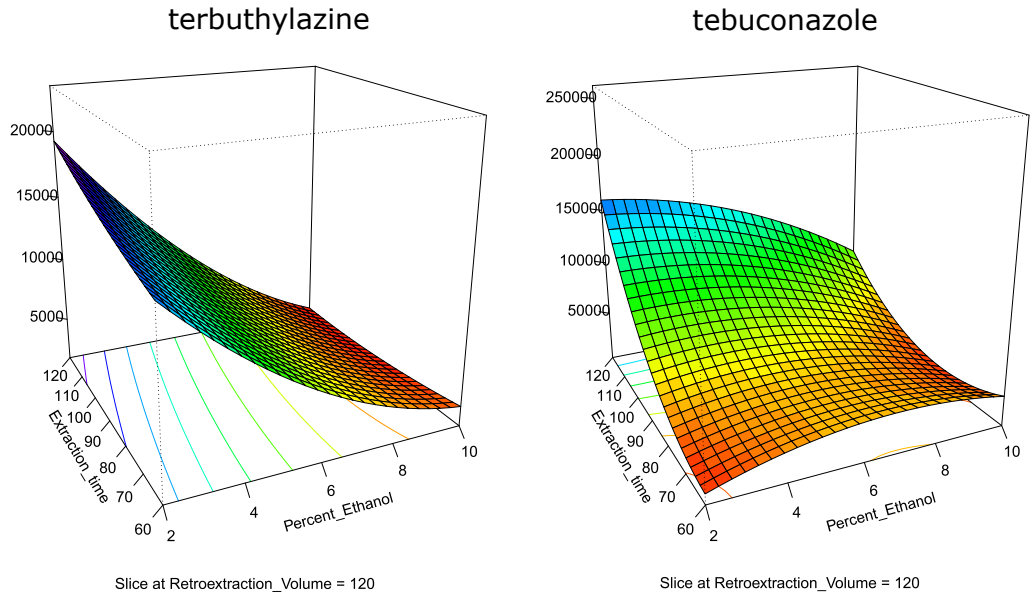

Figure 6.5: A response-surface example made with Chromapy. The two graphs were put side-by side and compound names added on top. Otherwise all features come directly from the software. The horizontal axes show extraction time and ethanol percentage. The vertical axis denotes the response variable (analyte signal). Coloring on the surface shows the same as the vertical axis (larger values in blue, smaller in red).

The program will also attempt to look for a local maximum within the experimental space and output it. However, it will sometimes find the maximum outside the experimental values (in the example above a maximum at over 120 min of extraction time). In this case, it was found that the extraction time was generally the most important variable, with the ethanol percentage also having an important contribution for most analytes.The

retroextraction volume was fixed at 120 µL for practical reasons, and a full-factorial experiment was conducted to optimize the ethanol volume and extraction time together, as described in Section 4.4 (*"Multiclass Pesticide Extraction by Immersion SPME"* in the "Results and Discussion" of the published paper).

Naturally, these methods of optimization all require a good knowledge of their workings in both application and data treatment. However, It could be argued that a failure in the optimization step of a method is not as critical as in the validation step, in terms of that method's reliability. At worst, an improperly optimized method will simply not be fit-for-purpose, whereas a wrongly validated method will yield erroneous results, which are often unrecognized unless tested for accuracy against a certified standard or another established method.

6.2.4 Quantification Assistant Module

Quantification calculations such as least-squares regression, internal standard ratios and recovery percentages are relatively easy to perform on any spreadsheet software, as long as the data is not extensive. In methods with a large number of analytes or samples (or both), calculations become tedious and sometimes prone to errors. Nowadays, most Instrument manufacturer's software provides automated quantification, and in most cases these work very well, so long as the system can be trusted to integrate the correct peak. Regardless, some analyses still required the analyst's trained eye and experience.

Chromapy takes `.csv` files with formatted data containing the calibration and unknown samples. It performs least-squares regression and calculates the concentrations for unknowns. Samples with the same name are treated as replicates, which are then outputted as a single average value and standard deviation. It will also estimate limits of detection and quantification from the calibration curve [62], even though this is usually not a very good method except when the calibration curve itself is close to these limits. It also supports internal standardization, although not surrogate standards (added to the sample before extraction) for precise recovery calibration. The module can also output a graphical representation of the calibration curve for visual inspection, but this is unnecessary in most cases.

In the future, other important functions might be added, such as Mandel's test of linearity, weighted regressions or the aforementioned recovery calculations. However, the greatest pitfall with this module is its limited usability, since as explained above most modern software (*e.g.* Agilent MassHunter or ThermoFisher Excalibur) is very capable of performing calibration directly from the chromatograms without any need to export to `.csv` and using a third-party tool such as Chromapy. Furthermore, simple calculations can often be performed in a spreadsheet software such as Microsoft Excel, which provides greater control of the data and is less prone to bugs or problems with incorrectly formatted data, something which Chromapy struggles with. In the end, this module will only be truly beneficial for researchers and engineers interested in creating automated workflows

for custom-made systems, where it can be changed and improved upon, unlike proprietary software, which offers virtually no possibility of building upon the allowed functions.

6.2.5 Chromatography Calculators

This very small module is a compilation of calculators which might be of use to those working in chromatography-related fields. In the original conception of the software package, it was going to be larger, with tools such as a calculator for GC linear retention indices [63] and an experiment randomizer, but these turned out to be impractical and more easily handled through the instrument's software or a spreadsheet.

At the moment, it provides calculations for GC carrier gas linear velocity and volumetric flow, as well as a calculator for the volume of solvent used by an HPLC, based on a chromatographic run. This was used extensively to obtain solvent consumption (and thus waste generation) of several published methods, in order to calculate the green analytical chemistry metrics' values used in the review paper on Section 3. The published works which were reviewed mostly state the HPLC eluent run in the same way, by giving the flow (in mL/min), and describing eluents A and B. Then, they commonly describe the run as increments in the percentage of B, *e.g.* starting with 5% B, then 95% B at 10 min held for 1 min, then a return to 5% B at 12 min and equilibrated for 2 min. Chromapy can give the volume of A and B eluents expended over that run. It requires the column flow and an array which contains the percentages at certain times. It then assumes a linear progression between those percentages (*i.e.* a constant slope) and performs integration to obtain the volumes.

6.3 The Future of Chromapy

Chromapy was created mainly to perform certain calculations related to this dissertation. There is currently no free software package which offers the same functionality in a chromatography-oriented way. Chromapy was released under the GPL Version 3, which means it can be modified and incorporated into other projects as long as those derivatives are also free software, but it cannot be incorporated into proprietary programs such as most chromatographic instrument software. It is also provided at no cost, and will remain so even if it reaches a level of maturity for which people might be willing to pay money (which it does not possess at the moment). If Chromapy proves useful to others, it is likely that different developers may take it on and begin working on improvements. The fact that any developer can take the source code and modify it is one of the major advantages of free software and a defining characteristic of its development: it is often a collaborative process [64]. If Chromapy does not find use, then it is likely to follow the trajectory of other discontinued software packages such as mMass [55], which is a common occurrence in the realm of software development. If analytical chemists are

satisfied with using proprietary software (which is naturally more polished and reliable, despite the disadvantages presented above), then they should continue to do so.

7

Conclusions and Future Perspectives

The work presented here is the culmination of a PhD which involved several different objectives and projects. The most important outputs of this work were several new methods for extraction and quantification of pesticides in soil, including a novel "on-the-fly" extraction which greatly simplifies sample treatment, as well as a new free-software package for analysis of chromatographic data. All of these are aimed at improving soil monitoring through the use of better, cheaper and safer methodologies.

The first work, presented in Chapter 2, helped classify pesticide packaging waste as hazardous or not, in order to dispose of it properly in line with EU regulations. The adapted method proved simple enough, although the biggest difficulty with this plastic sample has been pre-processing, especially grinding. The work also showed that after the campaign inciting the triple rinse, the waste has been classified as non-hazardous. Chapter 2 also explains the logic and research behind the choice of analytes, which was motivated primarily by frequency of occurrence in EU soils, but also by other factors such as chemical diversity within the list.

The major objective of developing a new method for analysing a multiclass group of pesticides along with glyphosate and its major degradation product AMPA was only partially successful, and it may be that a method fulfilling these expectations whilst also having adequate performance parameters by today's standards is currently unfeasible. The review of currently used methods for pesticide analysis in soil (Chapter 3) showed that although QuEChERS appears to be an excelent method when properly developed, neither it nor others currently in existence are likely to work well alongside glyphosate extraction. Nevertheless, the DI-SPME extraction as a standalone proved very interesting and it is likely that further improvements can be made, as well as increasing its scope to other analytes. Crucially, there is no reason why this extraction method should be restricted to pesticides, as several other organic compounds (polyaromatic hydrocarbons (PAH), flame retardants, etc.) can potentially be analysed as well. Organic contaminants selected by the European Commission for control under the Soil Monitoring Law (COM/2023/416) are the most interesting candidates for scoping and expanding this extraction technique. However, more polar analytes (*e.g.* pesticides commonly analysed by LC-MS) may not

sufficiently adsorb onto either of the SPME fiber chemistries available; or else the method may have to be optimized separately for polar and less polar pesticides, which would make it difficult to compete with QuEChERS in cases where a multiclass analysis of many compounds is required. More generally, the introduction of this new type of SPME extraction to environmental monitoring is perhaps the most important contribution of this thesis to the field.

The same SPME fibers also proved useful for "on-the-fly" pesticide extraction directly from the soil without needing extensive lab work. Admittedly, the method is still reliant on chromatographic-mass spectrometric determination, but currently existing simpler alternatives such as certain sensors [65] do not provide nearly the same level of selectivity in a single analysis. However, due to the method's inherent variability, it cannot accurately quantify these analytes, but with adequate validation it is likely to achieve a good level of reliability for detection of different organic contaminants. Since the determination has been performed with GC-MS/MS, false positives are unlikely lest the method is poorly validated. Field determination using portable GC devices might be an interesting avenue for further exploration, although sensitivity could be an issue. Nevertheless, given the SPME fibers portability, it might be possible to perform the extraction in the field and then mail them to a lab, where a screening using DART for direct desorption would result in a reliable form of contaminant detection at a fraction of the cost of traditional sampling and analysis. Furthermore, expansion of the method into other chemical classes of contaminants would be interesting and potentially very useful. As for the electrokinetic remediation experiment tested alongside the method, it's clear that it caused very significant changes in soil parameters, which regardless of degradation efficiency should warrant significant further development for this soil type before it may be applied in real conditions.

An interesting question which remained unanswered is to what extent these new SPME fibers truly reduce the environmental impact of this type of analysis. As explored in Section 4.2 (subchapter *3.5 Evaluation under green analytical chemistry* of the published article), the fact that the fibers are semi-disposable is an important issue, and the cost and waste generation of manufacturing them is unknown. Although the green analytical chemistry metrics calculated point to a good result, reusing the fibers for as long as possible (without compromising metrological reliability) is essential, even if their monetary cost is not high.

The software package entitled Chromapy is presented as a "work-in-progress", echoing the analytical methods developed for this work, which still have a great margin for experimentation and optimization. The software is free (as in freedom) and was written not only for the work, but also as a starting place to any chromatographers or analysts in general wishing to use and modify its capabilities. Naturally, it is mostly based on previously developed code, and for the most part I can be credited essentially with making an easier and more intuitive interface for chromatography related calculations. A great amount of good faith is evident in the extensive development of the code on which

Chromapy is based, and thus this work simply attempts to pay it forward.

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ANALYSIS OF PESTICIDE RESIDUES IN SOIL - NEW APPROACHES FOR ENVIRONMENTAL MONITORING