DETERMINATION OF KEY PARAMETERS INFLUENCING DISLODGEABLE

FOLIAR PESTICIDE RESIDUES (DFR)

PhD Thesis

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Abbreviations

ANOVA	*Analysis of Variance*
AOEL	*Acceptable Operator Exposure Level*
ARS	*Agriculture Research Service*
ARTF	*Agriculture Re-entry Task Force*
ASW	*Automated Surface Wipe*
BSR	*Bench Top Surface Roller*
CAKE	*Computer Assessed Kinetics Evaluation*
CDPR	*California Department of Pesticide Regulation*
CI	*Confidence Interval*
DA	*Dermal absorption*
DALA	*Day after the last application*
DAR	*Draft Assessment Report*
DAT	*Days after treatment*
DE	*Dermal Exposure*
DFR	*Dislodgeable Foliar Residue*
DFZ	*Difenoconazole*
DT50	*Half-life*
EC	*Emulsifiable Concentrate.*
EFSA	*European Food Safety Authority.*
EPA	*Environmental Protection Agency*
EU	*European Union*

EUROPOEM	*A concentrated action to construct generic databases of re-entry and bystander exposures to plant protection products and develop predictive models*
EXPO SAC	*EPA Science Advisory Council for Exposure*
EW	*Emulsion in water formulations*
FAO	*Food and Agriculture Organisation*
FIFRA	*Federal Insecticide, Fungicide, and Rodenticide Act*
GAP	*Good Agriculture Practice*
GLP	*Good Laboratory Practice*
GR	*Granule*
GRDC	*Grains Research and Development Corporation*
HAT	*Hours after treatment*
HILIC	*Hydrophilic interaction liquid chromatography*
IUPAC	*International Union of Pure and Applied Chemistry*
LCMS	*Liquid Chromatography-Mass Spectrophotometer*
LLE	*Liquid-Liquid Extraction*
LOD	*Limit of Detection*
LOG	*Logarithm Base 10*
LOQ	*Limit of Quantification*
MAF	*Multiple Application Factor*
MS	*Member states*
NOAEL	*No Observed Adverse Effect Level*
NOEL	*No Observed Effect Level*
PDE	*Potential Dermal Exposure*

PPDB	*Pesticide Properties Database*
PPE	*Personal Protective Equipment*
PPPS	*Plant Protection Products*
REI	*Re-entry interval*
RMS	*Rapporteur Member State*
RSD	*Relative Standard Deviation*
SAM	*European Commission's Scientific Advice Mechanism*
SAPEA	*Science Advice for Policy by European Academies*
SC	*Suspension Concentrate*
SL	*Solution*
SPE	*Solid-Phase Extraction*
STDV	*Standard Deviation*
TC	*Transfer Co-efficient*
TEHP	*Organophosphate tris(2-ethylhexyl)phosphate*
USDA	*The United States Department of Agriculture*
UV	*Ultraviolet*
VLCA	*Very-long-chain aliphatic*
WDG	*VETTABLE Dispersing Granules*
WG	*Wettable Granules*
WP	*Wettable Powder*
AOT	*Aerosol OT*
MC	*Measured concentration*
CDPR	*California Department of Pesticide Regulation*
CMC	*Critical Micelle Concentration*

DST   *Dynamic Surface Tension*

IP   *Internal pressure*

CI   *Confidence Interval*

AI   *Active Ingredient*

AS   *Active Substance*

Declaration

I hereby confirm that the work submitted in this Thesis entitled "Determination of Key Parameters Influencing Dislodgeable Foliar Pesticide Residues (DFR)" is an original piece of research. The presented work in this thesis and its associated publications listed at the end were submitted only to the University of Hertfordshire in partial fulfilment of the requirements for the award of Doctor of Philosophy with industrial experience in Life and Medical Sciences.

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Abstract

Pesticides have a crucial role to play in assuring food security for an increasing world population. Therefore, a risk assessment should be carried out for plant protection products (PPPs), covering both dietary and non-dietary routes and all possible exposure scenarios. Non-dietary risk assessments for workers, residents and bystanders mandate estimating the amount of pesticide residue transferred from plant foliage to the skin or clothes, known as dislodgeable foliar residues (DFR), along with the pesticide's half-lives (DT_{50}). However, both values were considered outdated and conservative by the industry and the public during the consultations and research (EFSA, 2014c; Kluxen et al., 2021).

Moreover, the DFR data in the literature are described as insufficiently reliable, limited, and encompass considerable statistical uncertainties. Thus, new data generation would allow for a more reasonable default value that reflects a more realistic exposure estimate and does not compromise human safety. To this end, the findings of this thesis explore some of the factors that affect DFR using a newly DFR developed lab method. This would enable more data generation and possibly refining of the PPP non-dietary risk assessment.

Possible correlations between dietary and DFR residue decline were investigated considering data from 177 dietary residue trials along with 56 DFR trials from outdoor studies on the same crops, besides residue decline data available in the Pesticide Properties Database (PPDB). The residue studies followed the non-

normal distribution, and the comparison between DT_{50} of both types of residues for most active substances revealed a statistical higher DT_{50} mean value of the dietary residue compared to the DFRs. Furthermore, the numerical back-transformed DT_{50} data for all tested active substances proved to be higher in an average of 5 to 23% in the dietary studies than in the DFR studies. Therefore, a DT_{50} value from dietary residue studies could act as a conservative surrogate DT_{50} for DFR, which could help determine the length of DFR studies and benefit both the agrochemical industry and the regulatory bodies in supporting non-dietary pesticide risk assessment.

The current work described a newly developed laboratory method for quantifying DFR. The laboratory method reflected the available field DFR methodology. It involved controlled application of droplets to the leaves and validation of the wash-off process used to completely remove the residue from the leaf surface before the analytical quantification using liquid-chromatography mass spectrometry. The aforementioned DFR technique was used to investigate the effect of leaf texture, formulation and co-formulants on the magnitude of DFR using the fungicide difenoconazole (DFZ) 10% (w/v). DFZ emulsifiable concentrate (EC 10%) and a wettable powder (WP 10%) with and without adjuvants on tomato, French bean and oilseed rape were tested. The findings showed that a comparable DFR% was observed from the WP and EC formulations on most sampled crop leaves, ranging from (82-74%) on French beans and (31-74%) on oilseed rape except for tomatoes (60-39%). No significant effect of adjuvants addition was observed for either formulation except when mixing TEHP (0.1% w/v) to the EC 10% on French bean, which resulted in a DFR recovery % decrease

from 82 to 74%. Changing the solvent system or the co-formulants in a DFZ EC formulation did not statistically affect the DFR recovery % in all crops. Still, a slight numerical increase in the DFR %, in that case, was observed on tomatoes from 60 to 65% and from 31% to 37% on oilseed rape. It was associated with low dynamic surface tension (DST) of the formulation, which was the reason for this enhanced uptake and low DFR.

The findings of this thesis also shed light on the effect of leaf texture on DFR, which showed a significant difference among all tested crops highlighting the importance of this factor. Moreover, grouping different leaves/crops based on their roughness (i.e., hairy, or waxy) proved to be relevant and applicable as hairy leaves were shown to have higher DFZ DFR (ranges from 82% to 52%) than waxy leaves (31%). However, an accurate classification approach would better involve the coverage degree and types of trichomes in different hairy leaves, as hairy leaves may act differently based on these characteristics. In conclusion, this work has demonstrated the importance of the newly laboratory developed DFR method and its application in studying the factors that could influence DFR to allow for refining the PPP non-dietary risk assessment.

Chapter 1 : Setting The Scene

1.1   Introduction

1.1.1   Pesticides - uses and hazards

The Food and Agriculture Organisation (FAO) defines pesticides as "any substance or mixture of substances intended to prevent, destroy, or control any pests. This includes vectors of human or animal disease, unwanted species of plants or animals, causing harm interfering with the production, processing, storage, transport, or marketing agricultural commodities, wood and wood products or animal feed"(FAO, 2003).

The term pesticide is broader than plant protection products (PPPs), although these terms are often used interchangeably. The European Commission differentiated between both by stating that plant protection products are 'pesticides' that protect crops or useful plants. They are used mainly in the agricultural sector, while pesticides are a broader term that also covers non-plant/crop uses, for example, biocides (European Commission, n.d.). Usually, plant protection products contain at least one active substance and have functions such as protecting plants or plant products against pests or diseases before or after harvest (Cabrera et al., 1985).

Pesticides are grouped and classified in different ways. One standard classification is based on pesticide function and the pest organism that they kill. This includes herbicides, fungicides, rodenticides, wood preservatives, garden chemicals, and household disinfectants used to kill or protect from pests. Whilst another classification is based on the mode of entry which describes how these

pesticides enter the target, such as systemic, contact or stomach poison. The last classification is based on the chemical properties of the pesticides, which include Pyrethroids, Organochlorine, Organophosphorus, Carbamates, and Pyrethrin (Rajveer et al., 2019)

There are huge benefits gained from using pesticides. At the same time, there are direct negative impacts on human and environmental health which should not be underestimated even with the significant benefits achieved. The adverse effects of pesticides are non-selective, and no segment of the population is considered completely protected from pesticide exposure. The United Nations reported that 200,000 people die annually from pesticide poisoning (DuVall, 2021).

The use of pesticides has increased dramatically since the early 1960s; in the same period, the yield average of wheat, rice, and maize, the primary sources of human nutrition, has more than doubled. Without pesticides, food production would drop, and food prices would increase (Popp et al., 2013). It has been estimated that without pesticides, 70 % of crop yields could have been lost due to pest infestations (Oerke, 2006).

Another benefit of pesticide use is vector control to help limit the spread of diseases by insects. Often the only practical way to control insects that spread deadly diseases, such as malaria and other possible endemic diseases, is by using pesticides. There is an intrinsic hazard and potential risk linked to pesticide exposure and its usage. Pesticide exposure has been linked to various health implications and diseases, such as endocrine disruption, carcinogenicity, genotoxicity, and immune system damage (Baltazar et al., 2014; Cocco, 2002; Ross, 2005). Also, long-term exposure to pesticides such as organophosphates and carbamates has been linked to a broad range of chronic health effects, including impaired neurobehavioral function (e.i., cognitive and behaviour

disorders), respiratory problems, obesity, and diabetes (Chakraborty et al., 2009; Kalliora et al., 2018).

Therefore, different nations have established regulations and rules to assess pesticide use in their countries and consequently minimize the risk associated with pesticide use. The variations in the pesticide regulations and rules among countries ranged from being primitive in some African and South Asian countries to very sophisticated regulatory regimes like the one in force in the EU, UK and the USA (Bozzini, 2017)

1.1.2 Route of pesticide exposure to human

Human pesticide exposure can occur occupationally and environmentally during manufacture and or after pesticide application indoors and outdoors. This could happen through the consumption of pesticide residues indirectly via residues in food and water or directly via occupational exposure to pesticides during the production or application of pesticides. Occupational exposure involves dermal absorption of pesticides due to pesticide mixing, loading, disposing or cleaning equipment, along with resident and bystander dermal exposure due to pesticide drift during the pesticide treatment or volatilisation and contact with contaminated surfaces after the treatment (Calliera et al., 2019). There are different routes by which pesticides can enter the human body: dermal, oral, ocular and inhalation (K. Kim et al., 2017).

Pesticide risk is a combination of toxicity and exposure. Therefore, the risk from a specific pesticide depends on the toxicity of the particular product in use, the amount and the form of exposure. However, other factors determine the risk of using pesticides, such as the concentration and intrinsic properties of the active

ingredient in a formulation, the length of exposure, and the route of entry into the human body (Damalas & Koutroubas, 2016).

Dermal exposure

Dermal pesticide exposure is one of the most relevant exposure routes for the agriculture industry. There is a potential for the worker to become exposed to pesticides during mixing and loading, crop application, or equipment clean-up (Anderson & Meade, 2014). Absorption of pesticides through the skin could also occur if agricultural workers enter an area recently sprayed and become directly exposed to foliar pesticide residues. The determinant factors of exposure, apart from the amount of residue on foliage, are the intensity of contact with foliage, the duration of contact, and the possible penetration of residue through the clothes. That explains the good agricultural practice of prohibiting the re-entry of workers to a treated field until enough time has passed to ensure that spray has dried or the airborne pesticide residue has been deposited (Kasiotis et al., 2017). The degree of pesticide toxicity via dermal absorption is influenced by the amount of pesticide reaching the skin and the duration of the exposure along with the innate toxicity of the pesticide active ingredient, the presence of other materials on the skin, temperature and humidity, and the use of personal protective equipment at the time of exposure.

Different pesticide formulations are absorbed differently through the skin; for example, emulsifiable concentrates (EC) are more absorbed than other formulations and absorption is also affected by varying levels of temperature and humidity (MacFarlane et al., 2013);. In addition, pesticide formulations vary

broadly in physicochemical properties and, accordingly, in their capacity to be absorbed through the skin (Beard et al., 2014).

EFSA guidance on dermal absorption described how variation in dermal absorption could be explained by physicochemical properties of the active substance, the type of formulation used, by properties of the skin sample exposed and experimental conditions. Such efforts helped to statistically group the pesticide formulations into four groups to set the dermal absorption default values being used in the regulatory risk assessment based on four categories (1) Primarily organic solvent-based, (2) Primarily water-based/dispersed, (3) Solid, (4) Others (EFSA et al., 2017).

Also, studies concluded that certain areas of the body (the genital areas and ear canal) are more susceptible to pesticide absorption than other areas of the body due to regional variations in the percutaneous absorption in human skin, as shown in Figure 1.1 below (Edwards, 1993; K. Kim et al., 2017).
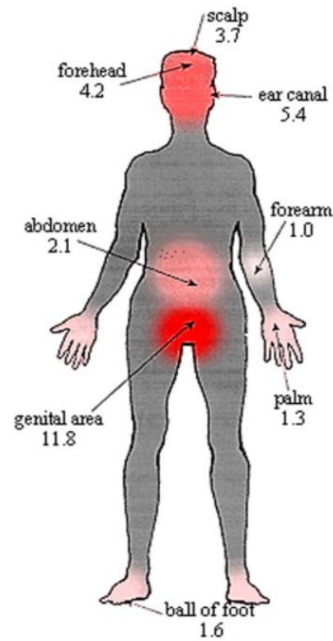
Figure 1.1: The Intensity of dermal exposure to pesticides on different body parts adopted from (Edwards, 1993; K. Kim et al., 2017).

Oral exposure

Most incidences of oral exposure are non-occupational and occur accidentally through exposure to pesticide residues in food, air, smoking, and drinking water, and this generally involves low doses (K. Kim et al., 2017). Due to pesticide use around homes and gardens, exposure to individuals can happen during the pesticide preparation or application or even after the application by exposure within the sprayed areas. Accidental poisoning from pesticides in the home environment is likely due to mishandling, improper use, poor storage, pesticide spillage, or accidentally drinking the pesticides due to keeping them in unlabelled contaminated bottles (EPA, n.d.). Workers handling pesticides or equipment for professional application could also be very vulnerable to pesticide exposure if they do not follow the rules and regulations or wash their hands before eating or smoking (EPA, n.d.).

Respiratory exposure

Respiratory exposure occurs through inhalation of pesticide-contaminated aerosols or particulate matter. The hygroscopicity and mass-mediated aerodynamic diameter of pesticide-containing particles are important in determining their local deposition in the respiratory airways and hence, potentially the site of toxicity (Ye et al., 2013).

In the same context, many studies have identified associations between respiratory symptoms and pesticide exposure. Respiratory symptoms such as coughing, wheezing, and airway inflammation are commonly observed among people working in the pesticide industry (K. Kim et al., 2017). The intensity of respiratory toxicity depends not only on the active ingredient toxicity level but also on the sprayed particle sizes of the pesticides, in general relatively large spray droplets that are produced by conventional application methods are unlikely to cause high toxicity compared to small droplets (Amaral, 2014).

Ocular exposure

Ocular toxicity from pesticide exposure, including the dose-response relationship, has been studied in different animal species. Cholinesterase enzymes have been detected in animal ocular tissue, with evidence of organophosphate-induced inhibition, and pathological effects of pesticides have been observed in the conjunctiva, cornea, lens, retina, and optic nerve. Pesticide exposure has been associated with retinopathy in agricultural workers and wives of farmers who used pesticides (Jaga et al., 2006). The best practice to avoid ocular exposure to pesticides is to follow the workplace's health and safety rules and regulations to minimize exposure incidents. Therefore, protective face

shields or goggles should be worn when spraying pesticides to prevent eye contact, depending on the risk and hazard assessment.

1.1.3 Pesticide policies and regulations

Pesticide regulation is a very complex, professional, and challenging process for nations like the USA, EU, UK, China, and Brazil, the largest agricultural producers in the world; each country has its regulations with separate and distinct regulatory bodies or systems that rule and assess the pesticide uses. The European Union (EU) currently has the most comprehensive and protective pesticide regulations among many major agriculture producers worldwide (Donley, 2019). For plant protection products (PPPs) to be approved in the EU, they need to pass through a process that involves all member states (MS), the European Food Safety Authority (EFSA) and the European Commission (EC). Additionally, members of the public and other parties can provide comments through the process to be considered. Currently, the registration of PPPs in Great Britain and Northern Ireland follows the same regulations and procedures as the EU (HSE, n.d.). As the EU has the most protective and comprehensive system, an active substance can only be approved if it ultimately meets the requirements and conditions stated in the Regulations (EC) 1107/2009 and 396/2005 (European Union, 2009; Harris & Tomerlin, 2002).

1.1.4 Non-dietary residue risk assessment of PPPs workers

Generally, risk assessment means the characterization of the potential adverse health effect of human exposure to environmental hazards. The risk assessment is considered an essential component of pesticide regulation in most pesticide regulatory laws and legislation. The current European risk assessment needed for plant protection products must be carried out for all possible scenarios of

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exposure to exposed groups: operators, workers, residents, and bystanders (Charistou et al., 2022; EFSA, 2014a).

The term operator is defined as any person involved in applying PPPs, including mixing, loading, repairing, or cleaning the machinery after the spraying activities. EFSA guidance also defines workers as" *any persons who, as part of their employment, enter an area that has been treated previously with PPPS or who handle any crop that has been treated with a PPP*" (Charistou et al., 2022; EFSA, 2014a).

Most of the exposure scenarios are covered by the EFSA guidance on the assessment of exposure of operators, workers, residents and bystanders to PPPs along with its calculator spreadsheet. This is except for some cases where the applicant or the assessor should follow an *Ad Hoc*, higher-tier assessment based on studies and evidence (Charistou et al., 2022). These scenarios describe the most possible circumstances of exposure during agricultural activities in conjugation with many parameters such as the amount of active ingredient used per day, the duration of the activity, formulation type, and the presence of some mitigation options to lower the prospected exposure. Such mitigation parameters are optional within the scenario to refine the risk of exposure, such as different levels of personal protective equipment (PPE) or types of machinery (e.g. drift reduction technology) (Charistou et al., 2022; EFSA, 2014a). The guidance also identified those exposure scenarios for the mentioned groups and recommended further research to reduce the uncertainties and overcome the data gaps in the assessment (EFSA, 2014a). In addition, it also included values and measurements, including default values such as the transfer coefficient, breathing rates, exposure duration, and different body weights for the exposed subgroups such as children and adults in each bystander or resident exposure scenario and

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differentiated in the endpoints among each of them to suits the scenarios of exposure.

In these scenarios that include worker exposure, dermal and inhalation are the main routes of exposure during post-application activities. The primary exposure sources are contact with foliage, soil, and possibly dust. Oral exposure may occur secondarily to dermal exposure through hand-to-mouth transfer; however, for workers, potential exposure by this route is generally assumed to be negligible in comparison with that via skin and inhalation (EFSA, 2014a). After the outdoor application of PPPs and after the spray dries, there will be rapid dissipation of the pesticide vapour leading to lower inhalation potential than from indoor treatment (greenhouses). Therefore, worker exposure estimates for the inhalation route after outdoor applications are only necessary in exceptional cases (e.g., for volatile substances). In this case, *an ad* hoc approach would be used (EFSA, 2014a).

When the worker re-enters the crop field after treatment and contacts the crop, a fraction of the remaining residue is dislodged and transferred from the crop to the worker, potentially resulting in dermal exposure. The extent of the exposure is mainly influenced by the intensity of the contact with the crop, the amount of dislodgeable foliar residue (DFR) on the crop, and the duration of the contact (T).

Clothing and PPE provide the worker with different levels of protection because only a fraction of the transferred residues migrates through the clothing or PPE and reaches the worker's skin. Only a fraction of the substance that reaches the skin will penetrate the skin and be absorbed into the worker's body. Therefore, the worker's risk assessment may be mitigated by instructing the workers to wear gloves for re-entry tasks on the PPP label (Butler Ellis et al., 2017; EFSA, 2014a). Ultimately, dermal exposure from contact with residues on foliage should be

estimated as per the equation below by quantifying the Dislodgeable Foliar Residue (DFR), the transfer coefficient (TC), and the task duration (T) (Charistou et al., 2022; EFSA, 2014a).

$$\boldsymbol{DE}[\textbf{mg a. s./day}] = DFR[\upmu\textbf{g}/\boldsymbol{cm^2}] \times \textbf{TC}[\boldsymbol{cm^2}/\textbf{h}] \times \textbf{T}[\textbf{h/day}]/\textbf{1000} \qquad \textit{Equ 1.1}$$

- Dermal exposure (DE)

The dermal exposure (DE) estimates the naked worker exposure and should be multiplied by a dermal absorption factor derived from the toxicological assessment to account for Intra and inter-species variations.

- Transfer coefficient (TC)

From the equation above, Transfer coefficient " *is a term used to describe the ratio of post-application worker exposure to the exposure time and the dislodgeable residue of the surface contacted by the worker, the derivation and use of transfer coefficients began following the development of a standardized foliar residue sampling methodology*" (Iwata et al., 1977). Generally, the transfer of residues from the treated plants to the skin or the human's clothes is considered by the transfer coefficient value used in the assessment regardless of the product applied, the level of exposure, and the time of exposure. It all depends on the type of activity the worker is performing and the duration of such activity (M. Dong & Beauvais, 2013; EFSA, 2014a).

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$$TC \ [cm^2/h] = PDE \ [mg/h]/DFR[mg/cm^2] \qquad \textit{Equ 1.2}$$

TC = Transfer coefficient

PDE= Potential Dermal Exposure

DFR= Dislodgeable Foliar pesticides Residues

Because of the agriculture diversity and different agricultural and cultivation practices, it is impossible to conduct exposure studies for all possible crop/crop growth stage/activity combinations to calculate the TC for each crop or scenario. Therefore, the Agriculture Re-entry Task Force (ARTF), a consortium of 31 agricultural chemical companies in the USA, was the first to generate a database that defines the TC for all crops/activity scenarios (ARTF, 2014; Charistou et al., 2022).

The ARTF worked with the authorities in North America (California, Canada, USDA, and the US EPA) to adopt a method of clustering crops, crop growth stages, and post-application activities into groups that are expected to result in comparable exposure. This was then reviewed by Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in 2008 and is currently used for the American pesticide risk assessment (EPA, 2017). Yet, the ARTF detailed data have not been published nor submitted to EFSA; therefore, EFSA considered this data to be indicative. Hence, its use is limited to a comparative check of the more restricted data. In the same context and until recently, the EFSA working group decided to apply TC from the EPA data due to the limitations of the data available and encouraged data generation in the future to allow for future moderation of TC values (EFSA, 2014c).

Furthermore, these values are set into different levels according to the level of protection used by workers as refinements that suit each scenario. Such TC values may be extrapolated to other re-entry scenarios, where the intensity and duration of contact with the foliage are judged to be similar (EFSA, 2014a). Recently and due to EFSA's belief that without access to the supporting data, it is impossible to adequately validate the information nor achieve the level of transparency required by EFSA's policy. The most current EFSA guidance on non-dietary exposure to pesticides included seven sets of TC recommendations, which are mainly 75th percentile-based values to guarantee protection.

- Activity time (T)

The time in the above equation is meant to be the activity time for the involved workers in the field performing any tasks; there are two default values according to EFSA guidance for two major scenarios. Two hours for crop inspection or irrigation and eight hours for harvesting and maintenance activities (Charistou et al., 2022; EFSA, 2014a; EPA, 2017).

1.1.5 Dislodgeable foliar pesticide residues (DFR)

Initially, the term dislodgeable foliar residue was first indicated and used by (Gunther et al., 1973) when reporting that some pruners, thinners, and pickers became ill after working in Californian fields where crops (citrus, grape vineyards, peach orchards, cotton, and tobacco) had had commercial applications of organophosphate insecticides. Despite the well-established fact that most organophosphate pesticide deposits can penetrate quickly into clean leaf surfaces. The formulation ingredient associated with the active ingredient, including the solid components of wettable powder (WP) formulation, is said to

mediate the full migration of the pesticide into the waxy and other subsurface layers of the foliage by strong sorptive action. As a result, the pesticides are left over on the surfaces for longer and are accounted to be residues rather than deposits (Whitmyre et al., 2005). Such residues are transferable to workers via dislodging from worker activity and transfer directly to the skin or clothes. Therefore, the dislodgeable foliar pesticide residue technique and definition were set as guidance for assessing, minimising, and mitigating the associated risk (Gunther et al., 1973).

There are different similar definitions of DFR in the literature; the historical and first definition was introduced by Iwata (1977), who described DFR as "*The amount of residues present on leaves' surfaces that can be washed from the leaf surface and DFRs are measured by using a weak detergent solution followed by a liquid-liquid extraction*" (Iwata et al., 1977). Another definition was then introduced by Korpalski (2005) "*as the amount of pesticide residue that can be dislodged from the two-sided foliar surface of a plant during a well-defined procedure. It is used together with worker exposure determinations to calculate transfer coefficients for workers re-entering treated crops*" (Korpalski et al., 2005).

Another definition was mentioned in the International Union of Pure and Applied Chemistry (IUPAC) glossary of terms related to pesticides: "*the portion of a pesticide residue on treated vegetation that is readily removable and may be used as an index for risk to farm workers. It is generally measured by the residue removed when leaf discs are shaken briefly in the water*" (Stephenson et al., 2006).

Historically, in the absence of DFR data from published studies and literature, the applied application rate divided by the leaf area index of the crop divided by the ground surface area on which the crop is growing was used to provide an estimate

of the foliar residue on crops. Such a method assumes a uniform residue distribution across the cultivated crop (Bates, 1990). Because only a portion of the total residue estimated using the leaf area index approach is actually dislodgeable to workers and not all the residue on the leaf, this kind of DFR estimation is concluded to be unrealistic and overestimating (Franklin & Worgan, 2005).

The only DFR method known currently is the one published in the EPA occupational and residential exposure test guidelines (OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation). This method is based on the method developed by Gunther *et al*. (1973), which stated that "*at present, these techniques are the most suitable for foliar residues*". Besides the available guidance for the determination of DFR issued by the Health and Safety report (HS 1600) published by California EPA, revised in 2002 (Edmiston et al., 2002). This EPA Guideline describes the technique and sampling methods used to quantify DFR. The technique (OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation) is based on this definition, "DFRs are the amount of chemical residues deposited onto the leaf surface that has not been absorbed into the leaf or dissipated from the surface, and that can be dislodged by shaking leaf samples in a detergent solution (Gunther *et al.,* 1973); the guidelines intended to meet testing requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) according to the Test Guidelines for Pesticides and Toxic Substances (EPA, 2016).

Whilst currently, there is no harmonized method or guideline for conducting the DFR studies as a part of the PPPs regulatory requirements within the European Union, the UK, or the OECD, the same method is currently used by most of the pesticide regulators with very limited variations. In 2020, the German regulatory

authority (BFR) endorsed and approved with some annotations the use of the EPA 2002 guidance (OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation) (BfR, 2020). Recently, EFSA published recommendations for designing, conducting, and assessing higher-tier field studies, including DFR studies, in Appendix J of the 2022 guidance (Charistou et al., 2022). The recommendation is to follow the sampling parameters first set and developed by Iwata et al. (1977) (Iwata et al., 1977). In addition to many other recommendations for the analysis, data interpretation, and study conditions (Charistou et al., 2022). The DFR technique is summarised in text box 1.1 below based on the original method developed by Iwata et al. (1977) and endorsed in many published sources (Charistou et al., 2022; EPA, 2017; Iwata et al., 1977; US EPA, 2016).

The targeted crop is to be sprayed with the pesticide questioned for the dislodgeable foliar pesticide residue quantification using the higher application rate recommended on the label. Treated samples (leaf discs) will be collected using a leaf punch sampler at 0 days before the first application (DBA1) and 0 days after the first application (DAA1) along with sample collections at 0 days before and after any following applications depending on the nature of the treatment and the number of applications recommend. Samples at 0, 8h, 24h, 48h, 3, 5, 7, 10, and 14 days or more after the last application (DALA) will be collected while untreated samples (leaf discs) will be collected at 0 DBA1 and 14 days after the last application (DALA) to act as a control.

After samples collection (40 leaf discs; 10 cm^2 /disc), the leaf disk samples will undergo dislodging procedure within 4 hours of sampling using 2 x 100 mL of washing solution (0.01% (w/v) Aerosol OT) and then samples will be shaken at approximately 200 cycles per minute for 10 minutes on a reciprocating platform shaker. The volume of each wash will be recorded and then 200 mL of acetonitrile will be added. Samples are then stored frozen until analysis and the leaf discs are discarded. If taking leaf punches for some crops is impossible or very hard thus, taking the whole leaf of these plants using forceps is recommended.

1.1.6 Re-entry interval (REIs) and dissipation time (DT$_{50}$)

The re-entry interval (REI); also known as restricted entry interval or re-entry time; is the minimum amount of time that must pass between the time a pesticide was applied to an area or crop and the time that people can enter that area without protective clothing and equipment (CCOHS, n.d.).

In the EFSA guidance 2022, a safe re-entry interval is defined as *"the specific time point post-application, after which the worker exposure levels calculated for the relevant re-entry tasks are lower than the Acceptable operator exposure level AOEL considering the different clothing and PPE cases and depending on the TC availability"* (Charistou et al., 2022). A re-entry interval can be established to allow the pesticide to degrade to levels that do not cause an unacceptable risk to workers (FAO, n.d.). Nevertheless, the REI is not mandatory in the EU regulations and is only set where immediate re-entry is not acceptable. Additionally, the current exposure assessment methodology takes into account principal parameters such as the dissipation time of the active substance in the PPPs (DT$_{50}$), the transfer coefficient (TC) (from crop to worker) and the dermal absorption (DA) along with (DFR) (EFSA, 2014a; Markantonis et al., 2018).

Calculation of the re-entry intervals and dissipation time:

REIs are established by determining the time at which the daily exposure for given work activity and DFR level is equal to an established safe level for the pesticide-active substance in question. A safe exposure for a given pesticide is estimated theoretically by dividing an appropriate toxicological *no observed adverse effect level* (NOAEL) by a safety or uncertainty factor (usually 100) when the NOAEL is from an animal study and depending on the severity of the endpoint. A higher safety factor may be used (Franklin & Worgan, 2005).

In the USA, during the risk assessment, the applicant or the registrar will calculate the re-entry interval for a specific PPP sprayed on a particular crop using the residue dissipation studies available. Then, the applicant will review the dissipation of the residues on specific crops for a period of time to indicate at which time the residue will decline to an acceptable Re-entry (*Rs*) level that will not cause harm to workers while performing specific tasks in the treated area; (Worgan & Rozario, 1995).

During some re-entry studies, a TC values calculation could be done to a crop treated with the pesticide formulation, and at designated intervals after application, workers are sent into the field to conduct specific work tasks (e.g., pruning, thinning, harvesting, etc.). At each re-entry time corresponding to particular work activity, foliar pesticide residue is measured by sampling the leaves in the treated area around the activity and washing them off in the laboratory to estimate the DFR. In addition, pesticide residues caught by the worker dosimetry clothes are sent for analysis to indicate the transfer coefficient (TC) that represents each body part and the whole body of the worker. Typically, the worker does not enter a treated field immediately after the application, and that is why it is necessary to experimentally determine the decay rate of the residues on the leaves to estimate the DFR at the anticipated entry time (Franklin & Worgan, 2005). This decay rate is affected by many factors, including the chemical nature of the pesticides, the degree of uptake by the crop, the characteristics of the foliage, and climatic conditions such as sunlight, rain, wind, and temperature (Bates, 1990; Charistou et al., 2022; H. Choi et al., 2013; Ebeling, 1963; Willis & McDowell, 1987). The correlation between the residues collected and the time in a log relationship will then be drawn, which enables calculating

the accurate re-entry interval (*Ts)* (see Figures 1.2 and 1.3) (Worgan & Rozario, 1995).
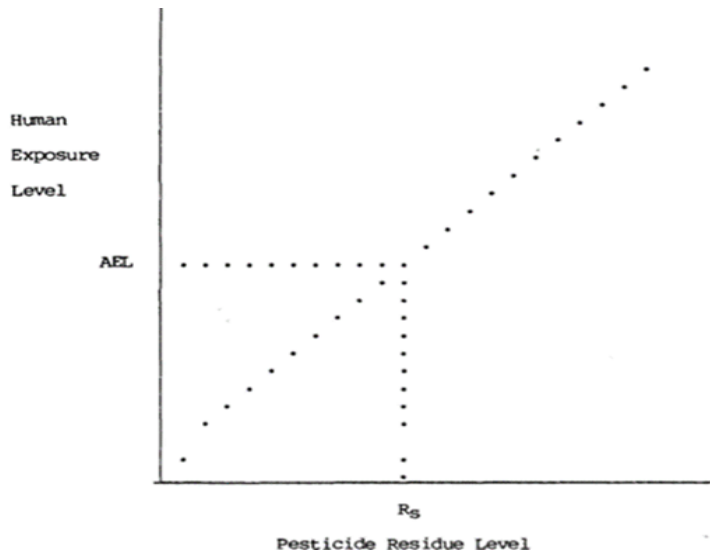
Figure 1.2: Estimation of exposure against the increasing level of residues.
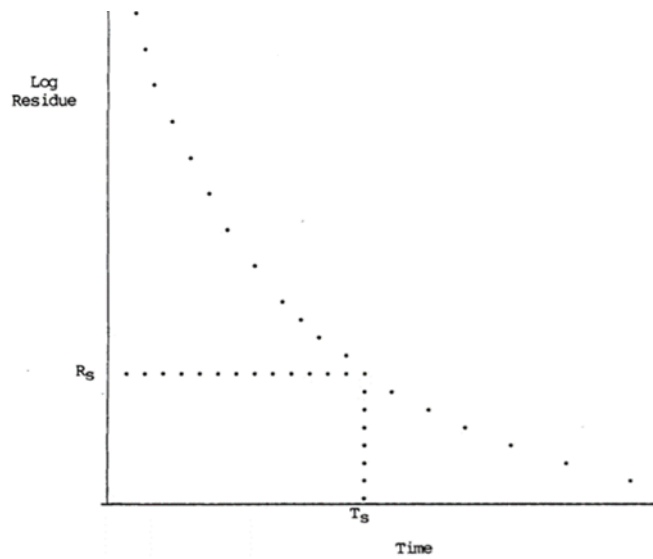
Figure 1.3: The residue dissipation curve.

Recently, EFSA guidance 2022 introduced an equation to estimate the safe re-entry interval, with or without workwear and or /gloves, to the online calculator

to facilitate the calculation for the safe re-entry into treated crops as per Equation 1.3 below.

$$t = \left(ln\left((PDE * 100{,}000/DFR0 * TC * T * MAF)\right)\right) * (-1/K) \qquad \textbf{\textit{Equ 1.3}}$$

Where,

- t= safe re-entry interval (days)
- PDE= potential dermal exposure (mg a.s/day)
- DFR0= initial DFR just after application, assuming that no dissipation time point (μg cm^2)
- MAF= multiple application factor
- K= natural logarithm of 2 divided by the half-life-ln (2)/DT$_{50}$ (rate constant)
- TC= transfer coefficient (cm^2/h)
- T= task duration (h/day)

The current European risk assessment uses the *Acceptable Operator Exposure Level* (AOEL) as a reference value against which dietary pesticide exposure is currently assessed. AOEL defines the level of daily exposure throughout the spraying season at or below which no adverse systemic health effect would be expected. The AOEL is derived from the correction of the *No Observed Effect Level* (NOEL) often by using a safety factor of (100) because the NOEL is often derived from toxicological studies in which animals were dosed for at least 90 days and to allow for the inter and intra-species variabilities. (EFSA, 2014d).

The regulatory risk assessment follows a tiered approach for the assessment of re-entry exposure, where *Tier 1* involves using all the default values proposed by

EFSA to give a predicted value of the expected re-entry exposure. If this value is within the AOEL, no further action is required, and approval could be granted. Alternatively, *Tier 2* involves the use of PPE to refine the value gained in the first tier. Finally, further tiers are advised if this value is still beyond the AOEL. This could include using product-specific data from re-entry exposure studies such as DFR studies or REIs as a mitigation measure. This provides robust exposure data for granting the PPPs' approval (van Hemmen et al., 2006).

1.2 Dislodgeable foliar pesticide residue data between gaps and limitations

According to the most recent EFSA guidance 2022, Regulation (EC) No 1107/2009, the risk assessment for PPPs must be carried out for all scenarios of exposure for operators, workers, residents, and bystanders that can be expected to occur as a consequence of the proposed uses of a PPP. Most of these scenarios will often fall into a category for which standardised first-tier exposure assessment should be applied using the guidance. While for those scenarios not covered in the first-tier assessment, the applicant may also use an ad hoc, higher-tier exposure assessment (Charistou et al., 2022; EFSA, 2014a).

The guidance also identifies those scenarios for which exposure estimates are least satisfactory with data gaps and makes recommendations for further research that would reduce current uncertainties. For those related to worker exposure, the guidance made it clear by reporting the following statement *"Available data are not reliable enough to proceed with the acute exposure assessment (in particular concerning the DFR values)." The ad hoc EFSA working group (hereafter "WoG") strongly recommended further collection and production of data on specific TC and DFR values to produce more realistic exposure assessments* (Charistou et al., 2022; EFSA, 2014a).

To be more specific, in the absence of experimentally determined DFR data, the applicant or the risk assessor should use the EFSA default value for the DFR, which is 3 µg active substance (a.s.)/cm^2 of foliage/kg a.s. applied/ha; the value provided is regarded as highly conservative (Charistou et al., 2022; EFSA, 2014a; Kluxen et al., 2021). EFSA 2014 guidance introduced several changes in the generic DFR default values, for example, the default DFR of 1 µg cm^{-2} per Kg a.i. ha^{-1} was substantially increased to the current 3 µg cm^{-2} per Kg a.i. ha^{-1} (Krebs et al., 2000). The current DFR default value comes from the EUROPOEM II project (van Hemmen, 2001). The term EUROPOEM is an abbreviation for a project called "a concentrated action to construct generic databases of re-entry and bystander exposures to plant protection products and develop predictive models", which was compiled at that time through the available scientific literature and the authority-generated data without using proprietary studies. The database consists of 55 studies from 1958 to 1999, including 46 active substances and 28 crop types. EUROPOEM II suggested that for a highly conservative assessment of the initial DFR (DFR0), in a first-tier assessment, 3 µg cm^{-2} active substance on foliage, which is about the 90th percentile of the distribution, can be taken as a default value when no relevant/appropriate data on leaf area index can be used to estimate worker dermal exposure. However, according to the publication, the complete list of studies was not presented "due to the size", which makes the derivation of the DFR value non-transparent (Kluxen et al., 2021).

For that reason, a literature review was done by Lewis et al. (2017), which concluded 27 more studies of acceptable quality criteria set by the authors for inclusions and exclusion of the studies in that report. The main purpose of such a review was to collate all reliable data related to DFR from the literature, with no further analysis intended for such a review. Additionally, the results of a literature review performed during the BROWSE project were also considered and revealed

35 more studies of accepted quality (Charistou et al., 2022; Doan Ngoc, 2014; Lewis & Tzilivakis, 2017b)

The EFSA working group assessed the collated data in the review, and regardless that some of the studies have already been taken into account during the 2014 EFSA guidance on worker exposure and default values, the conclusion couldn't conclude any direct comparison or links between the studies. The reason for such a conclusion was due to the high variability in reported DFR; most of the studies were conducted on turf, with a focus on golf and other recreational activities (Charistou et al., 2022).

Nevertheless, the collated data confirms the complexity of the involved factors, such as the Physico-chemical properties of the chemical and the co-formulants, properties of the formulation, application techniques, cultivation, weather conditions, crop-specific factors, and many more. These factors could influence the level of DFR. In conclusion, there was no single parameter that could be considered a major driver for the level of DFR in the field (Charistou et al., 2022).

In Europe, to minimize the uncertainties and ease the registration procedures, an updated guidance document on work-sharing in the northern zone in the authorization of plant protection products was published in June 2019 (European Commission, 2020). The guidance included criteria for the DFR studies that could be submitted by the registrants where the study should cover all the intended uses (GAP). This includes the application rate, number of applications, application efficiency, equipment, environmental conditions (i.e., appropriate time of year and geographic location), crop type, physical and chemical properties of the applied PPP, etc. The same approach was taken by the EFSA working group when it recently published the guidance on the assessment of exposure of operators, workers, residents, and bystanders (Charistou et al., 2022). EFSA highlighted that

the higher tier experimental DFR should follow the initial guidelines. These guidelines are of the EPA (e.g. US EPA OPPTS Guidelines 875.2000; 875.2100, guidance for the determination of dislodgeable foliar residue) and strictly conditioned the study to follow the good laboratory standards (GLP) (Charistou et al., 2022; European Commission, 2020).

Despite the presence of US EPA OPPTS guidelines 875.2000; 875.2100; guidance for the determination of DFR; there is no harmonised method of conducting DFR studies throughout the literature, and there are some variabilities in the methodology of the DFR (EPA, 2009). This has also been recognised by the EFSA working group, and in response, a non-exhaustive list of test guidelines has been proposed in appendix J of the EFSA 2022 guidance for the DFR field studies (Charistou et al., 2022). The DFR default value decided by EFSA (3 μg active substance/cm^2 of foliage/kg a.s. applied/ha) was regarded as highly conservative, unlike many other countries (EFSA, 2014c). In countries such as the USA and Canada, the regulatory authorities are assuming to represent a DFR default value protective of health without being unreasonably conservative. The registration authorities decided a 25% of the application rate to represent the initial DFR with a 10% dissipation or decay rate per day in the absence of chemical-specific DFR studies according to the most updated EPA advisory council policies and Canadian Pest Management Regulatory Agency (PMRA) (H. Choi et al., 2013; PMRA, 2014).

In the same context, the technical report published by EFSA 2014 discussing the outcome of the public consultation on the latest published EFSA guidance 2014 regulation (EC) No 1107/2009 pointed to the gaps present in the current system along with the EFSA comments on them. The public comments raised several points considered as data gaps and limitations. Concerning public comments related to DFR and TC values, they identified that the only parameter associated

with the plant is the surface of the foliage, and the guidance did not mention any other criteria that could play a role, like the type of leaf and its morphological or physical characteristics: smooth, rough, toothed... EFSA commented on that very clearly *"No data on these parameters is currently available to the WoG."* This indicates the need for more advanced data to study the behaviour of the foliar residue on different leaf textures and/or in conjunction with the environmental conditions (EFSA, 2014c).

Another data gap addressed by the public was the suitability of using USA DFR and TC data (ARTF data) which is not well published for public review. EFSA WoG confirmed this limitation of ARTF data to the public. However, EFSA decided to make use of such data to fill the gaps, providing that ARTF used the most conservative data available (EFSA, 2014c). Another point of applied conservatism in the guidance is using 30 days as a default value of the decay or dissipation time for the PPPs if there is no available experimental data. The value comes from a published data set in 1987 by (Willis & McDowell, 1987). Such data set is old and lacks the currently used active substances in the market. The public consultation report considered this proposal overly conservative when used with the high-protective default DFR value, high transfer coefficients, conservative exposure periods, highest dermal absorption values, and low body weight leading to predicted worker assessments which are considered to be unrealistic (EFSA, 2014c).

Despite the efforts that different scientists and researchers have made to collate the available data in the literature, the DT_{50} decline data available is still problematic with many variabilities observed (Charistou et al., 2022). First, the data collated by Frantke and Juraske for 346 pesticides using 811 published studies in 2013 was considered, followed by the Pesticide Properties Database

(PPDB) (Lewis & Tzilivakis, 2017a). PPDP is the extensive collative database available to date and used worldwide after being endorsed by many regulatory and scientific bodies. Unlike the previous data available, it is managed and maintained on an ongoing basis to assist regulatory bodies along with the risk assessors. The reason for such variability in the data is not just due to the physicochemical behaviour of the active substance but also to the type of plant leaf, texture, and whether the collected data depends on the foliage residues or other parts of the plants. Also, the data variability is due to differences in the commercial formulations of the pesticides used and many other environmental factors surrounding the data collection (Lewis & Tzilivakis, 2017a). A different variability and data uncertainty level is observed during the interval between the application and the sample collection, where several events could take place, which could affect the DFR significantly. These events, such as rainfall, irrigation or during that time, the weather could unexpectedly become wet or humid, and this adds more uncertainty as to how comparable the data is and to what degree the sample chosen for the DFR study is genuinely representative (M. Dong & Beauvais, 2013).

Data collected from DFR studies may also vary in the same field according to variations in climatic conditions. This could be during or before the residue collection as the wind may cause drift of the spray droplets during the spray, causing changes in the amount of residues left over on the surface from row to row. In addition, changes in the temperature and humidity may occur on the same day during the experiment resulting in possible changes in the residue intensity. As a result of the above data gaps, EFSA clearly stated that the available guidance should be reviewed periodically and amended whenever available data become available and appropriate, with the strong recommendation from the working group for further collection and production of reliable data on specific

TC and DFR values to produce more realistic exposure assessments (Charistou et al., 2022; EFSA, 2014a).

1.3 Industry challenge and perspective change

In the literature, various studies have come to light assessing dermal exposure to pesticides during the re-entry of workers, including DFR studies (Suganthi et al., 2008). However, it is clear that studies that will address and include critical factors that may be important to the concentration of DFR on the plant, such as crop type, pesticide formulations, the timing of application and sampling, leaf texture and shape, environmental conditions such as temperature and humidity are needed to understand the behaviour of the DFR. This would improve the knowledge of the crop protection industry, regulatory bodies, stakeholders, and environmental scientists, leading to more robust regulation of pesticide use in a way that does not compromise human or environmental safety.

In general, according to the PPPs registration requirements in the EU or the USA, GLP standards for conducting DFR studies are essential, especially to refine the regulatory risk assessment, If the default values used are conservatively associated with the risk with the actual use. Although monitoring residues on plants is considered the gold standard for evaluating pesticide safety, experiments are generally expensive, seasonal, and time-consuming (BfR, 2020). On the other hand, extrapolation between DFR studies is often not allowed by regulatory authorities (i.e., EFSA), and its data limitation is still considered a gap in residue science. EFSA acknowledged this gap in its latest guidance on non-dietary risk assessment and recommended generating more good quality DFR

and TC experimental data to identify and conclude the possibility of extrapolating results between crops and formulations (Charistou et al., 2022).

Therefore, developing a precise laboratory technique to investigate these factors that may affect DFR is crucial, especially with the high costs and seasonal nature of DFR field studies. Investigating these factors that might affect the DFR is expected to influence the PPPs registration significantly. Furthermore, linking the studies based on scientific evidence enabling merging between DFR studies will facilitate the registration based on an actual correlation between the factors and the level of residues without overestimating or underestimating the associated risk.

The prospected findings of the correlation between these factors and the residues will also allow the regulatory bodies to make a fast and robust decision on the possibility of extrapolating between different studies when needed. Such extrapolation could include different formulations, crops, and environmental conditions, saving time, resources and effort. Moreover, the expected proven influence of the factors would enable other researchers in the field of pesticide residue science to build up knowledge on the nature of these residues on plant foliage, besides helping further research in combining the factors that influence DFR to explore the effect of those factors in combination.

Furthermore, using the abundant dietary dissipation data available in the open literature and correlating it with the available DFR decline data could conclude the use of dietary decline data as a surrogate for the DFR decline data. On such occasions, the dietary dissipation values could be helpful if there is a lack of DFR data and there is no opportunity to generate any (perhaps due to regulatory timelines) or the dissipation behaviour of the active substance is not well known. In these instances, using dietary residue decline as a reference to determine the

appropriate length for a DFR study or even under some circumstances to allow the limitation of the DFR study to measure only the dislodgeable fraction just after application (DFR0) could be possible. Such an approach could save time and resources without carrying out long-tailed DFR studies while adhering to the precautionary principle. This would benefit the agrochemical industry and the regulatory bodies and ease the registration process accordingly.

Chapter 2 : Literature Review Methodology and Findings

2.1 Introduction

A systematic review in research is an organised, structured approach adopted to identify, gather and synthesise all the documented evidence that could answer the research question. According to the project aims and objectives, there are usually eligibility and quality criteria associated with this documented evidence to be included or excluded from the review process. The reviewing process is known as the "review protocol". It describes in detail the approach that was implemented to search for the most relevant materials and evaluate them against some predefined criteria that suit the project objectives. A research question is also a vital part of the systematic review. It should be formulated to define and answer the research aim and answering such questions in a conclusion-based approach should be the purpose of the planned, systematic review.

This PhD project is designated mainly to identify critical factors that influence the dislodgeable foliar pesticide residues (DFR) and to get these factors studied or explored; different questions have been formulated around it, which are:

- Are there already any clear pieces of evidence in the literature on the methodology of investigating factors that may affect DFR?

- Are there any current proven correlations between the dietary and non-dietary (DFR) decline data?

- Are there any published laboratory studies or experiments in place that investigated the effect of different factors that could influence the DFR (i.e., different leaf textures, pesticide formulation types, co-formulants, and the physicochemical properties of pesticides)?

The literature review included data from all over the world and was not restricted to the United Kingdom. Any published paper discussing factors that may affect DFR has been collected with no limitation to specific crops, leaf type, meteorological conditions, or other factors that might be present in the studies. The presence of any evidence that proves existing factors that may affect DFR and /or any correlation between the dietary residue decline data and the DFR decline data was investigated. Besides, collecting any pre-work related to laboratory studies that were designed to study potential factors that may affect DFR.

All peer-reviewed data published in reputable journals or governmental authorities' repositories were included. This included any related data published between 1950 till 2022. The search was restricted to the English language and limited to manuscripts published in English or any of those written in other languages but had at least an English abstract available. Also, any published article before 1950 was disregarded. Any collected studies related to the review were considered regardless of the laboratory standards used in these studies, whether it follows the Good Laboratory Practice (GLP) or non-GLP standards.

2.2 Literature review methodology

The search methodology was performed using the University of Hertfordshire Studynet Online Library, which provides access to several library databases along with the continuous check of any announcements or publications made available

by the worldwide pesticide regulatory authorities and their journals (i.e., EFSA, EPA websites, Canadian Pest Management Regulatory Agency (PMRA), etc.). Studynet provides instant access to many published papers, study summaries, and databases. In addition, in case of instant access was not available, papers and articles were ordered for electronic delivery within 48 hours from worldwide sources.

Specific parameters were used to select and identify the relevant databases for the scooping technique. This has been achieved by checking the manuscripts and data from the search in every database used and assessing them based on some criteria. These retrieved resources were not counted directly to be included in the literature review but were tested on how efficient they were in answering the literature review questions using the below criteria:

1-   How many articles or manuscripts were retrieved in the first 10 pages of the search for each database using logical keywords?

2-   The key articles appear to be related to the aim of the literature review and how efficiently they answer the question.

3-   The duplication and overlap between articles retrieved from each search using the exact keywords.

Based on the above, databases that covered most of the related journals and articles were chosen. Reference snowballing strategy was performed using the reference list in relevant publications (including review and pre-print articles) to help identify other suitable related work. Also, the search included the identification of key researchers, research groups, and organizations and checking their online publication resources for any updates. Furthermore, the literature review included searching for articles using different electronic

databases such as PubMed, Medline, Cochrane Library, Web of Science, Science Direct, Scopus, EFSA and EPA Journals, Google Scholar, Journal of Regulatory Toxicology and Pharmacology, and the Journal of Toxicology.

All the literature search results using the chosen databases were individually downloaded into the Endnote and Mendeley software. Exact article duplicates were removed from this software, and all the articles were stored in the online clouds as a backup. Data extraction was done on Excel documents, while Mendeley software was used to keep track of references, insert the citation in the whole thesis, and create the final bibliography.

After testing the logical combination of search terms, different keywords and phrases were used to perform trial searches, using titles and keywords from retrieved literature to identify additional words. In addition, using plurals and singular of the selected words, wildcards, etc., Table 2.1 below states the most useable keywords used in the review protocol. All these phrases and keywords were used single and in combination with one another to retrieve the most comprehensive relevance of the articles.

Table 2.1: Keywords used in the literature review.

words	Phrases
Dislodgeable	Dislodgeable Foliar Pesticide Residue
Foliar	Plant Protection Products
Residue	Pesticide residue
DFR	Risk assessment
Pesticides	Non-dietary exposure
Herbicides	Dietary exposure
Fungicides	Pesticide half-life
Insecticides	
Plant protection products (PPPs)	
dissipation	
Retention	
Leaf texture	
Worker	
DT_{50}	

Table 2.1 lists the most used keywords and phrases alone or in conjunction with each other that retrieved the most relevant sources that answered the review question.

2.3   Literature review findings

The literature review findings were sectioned into three subsections that answer the review's main questions. Despite that there has been an overlap between the findings that could answer the review questions, it was evident that there was a scarcity in the DFR data available in the literature.

2.3.1   Findings on the methodology of investigating factors that may affect DFR

Despite the solid recommendation from the EFSA to generate more data related to the DFR and the clear call to investigate the influencing factors, there is no shred of evidence in the literature on any adopted methodology to study these factors. Notwithstanding the OPPTS Guidelines 875.2000 and 875.2100, there is still no harmonised method for conducting DFR studies throughout the open literature. Most studies follow the previously mentioned method with minor differences (BfR, 2020).

The EFSA working group ("WoG") strongly recommended further collection and production of data on specific (TC) and (DFR) values to produce more realistic exposure assessments (EFSA, 2014a). The same recommendation has been made recently in The latest EFSA guidance (2022) on the assessment of exposure of operators, workers, residents, and bystanders by acknowledging the need for further collection/production of good quality DFR to enable the evaluation of the factors that could influence the DFR (Charistou et al., 2022). In addition, the same guidance highlighted some parameters for the acceptability of the field trail DFR studies. This has been added to create a sort of homogeneity among all the prospected DFR trials in the future (Charistou et al., 2022). The scarcity of the DFR data in the literature could be due to the seasonal nature of the studies, the expenses of these field studies and the data's privacy.

2.3.2 Findings on the correlations between the dietary and non-dietary (DFR) decline data

Pesticide residue studies have always been an essential components of the pesticide registration process. These studies support the dietary risk assessment and ensure that the residues found in the edible parts of the plants are below the acceptable limits. Recently, there has been a surge of new data from dietary residue studies published in peer-reviewed literature. Fantke & Juraske (2013) collated a dataset of 346 pesticides. This dataset, as well as that gathered and reported by Wills & McDowell (1987), was updated by Lewis & Tzilivakis (2017) and called Pesticide Properties Database (PPDP) (Fantke & Juraske, 2013; Lewis & Tzilivakis, 2017b; Willis & McDowell, 1987). However, the latter is considered comprehensive and the most up-to-date dataset available, the majority of this DT_{50} data is associated with total residues (as used in dietary risk assessments) and not necessarily the DFR fraction.

Recently EFSA WoG decided to consider the PPDB for possible refinement of the default DT_{50} after rechecking and assessing the available data from the gathered literature against quality criteria (i.e., crop growth stages, GLP status, sampling strategy, number of replicates, limit of quantification (LOQ), and limit of detection (LOD), calculation of the DT_{50}, etc.). The review of this data revealed 32 publications to be reliable; from this data, different uncertainties were flagged. It was then recommended to use high-quality data submitted for regulatory purposes to refine the DT_{50} default value rather than depending on publicly available data from the literature (Charistou et al., 2022).

Nevertheless, to date and from this systematic review conducted, there was no evidence supporting the relationship between the decline rate of dietary and non-dietary pesticide residue. The reason for the non-existence of such correlation could be due to the limited number of DFR studies found in the literature to draw such a conclusion.

If it existed, this correlation could be used to promote using the dietary half-live as a conservative surrogate to the DFR half-lives in the case of the non-availability of the DFR field trial data. This approach could save time and resources without carrying out long-tailed DFR studies whilst still adhering to the precautionary principle of the risk assessment.

2.3.3 Findings on the factors that influence the DFR

From the systematic review, despite the limitation in the DRR studies available, there were several examples that shed light on the presence of some evidence on factors that could affect DFR. Therefore, these sources were collated together, and an interpretation of the existing evidence was stated below.

EFSA Guidance 2014 pointed to the factors that might affect the DFR of plant protection products (PPPs). For example, the report states that "*the amount of residue on foliage depends on several factors, including the application rate, application efficiency (how much reaches and left over on the target leaves), crop type, and the amount of foliage (leaf area index). Also, "Dissipation of residues on crop foliage over time depends on the physical and chemical properties of the applied PPP, and also on environmental conditions*". These are some of the factors that may affect DFR, and those have been mentioned in many articles in the open literature with no extensive investigation (EFSA, 2014a).

In addition to the above, (Iwata, 1980a) reported that the amount of foliar residue will differ in different geographical areas. The amount will vary with the nature of the crop, the irrigation method, the amount of rainfall, air temperature, wind movement, and or the sunlight intensity, affecting pesticide vaporisation and photodegradation accordingly (Iwata, 1980a).

In the same context, The Agriculture Research Taskforce (ARTF) critical findings during the statistical analysis of 196 residue studies are that the initial DFRs significantly appear to be proportional to the application rate even though the quantity of data is insufficient to draw a firm conclusion due to the high variability existing. In addition, ARTF reported that the DFR might not be consistently proportional to the application rate for all chemicals and crop combinations studied; for instance, the DFR appeared proportional to the application rate in cabbage but not in lettuce. Therefore, the application rate alone is clearly insufficient to predict the initial DFR (Bruce & Korpalski, 2008). These findings align with (Van Drooge et al., 2001), where the application rate has not influenced DFR studies on cucumber when sprayed with bupirimate pesticides used to control Mildew disease.

In another study, the formulation type and formulation ingredients (co-formulants) have been clearly reported by Gunther& Blinn (1955) to affect DFR. The report states that *"the formulation ingredient associated with the active ingredient including the solid components of WP formulation may mediate the full migration of the pesticide into the waxy and other subsurface layers of the foliage by strong sorbtive action and as a result of that, the pesticides left over on the surfaces for a longer time is accounted to be residues rather than deposits"* (Gunther & Blinn, 1955b).

As a result of the historical concept of the wettable powder (WP) formulation considered the worst-case scenario of the end-product used, the EPA recommended considering the range of formulations available for the active ingredient during the registration being assessed. Thus, data may be required for each formulation type, and using the WP end product formulation on its maximum application rate with minimum dilution is recommended for plant protection product registration (EPA, 2017; Gobierno de España, 2020).

Different studies were conducted by Whitmyre et al. (2004) and the California Department of Pesticide Regulation (CDPR) to measure the DFR from two formulations of Endosulfan (I.e., emulsion concentrate (EC) and wettable powder) (Whitmyre et al., 2004). The WP formulation in all of these studies resulted in higher DFR (Beauvais et al., 2010). Comparably, for other types of solid formulations, such as granular formulations, Thompson et al. (1984) also reported a 15 times reduction in 2,4-D granular applied herbicide residue compared to liquid-applied 2,4-D which also points to the effect of formulation type on the degree of residue dislodgeability (Thompson et al., 1984).

Different pesticide formulations are reported to transfer from the turf at different rates, even with the same active ingredient in them (Hurto & Prinster, 1993).

Transferability from turf to people is influenced by different factors, including the formulation type (e.g., liquid versus granular), the water solubility of the active ingredient applied, environmental stability of the active ingredient, and additives to the formulation such as stickers, stabilizers, and surfactants (Krieger, 2010).

In addition, the time of pesticide application could be a good contributor as a common factor that affects DFR across different formulations. That was highlighted in a study performed by Patrick Jerome Maxwell (2017) concluded that the liquid formulated Azoxystrobin applied in the afternoon resulted in a greater DFR compared to the morning time (Maxwell, 2017). However, the opposite trend was observed within granular formulated Azoxystrobin. The low DFR associated with the liquid formulation sprayed in the morning is due to the moisture present on the canopies. This moisture could dilute Azoxystrobin left over on foliage and increase its potential absorption into the leaf. Conversely, liquid formulated Azoxystrobin applied in the afternoon with no canopy moisture allowed the spray solution to dry faster on the foliage (≥4 hours of sunlight), which promoted greater retention. In addition, granular formulation applied in the afternoon without canopy moisture resulted in fewer granules adhering to foliage and, thus, less DFR quantified on the upper turfgrass canopy available to dislodge (Maxwell, 2017).

In the same context, a study of leaf sampling timing during the DFR technique between morning and evening times suggested the sampling time could be a factor that affects DFR. This factor was proved to affect the intensity of DFR as samples collected in the morning showed a 5-to-10-fold increase in dislodgeable 2,4-Dimethyaine salt (herbicide used in turfgrasses). The result suggests that pesticide residues may be influenced by conditions favouring canopy moisture development (Jeffries et al., 2016; Maxwell et al., 2018). On the contrary, another

suggested process that may increase dislodgeability in mornings is plant guttation or the exudation of aqueous materials from hydathodes of the plants as pesticides were detected in guttation following pesticide applications and reported in many reviewed articles. Nevertheless, it concludes that the canopy moisture may be a determining factor affecting DFR, not the time of application within the day (Gannon & Jeffries, 2014).

Despite the historical evidence that the formulation types influence DFR, especially for WP formulation, no significantly different effect was reported for Bupirimate and Tebufenozide EC and SC formulation on two different crops such as tomato and peppers (Kasiotis et al., 2017).

The formulation type and the leaf texture together could be influencing factors affecting the intensity of the DFR on leaves. The influence of pesticide formulation type and epicuticular waxes were addressed in many studies. Epicuticular waxes were reported to decrease photodecomposition for most pesticide–plant species combinations (Fantke & Juraske, 2013). On the other hand, the large lipid-covered plant surface forms an ideal sink for accumulating hydrophobic pesticides and may result in higher DFR (K. Sundaram & Curry, 1994).

The morphological variations that exist among leaf surfaces and structures make it possible to classify the leaves into two main categories: leaves that are easy to wet and those that are difficult to wet. The specific characteristics and structures that help delineate the leaf variations are the cuticular membrane, waxes, veins, stomata, and trichomes. These leaves which are difficult to wet are those with waxy and hairy surfaces (water repellent surfaces). Successful application of any pesticides on these kinds of leaves is considered challenging because of the droplet rebounding and rolling off the leaves (Wang et al., 2015). However, some

droplets do remain on the surfaces of difficult-to-wet leaves, but they form high contact angles providing a minimal interface between the droplet and the leaf surface (Wang et al., 2015). Consequently, application efficiency is decreased, and spray usage is increased (Bhushan & Jung, 2008).

The leaves that contain crystalline wax are often more hydrophobic and, therefore, more challenging to capture and wet when sprayed with an aqueous solution. In addition, leaves with trichomes are usually more water repellent than leaves without trichomes, especially when the trichome density is greater than 25 mm^2, preventing water droplets from reaching leaves' epidermis (Brewer et al., 1991). Thus, the wettability of a leaf surface is an essential factor in the deposition, retention, and spread of spray droplets on the leaf surface and the penetration of pesticides into the leaf (L. Xu et al., 2011).

In the same context, a classification has been set by the USA (ARTF), which grouped the leaf types statistically based on their texture effect on the DFR into three categories smooth, waxy, and hairy (Bruce & Korpalski, 2008). The degree of DFR was reported to follow a statistical pattern of hairy> smooth >waxy leaves, as shown in Table 2.2. In other words, hairy leaves tended to yield higher DFR values than smooth and waxy leaves providing that all other factors were constant (Bruce & Korpalski, 2008). The report does not mention the variability of leaf texture within the classified groups in responding to the residue on the surface or the variation of the pesticides used to spray these leaves.

Table 2.2: The ARTF statistical classification of DFR data against the leaf type (Bruce &
Korpalski, 2008).

Data Subset	Number of studies	Geometric Mean Initial Normalised DFR ($\mu g/cm^2$/lb-ai/Acre)
Hairy leaf	13	2.48
Smooth leaf	107	1.20
Waxy leaf	76	0.78

Table 2.2 shows the USA Agriculture Research Taskforce (ARTF) statistical classification of the
leaf type based on the number of studies available, as shown in the table. The normalised DFR
was higher in hairy leaves, followed by the smooth and waxy leaves (Bruce & Korpalski, 2008)*.*

Dislodgeable foliar pesticide residue studies on two crops belonging to the
Solanaceae family, tomato and pepper, were conducted using two different
pesticides, Bupirimate and Tebufenozide, where the amount of pesticide applied
on the crops were the same. The study concluded the effect of the crop type on
one of the active ingredients sprayed (Bupirimate) was significant. At the same
time, the other pesticide (Tebufenozide) did not affect the crop type of both
tomato and pepper (Kasiotis et al., 2017).

In the open literature, the crop type seems to be a critical parameter that
profoundly affects DFR values (Cabras et al., 1988; Lu et al., 2014b). In the
previous study, Bupirimate yielded more DFR value in pepper crops than the DFR
value of tomato crops. The author explained this finding due to the presence of
high-density trichomes (hair-like structure) on tomato leaves that interacted with
the pesticide and increased its affinity to tomato and, consequently, its
absorption compared to pepper leaves that lack such a morphological defensive

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characteristic (Kasiotis et al., 2017). Although trichomes generally are water repulsive, hydrophilic trichomes are also reported (Lusa et al., 2015b).

In another study, the effect of crop type was highlighted when six different vegetables were treated with chlorpyrifos. This treatment showed a different chlorpyrifos deposition level among the six vegetables after the greenhouse foliar treatment. The initial foliar deposited concentration of chlorpyrifos on the six vegetables followed the increasing order of brassica Chinensis < lettuce < celery < asparagus lettuce < eggplant < pepper. The author regarded such findings as the effect of the leaf characteristics of the selected vegetables on DFR (Cabras et al., 1988; Lu et al., 2014a). In conclusion, even though the crops may belong to the same family, they exhibit different morphological and physiological characteristics that enable them to react differently to chemicals. Thus, there is strong evidence that the crop type may be an influencing factor that could affect the intensity of DFR. Therefore, there is a significant need to study the leaf types within the same categorised texture group (e.g., hairy, smooth, and waxy)

Practitioners usually apply herbicides with various commercially available adjuvants and surfactants to increase the efficacy of the applied herbicides. Adjuvants usually include performance-enhancing agents such as drift retardants, suspension aids, spray buffers, and wettability. On the other hand, surfactants are designed to improve the spray mixture's dispersing, emulsifying, spreading, sticking, and/or penetration (Chow, 2017). For example, a study investigating the effect of non-ionic surfactant combination with 2,4-D herbicide on DFR showed that the surfactant inclusion might slightly reduce the DFR and reduce the potential human dermal exposure (Maxwell et al., 2018). On the contrary, the DFR of Chlorothalonil and Chlorpyrifos in a Cranberry Bog proved to increase in the presence of a spreader-sticker adjuvant which was also previously shown to

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reduce off-site drift during pesticide applications to cranberry bogs (Putnam et al., 2003).

In contrast, the influence of different adjuvants inclusion in a Fenitrothion formulation was studied by Sundaram & Sundaram, 1987. The formulation containing polymeric adjuvant reported significantly larger droplets during the treatment and higher DFR in balsam fir needles than those formulations containing surfactants and co-surfactants (non-ionic/anionic) (K. Sundaram & Sundaram, 1987). The same report provides a higher ratio of dislodgeable foliar residue to the penetrated residue and a slower dissipation rate for the formulation with polymeric adjuvant than other formulation combinations.

The EPA Science Advisory Council for Exposure (Expo SAC) policy 3 revised in January 2017, emphasized the environmental condition effect on the dissipation of pesticides and consequently on the degree of dislodgeability. The report stated that "*DFR studies conducted on greenhouse-grown vegetables or ornamentals are also considered worst-case, provided overhead irrigation is not used, and the Ultraviolet (UV) which can be reduced by the glass or plastics used in greenhouses can result in slower dissipation by photolysis for certain synthetic pyrethroids*". Many other studies on different crops, vegetables, or turf have proved the same; studies on peaches, for instance, suggested that environmental conditions such as temperature, humidity, rainfall, sunlight, as well as cultural practices can significantly affect the rate of pesticide decay (Hernandez & Fredrickson, 1998).

 A study by Schipper *et al*. (1999) concluded that the dissipation of bupririmate pesticides on cucumber leaves could be seasonal. That was concluded after studying the DFR level during two harvesting seasons in June and October, the latter season showed less foliar residue when compared (Schipper et al., 1999). Other studies on turf concluded that residue dissipation was found to vary as a

function of weather conditions, with more rapid dissipation occurring in warm, dry conditions and a slower dissipation occurring in cool, wet conditions (Cowell et al., 1993).

The above findings clearly indicate the effect of environmental conditions such as sunlight intensity and the impact of irrigation or rainfall on the degree of dislodgeability. THE rain has been reported in many research papers to have markedly reduced DFR (Guthrie et al., 1976; Maxwell et al., 2018). Irrigation immediately after the treatment also has been reported to significantly increase the dissipation rate of pesticide residues on foliage (Murphy et al., 1996; Snyder et al., 1999). These studies suggested that irrigation influence on pesticides will vary more with formulation than with active ingredients. As a result, authors warrant further investigation to compare dislodgeable residue levels among formulations of the same pesticide due to the expectation that irrigation can reduce residue levels of some pesticides (i.e., dry or aqueous-based formulations) yet not others such as EC formulations (Hurto & Prinster, 1993)

The intensity of sunlight has also been proved to be an influencing factor determining the amount of DFR, as reported by Iwata (1980) on fruit residues and foliage residue (Iwata, 1980b). One can reasonably speculate that the fruit, which is located somewhat inside the canopy of foliage protected from the sunlight and air movement, can retain residues longer and the same with foliage where the big trees can shade their leaves from the sunlight and air movement similarly results in more residue than smaller unshaded exposed tree leaves. In agreement with Iwata (1980), the sunlight intensity had proved to degrade the herbicide (Agent Orange) used during the Vietnam war resulting in rapid decomposition of the residue on plant leaves after one hour from the spray (Young et al., 2004).

Post-application irrigation can also significantly affect the transferable residue on turf. Although it usually decreases the transferable residues from applied liquid formulations, it can increase the residue from the granular formulation because it releases the active ingredient from the carrier in the formulation (e.g., clay) on which it is coated (Thongsinthusak & Dong, 2010). Moreover, previous research has shown evidence that post-application irrigation can reduce pesticide (Azoxystrobin) DFR from turfgrass. The study showed that the sooner the irrigation after the treatment, the more the residue collected was reduced (Maxwell et al., 2018). Besides, delaying post-application irrigation until 48 hours after treatment (HAT) resulted in a 4-fold increase in Azoxystrobin dislodged compared to irrigation 4 HAT (Maxwell et al., 2018).

Another critical factor is the method of application which is reported to influence the amount of DFR. Field treatment data in California was gathered by Iwata (1980) using Dioxathion on orange trees using a different method of application; low volume application versus boom application proved that the method of application would greatly influence the residue levels when the same amount of pesticide is applied on a specific area of cultivated crops. The low-volume application method revealed more residue than the boom conventional application method (Iwata, 1980a). In agreement with the collected data by Iwata (1980), a study by Giles *et al*. (1992) proved that the use of reduced volume electrostatic more concentrated application resulted in approximately 3.7 times more foliar residue than the use of the conventional wet-spray technique (Giles et al., 1992). Additionally, the application technique (i.e., high volumes vs ultra-low volume fogger) as a significant factor affecting DFR was reported by Edmiston et al. (1991) when they studied the dissipation of Methomyl on grapes foliage in the field versus greenhouse treatments. These findings concluded that various application techniques could affect the amount of pesticide deposition, which

may ultimately affect the pesticide's dislodgeability (Edmiston et al., 1991). Moreover, the relationship between increased efficacy and smaller, more concentrated spray droplets occurs commonly in the literature (Hislop, 1988).

In the same context, a study by Schneider and Fredrickson (1999) elucidated the effect of the application method on the DFR of Abamectin sprayed on Gerbera flowers in a greenhouse. In this study, the low volume application resulted in a lower residue deposition and lower DFR compared to the conventional spray (Schneider & Fredrickson, 1999)

Among the influencing factors is the maturity of the leaf, which was first suggested by Iwata *et al*. (1977) and reported in his DFR developed technique by avoiding the smaller leaves of citrus and choosing the mature leaves only. The reason is the residues on young leaves are subject to dilution due to growth and may influence the DFR measurement accordingly (Kasiotis et al., 2017). The same has been supported by the EPA Science Advisory Council for Exposure (ExpoSAC) Policy 3 Revised January 2017(EPA, 2017).

Sutherland (1971) listed the crucial factors that affect foliar dissipation of pesticides by describing foliar dissipation as "the *summed effects of ultraviolet degradation, volatilization, metabolism, mechanical dislodgement by wind and rain, atmospheric oxidation and hydrolysis by plant or atmospheric moisture* " these factors have been well illustrated in a figure published in 2013 by Dong and Beauvais (M. Dong & Beauvais, 2013). These crucial factors are summarised in Figure 2.1below.
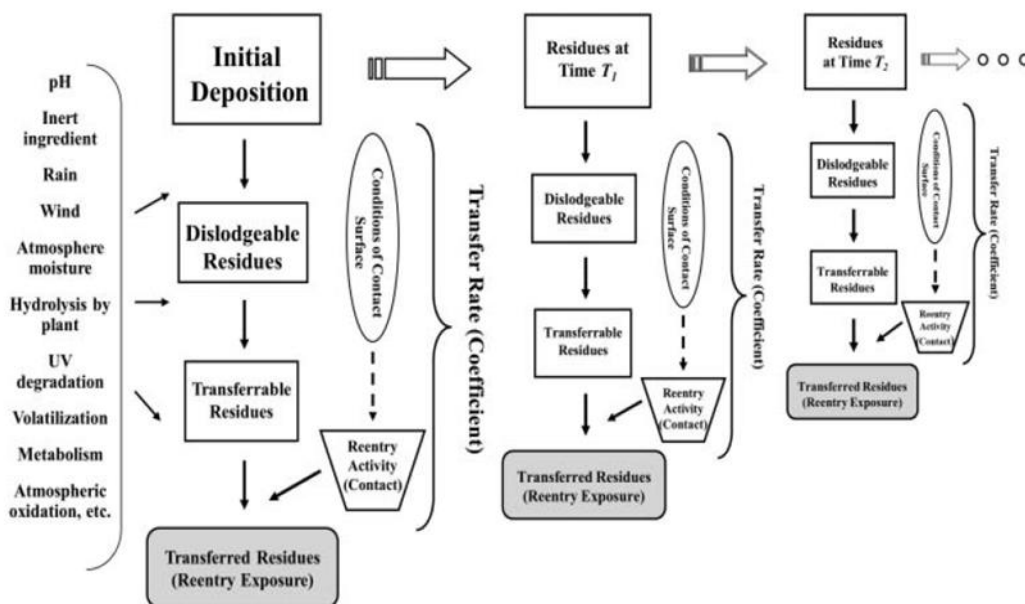
Figure 2.1 represents a descriptive figure published in Dong & Beauvais's (2013) article that lists all the factors that could affect foliar dissipation and consequently could affect DFR as illustrated in the " (Dong & Beauvais, 2013).

Figure 2.1: Crucial factors that affect the dislodgeability of pesticides

In the same context, Cornelissen et al. (2006) reported the pH of the foliage as a significant factor affecting the concentration of DFR by emphasising the possibility of the foliar residue degrading at a faster or slower rate (Cornelissen et al., 2006). During the treatment, some pests could release a reacting acidic or alkaline substance in an amount sufficient to alter the foliage pH, and some pesticides tend to be hydrolysed more rapidly in an acidic medium, such as Diazinon. In contrast, others need an alkaline medium to do the same such as Phosomet. Formulation inerts sometimes are intended to acidify or alkalize the spray for the same reason (M. Dong & Beauvais, 2013).

In contrast, the level of fungicide Chlorothalonil residue on celery monitored at 2 hours post-application at most sites was found to be significantly lower than those measured on day 1 post-application. Also, in another study, the fungicide

Propargite was applied to Thompson grapes in two vineyards in California at the same time with the same application rate. The DFR level measured at 12 hours post-application was two times lower than the level estimated 1-day post-application in one vineyard. In the other sprayed vineyard, the difference between the two-time points was in the opposite direction (the level at 12 h post-application was two times higher). The authors attributed these findings to either different possible rates of pesticide residue settlement on the plant leaves or different levels of pesticide residue hydrolysis due to pH level changes on the treated foliage; in the two locations; in agreement with Cornelissen *et al*. (2006) (Cornelissen et al., 2006; M. Dong & Beauvais, 2013).

A study conducted by Kurtz (1990) investigated estimating daily airborne flux from foliar surfaces (Kurtz, 1990). The study data showed a rapid attenuation of the foliar residues for the first 5 days, where airborne residues account for approximately 10 % of the daily foliar dislodgeable residue attenuation, while at the end of the study; after 15 days; this percentage has dropped to 5 %. The study suggested that the residue remaining on the canopy after 15 days is considered tightly bound and less available for airborne loss. This evidence suggested that the drop in the residue percentage at the end of the study is due to reaching the canopy equilibrium phase, as penetration of the residue into the plant tissue will reduce the amount of residue that is susceptible to volatilisation. As surface residues are depleted, penetrated residue may diffuse back to the plant surface following the concentration gradient. Thus, volatilisation may be an influencing factor affecting DFR. This finding agrees with the Cooper et al. (1990) article, which concludes on the significance and direct relation between volatilisation and DFR (Cooper et al., 1990).

2.4 Conclusion

From the performed systematic review, it is obvious that the available articles related to the DFR are either regulatory or some research articles conducted years ago. Nevertheless, these articles represent the legacy of science and the first attempts to understand the nature of DFR and should not be overlooked. From this review, the area of defining factors that could affect DFR required more research and data collection for conclusions on the influencing factors to be drawn. Data collection could be challenging with the existing variability in the methodology of the DFR and the variabilities that exist due to many existing factors (i.e., different crops, meteorological conditions, spraying patterns, etc.). In addition, the review did not reveal any sources that could give an appropriate answer to the findings on the methodology of investigating factors that may affect DFR, nor the correlation that could exist between the dissipation of dietary and non-dietary residues. To this end, investigating factors that could affect DFR, developing a standardized method to study these factors, and creating a correlation between the decline of both residues (dietary and non-dietary) would help to fill the gap in the pesticide residue science and pave the road for generating more data related to DFR.

Chapter 3 : Correlation Between Dietary and Dislodgeable Foliar (DFR) Crop Residues Decline Data; A Proposed Approach to Refine Non-Dietary Risk Assessment.

3.1 Introduction

Pesticides have a crucial role to play in assuring food security for an increasing world population (Bonner & Alavanja, 2017). However, despite the application of good agricultural practice, pesticide residues may persist on or within the treated crop, subsequently leading to human exposure. The intensity of exposure to such residue depends on many factors, including the pesticide application rate, dissipation rate, environmental conditions, transfer coefficients (crop to skin transfer), and the physicochemical properties of the pesticide applied (National Research Council, 1993). Consequently, pesticide residues on or in food or feed crops can potentially impact human health if the exposure results in an unsafe dose (EFSA, 2010; Rani et al., 2021).

Assessing risk from dietary exposure to pesticide residues in food crops has become essential in authorising and regulating pesticides. It is based on standard procedures required by international pesticide regulation bodies (Damalas & Eleftherohorinos, 2011). Such assessments typically include detailed information about the pesticide residue dissipation on target plants and the quantification of pesticide residues found in plant components harvested for food and animal feed (EFSA, 2011). Understanding and quantifying pesticide residues is also invaluable to supporting non-dietary risk assessments, which are also mandated by the registration process. Part of this risk assessment requires estimating dermal pesticide exposure, which is used to help ensure agricultural workers, residents, and bystanders are protected (Charistou et al., 2022). This is driven by quantifying the part of the pesticide residue that can be dislodged when the treated surface

is touched or brushed against and is referred to as the dislodgeable foliar residue (DFR) (EFSA, 2014a).

Residue decline studies are an essential part of risk assessment processes and, despite the high associated costs of field studies, they offer numerous advantages over studies that just measure residues at harvest. The former provides data on residue behaviour over time, allowing reliable estimation of residues at any point up to and including the point of harvest, and so are used to determine Maximum Residue Limits (European Commission, 2019b). Maximum Residue Limits (MRLs) are defined as "*the upper levels of pesticide residues that are legally permissible in or on food or animal feed, based on good agricultural practice and the lowest consumer exposure necessary to protect vulnerable consumers*" (Markantonis et al., 2018). The decline of residues on crop foliage, quantified as the foliage dissipation half-life (DT_{50}), depends on the applied PPP's physicochemical properties and environmental conditions (EFSA, 2014a). According to the 2014 and 2022 EFSA guidance (EFSA, 2014a), if no experimental DFR data are available and data for the active substances in question are not included in the guidance appendices, default values for DFR and DT_{50} should be used in the first-tier assessment. These default values are 3 μg active substance/cm^2 of foliage per kg active substance applied/ha for the DFR and 30 days for the DT_{50} (EFSA, 2014a).

The EFSA default value for the DT_{50} was estimated from the data included in appendices C and D in the 2014 EFSA guidance on pesticide exposure assessment of operator, worker, resident, and bystander (EFSA, 2014a). The value of 30 days was estimated from a dataset of 130 values for 48 compounds reported by Willis and McDowell (1987) and included in appendix C (Willis & McDowell, 1987) along with the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) which reported a dataset of foliar DT_{50} values for 277 compounds

in appendix D (EFSA, 2014a). Although the 95th percentile of the DT_{50} values in Appendix C are around 10 days, a high percentile value from USDA data (Appendix D) has been used to derive the default value (30 days).

Despite the importance of this data, it is relatively old, dating back more than 30 years, and more importantly, some active substances are not on the market anymore, and many modern pesticides currently authorised for use in agriculture are missing (Fantke & Juraske, 2013). Although the EFSA guidance (EFSA, 2014a) states that the 30-day default should only be used in the absence of other data (referring in part to the Willis & McDowell dataset), the subsequent use of the data in the EFSA 2014 guidance appendices has largely been rejected by regulatory bodies (Kluxen et al., 2021).

Moreover, the technical report published by EFSA (EFSA, 2014b) discussing the outcome of a public consultation on the latest published EFSA guidance under 2014 Regulation (EC) No 1107/2009 identified weaknesses and gaps in the current system and several comments suggested that the 30 days default value may be unrealistic and overly conservative (EFSA, 2014b). This may be justified considering that experimental data supporting such a default value was limited and relies on "a now-outdated" statistical analysis (Kluxen et al., 2021). In addition, residue dissipation data tends to be highly variable depending on how the dissipation rate was measured (e.g., on the crop surface or inside the crop tissues) and on the part of the plant tested (foliage, stems, fruit, etc.). Climatic and other environmental factors also influence the dissipation rate (EFSA, 2014d). In addition, just 13% of pesticides in the The United States Department of Agriculture (USDA) data set had DT_{50} values reported as 30 days or more (EFSA, 2014a).

There was a flood of new data from experimental field studies published in the peer-reviewed literature in recent years. For example, (Fantke & Juraske, 2013) collated a data set of 346 pesticides. This dataset, as well as that gathered and reported by (Willis & McDowell, 1987), was updated by (Lewis & Tzilivakis, 2017a) for inclusion in the Pesticide Properties Database (PPDB) (Lewis & Tzilivakis, 2017b). However, the majority of this DT_{50} data is associated with total residues (as used in dietary risk assessments) and not necessarily the DFR fraction.

Recently EFSA working group decided to consider the PPDP for possible refinement of the default DT_{50} after rechecking and assessing the available data from the gathered literature against quality criteria (i.e., crop growth stages, GLP status, sampling strategy, number of replicates, LOQ, and LOD, calculation of the DT_{50}, etc.). The review of this data revealed 32 publications to be reliable. From this data, different uncertainties were flagged as follows; (Charistou et al., 2022).

- This data included only 7 active substances out of 32 approved for use in the EU.
- The evaluated data was not representative of the majority of pesticide chemicals classes.
- Due to limitations on the information available in the public paper, there were many cases with wrong/mistyped reported DT_{50} values. Moreover, different software were used to calculate the DT_{50},
- Lack of methodological information due to the non-existence of a harmonised method for conducting DFR studies.
- The most recent data from the evaluated database are from 2007, while the oldest was dated back to 1971; thus, there is uncertainty in comparing methodologies across 40 years.

Based on the above findings, The EFSA working group could not conclude any refinement of the default value of 30 days from the reliable data points despite acknowledging the conservation of the value due to lack of reliable data. Notwithstanding that, some of the data that flagged uncertainties partially exceeded the 30 days value; It was then recommended from the above findings to use high-quality data submitted for regulatory purposes to refine the DT_{50} default value rather than depending on publicly available data from the literature (Charistou et al., 2022).

Nevertheless, it has been unclear whether there is a relationship between these two residue types (dietary and non-dietary). Consequently, this particular study's aim is to consider the abundance of dietary residue decline data to explore if there is a statistical correlation between the decline of dietary and DFR data expressed as DT_{50} and/or if dietary decline data could be beneficial in refining non-dietary risk assessments and used as a more appropriate value to the current 30-day default.

3.2   Aims and objectives

This chapter investigates the correlation between the decline data available for both dietary and non-dietary residue data. First, it explored the decline of the abundant dietary residue data (DT_{50}) and compared it to the decline in non-dietary residue from a non-publicly available source. Syngenta UK has provided this residue data to support this research from their high-quality, good laboratory studies (GLP). Furthermore, investigate the publicly available data in the open literature (from the latest, most comprehensive dataset, PPDP) to compare the decline of dietary and non-dietary residue on the same tested crops.

3.3 Material and Methods

The analysis to investigate the correlation between the two types of available residue data (i.e., DFR and dietary/total residue) was undertaken in two stages. Firstly, on high-quality, detailed field trial data and secondly, based on data extracted from peer-reviewed studies identified in the literature.

3.3.1 Analysis of field trial data

In the first stage, the analysis was conducted on a set of paired studies owned by Syngenta for PPPs registration purposes to support the dietary and non-dietary risk assessment requirements. Studies were paired based on providing data for both residue types for the same specific crop/active substance. DFR studies were conducted on crop foliage, whilst the total residue studies were conducted on the edible part of the crops. These studies used six pesticide-active substances: lambda-cyhalothrin, adepidyn™ (pydiflumetofen), cyantraniliprole, cyprodinil, emamectin-benzoate, and difenoconazole (see also Table 3.1). These studies had detailed data available such that there was confidence that the paired studies were conducted under very similar experimental conditions. All these studies followed both Good Laboratory Practice (GLP) standards and the EPA occupational and residential exposure test guidelines (EPA, 2009). Both types of trials (DFR and total residue) included data on the dissipation of the active substance residue over time (at least 5 points per trial) to allow the calculation of each active substance's DT_{50} for each trial independently.

The calculation of trials' DT_{50} from the residue dissipation points over time was carried out using Computer Assessed Kinetics Evaluation (CAKE) software version 3.4 by Tessella Technology and Consulting. The software is available online with public free access (*CAKE Showcase | Computer Assisted Kinetic Evaluation*, n.d.).

This software has been used several times by the EFSA working group and other regulatory authorities to calculate the DT_{50} (Charistou et al., 2022). The degradation of the pesticide active substance was governed by first-order degradation kinetics using the software. This was checked by fitting a first-order equation to the data tested.

Table 3.1: *Active substances and crops used in Syngenta trial data.*

Pesticide active substances	Trial's crops
Lambda-cyhalothrin	Corn, Cotton, Pepper, Grape, Apple, Citrus
Adepidyn™or (pydiflumetofen)	Apple, Grape
Cyantraniliprole	Lettuce, Pepper, Apple
Cyprodinil	Barley, Grape
Emamectin-benzoate	Apple, Peach
Difenoconazole	Apple

Table 3.1 shows 6 pesticide-active substances on different crops that were studied to conclude the correlations between the DT_{50} of DFR and dietary residue. These studies were conducted under similar experimental conditions that allowed a good quality comparison.

The rate of pesticide transformation in the environment is commonly described using first-order kinetics even after being deemed a conservative approach to calculating the dissipation (Charistou et al., 2022; EPA, 2015). Yet this is generally considered the most appropriate because the rate is calculated using a single parameter (the rate constant (K), and the transformation rate is independent of the initial concentration used in the studies). The DT_{50} is used to express the rate of decline of first-order degradation and is calculated using the dissipation rate constant as per the equation below. The DT_{50} is the time required for the concentration to decline to half of the initial value (EPA, 2015; European Commission, 2014). Using the single first-order kinetics, the time for the decrease in the pesticide concentration from 100% to 50% of the initial amount is identical to the time for a decline from 50% to 25% of the initial concentration (European Commission, 2014).

$$C_t = C_e e^{-kt}$$ <div align="right">*Equ 3.1*</div>

Where C_t is the concentration of the pesticide at a time (t); C_0 is the initial pesticide concentration,

The natural logarithm (Ln) of 2=0.693, and k is the pesticide dissipation rate or rate constant of decline. The dissipation half-life, DT_{50}, is then calculated from:

$$DT50 \frac{Ln2}{k}$$ <div align="right">*Equ 3.2*</div>

Pesticide residue data, especially for DFR, often follow a non-normal distribution (Korpalski et al., 2005). Therefore, all the field trial data were converted either to the logarithm base 10 (Log 10) or square rooted and tested for normality using the Kolmogorov-Smirnov test using SPSS (Gravetter & Wallnau, 2009). The significance values (P-value) ≥ 0.05 of with ensuring that the skewness and Kurtosis values are in the tests indicated normality along acceptable range of the normal distribution (-1,1) and (-3,3), respectively (Shapiro & Wilk, 1965). All the raw data were analysed using SPSS, IBM version 27.0 (BM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). The variables were analysed using an independent T-test to compare two independent means from two different samples with different sample sizes and, consequently, different variances. The P-value, assuming that the variance of the groups was not equal, was used (Welch's test), and the significance was defined as ($P \leq 0.05$) with a confidence interval (CI) of 95%. The power analysis has been confirmed for the independent T-test using SPSS for all tested groups and revealed $\geq 85\%$ power except for difenoconazole, where sample power was 17%,

and this is justified by the very small sample used for both types of residues on only one crop (i.e., apple). The overall conclusion on the statistical relation between both types of residues selected from the literature was drawn by calculating the correlation coefficient (r).

To better assure the above analysis, all the residue data were also analysed using the non-parametric statistical test Mann–Whitney U-test to avoid any bias toward the small sample size of the DFR trials available. This test is commonly used to compare two independent samples when the outcome is not normally distributed and the samples are small (Nachar, 2008).

3.3.2 Analysis of literature sourced data

The second stage of analysis was conducted to support the first stage, particularly as the number of data pairs in Stage 1 was low. Data pairs (DFR and total residue data for the same crop/active substance) were extracted from the Pesticide Properties Databases (PPDB) supplemented by additional data sourced from published studies post-2017. The methodology followed that reported by (Lewis & Tzilivakis, 2017a), which reported high variation in plant dissipation DT_{50} values, probably due to these studies being conducted using different measurement and analytical techniques under various meteorological conditions and using different parts of the plants. Nevertheless, it was possible to select study pairs of active substance/crop combinations which could be beneficial in helping to elucidate the correlation between both types of residues using the open literature data despite the variabilities that exist. For this work, 43 examples of decline involving DT_{50} data points for 30 active substances on matching crops were identified. However, it should be noted that the data pairs were not necessarily from the same study. In some instances, more than one study was recognised for a crop/active substance combination, and in these instances, the varying DT_{50}

values were averaged. For comparison, the calculation of residue data's 90% and 25% existence was done across all residue studies. Calculation of the DFR and dietary residue DT_{50} mean values and the independent analysis of mean differences (independent T-test) and the normality test (Kolmogorov-Smirnov test) using SPSS, IBM version 27.0 were carried out. The overall conclusion on the statistical relation between both types of residues selected from the literature was drawn by calculating the correlation coefficient (r).

3.4   Results

3.4.1   Analysis of field trial data

The selected field trial data covered six pesticide active substances on nine crops, as shown in Table 3.1. Although the trial protocols were similar, they were carried out in Europe, Brazil, and the USA in different geographical areas.

As illustrated in Table 3.2 below, the statistical analysis, using both parametric and non-parametric approaches, showed a significant difference between the dietary and DFR DT_{50} values for lambda-cyhalothrin, cyantraniliprole, cyprodinil, and emamectin-benzoate (P<0.001) with a 95% confidence interval (CI). However, significance was achieved for adepidyn™ (pydiflumetofen) using the non-parametric test only (P=0.04), while the non-significance existed (P=0.053) in the case of the parametric test. In the case of difenoconazole, the significance was not achieved using the parametric or non-parametric analysis (P=0.3, P=0.7, respectively).

For the back-transformed data, the calculated dietary DT_{50} mean values for the six tested pesticides active substances from field studies are higher than the DT_{50}

of the DFR, as shown in Table 3.3. This was statistically significant (P≤ 0.05) for 5 of the 6 tested active substances (i.e., lambda-cyhalothrin, adepidyn™, or Pydiflumetofen, cyantraniliprole, cyprodinil, emamectin benzoate) using the non-parametric statistical test (i.e., Mann–Whitney U-test). Adepidyn™ or Pydiflumetofen dietary DT_{50} was not statistically different (P=0.05) to the DFR DT_{50} using the parametric T-Test, despite the significant difference observed with the non-parametric test. For difenoconazole, there was no significant difference between dietary residues and DFR using either statistical test. An overall correlation coefficient (r) was calculated between the mean of both types of residues (dietary and DFR) for the available 6 active substances from field data which showed a medium positive correlation (r= 0.5).

Figure 3.1 below compares the dietary and DFR DT_{50} mean decline values for the six pesticides tested from available Syngenta studies. The box and whisker plot in Figure 3.1 shows a significantly higher DT_{50} for dietary residue values than for DFR. Mean +/- standard deviation (SD) is plotted for each pesticide active substance, and the exact values are presented in Table 3.2.

Table 3.2: Statistical data for field trials analysis

Active substance	Residue type	Number of trials	Mean DT_{50}	Standard deviation Mean	Standard error Mean	Parametric T-test P-value (2-tailed)	Non-parametric U-test P-value (2-tailed)
				Statistics			
Lambda-cyhalothrin	Dietary residue	37	4.5	2.2	0.4	*<.001	*0.00002
	DFR	21	2.2	0.9	0.2		
Adepidyn™ or (Pydiflumetofen)	Dietary residue	21	1.5	0.2	0.1	**0.05	*0.04
	DFR	7	1.1	0.4	0.1		
Cyantraniliprole	Dietary residue	47	2.8	1.0	0.1	*<.001	*0.003
	DFR	7	1.8	0.2	0.6		
Cyprodinil	Dietary residue	17	4.9	1.7	0.4	*<.001	*<.0001
	DFR	7	0.9	0.1	0.5		
Emamectin-benzoate	Dietary residue	48	2.4	0.8	0.1	*<.001	*<.0005
	DFR	8	0.6	0.2	0.1		
Difenoconazole	Dietary residue	7	4.7	1.0	0.4	**0.3	**0.7
	DFR	6	3.8	1.8	0.7		

Table 3.2 elucidates the descriptive statistics for the 6 active substances tested on both DFR and Dietary residue DT_{50}. Parametric and non-parametric statistical analyses have been carried out to compare both types of residues' half-lives. *P-value <0.05 indicates significance among values compared. **P-value ≥0.05 indicates non-significance among values compared. Mean DT_{50} is the DT_{50} mean value for the transformed data (square root, or Log 10).
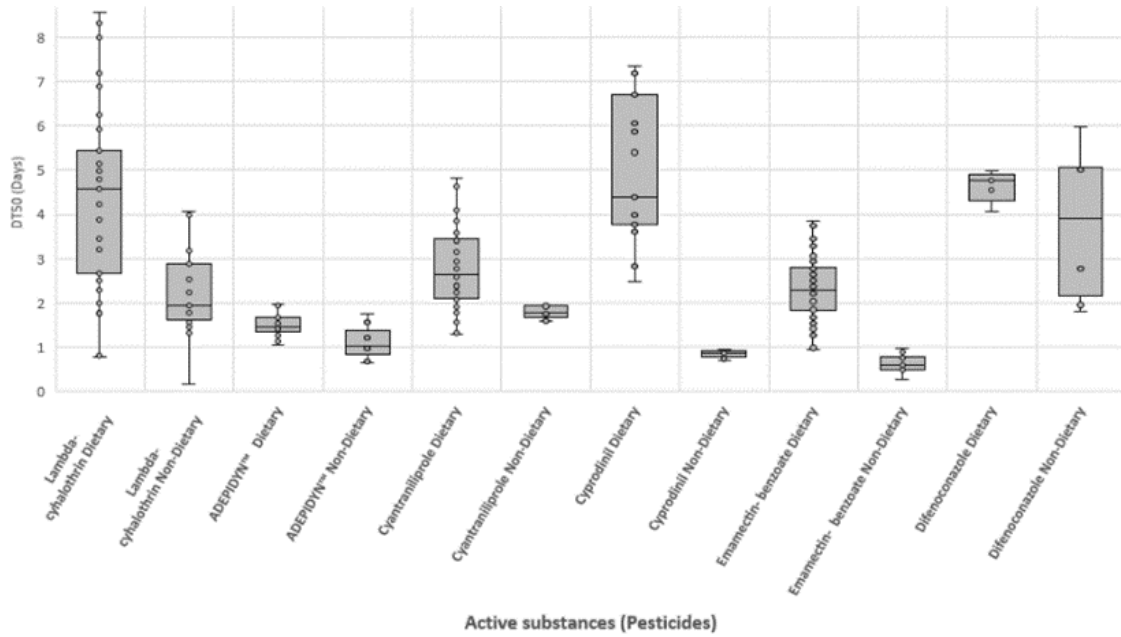
The box and Whisker plot in Figure3.1 shows an increased dietary residue DT_{50} among tested active substances compared to the DFR DT_{50} except for difenoconazole. The X-axis shows the 6 active substances tested on matching crops for field trials DFR and dietary residue studies, while the Y-axis shows the transformed DT_{50} (days).

Figure 3.1: Comparison between dietary and DFR DT_{50} mean values of field trial data.

3.4.2 Analysis of data sourced from open literature

The residue data sourced from the literature (PPDP) were analysed and included data for 31 pesticide-active substances on 20 different cultivated outdoor crop types. The trials were carried out in different worldwide geographical areas in Europe, Africa, Asia, the Middle East, North and South America, and on different numbers of crops, as shown in Table 3.4 below.

The analysis revealed that 75% of the pesticide-active substance/crop pairs extracted (n=43 residue decline data pairs) showed higher mean dietary or total residue DT_{50} values compared to the DFR DT_{50} on the same crops, as shown in Figure 2. However, a non-statistical difference was proved between both dietary and non-dietary residue using the independent T-test (P=0.1). The total DT_{50} mean value for the DFR data was 4 (SE+/-0.5) days, while the total DT_{50} mean value for the dietary residue decline data was 6 (SE+/-0.7) days among all the collected studies. The 90th percentiles of the data points in the extracted data were 9 days and 12 days for the DFR and dietary residue DT_{50}, respectively. In addition, amongst all half-lives data, there were no single DT_{50} values above 30 days (EFSA estimated DT_{50} default value) for both types of residues. The data proved to be not normally distributed even after being transformed to Log10 and square root values. Moreover, the correlation coefficient (r=0.1) between both kinds of residue decline extracted from the PPDP showed very week to no correlation.
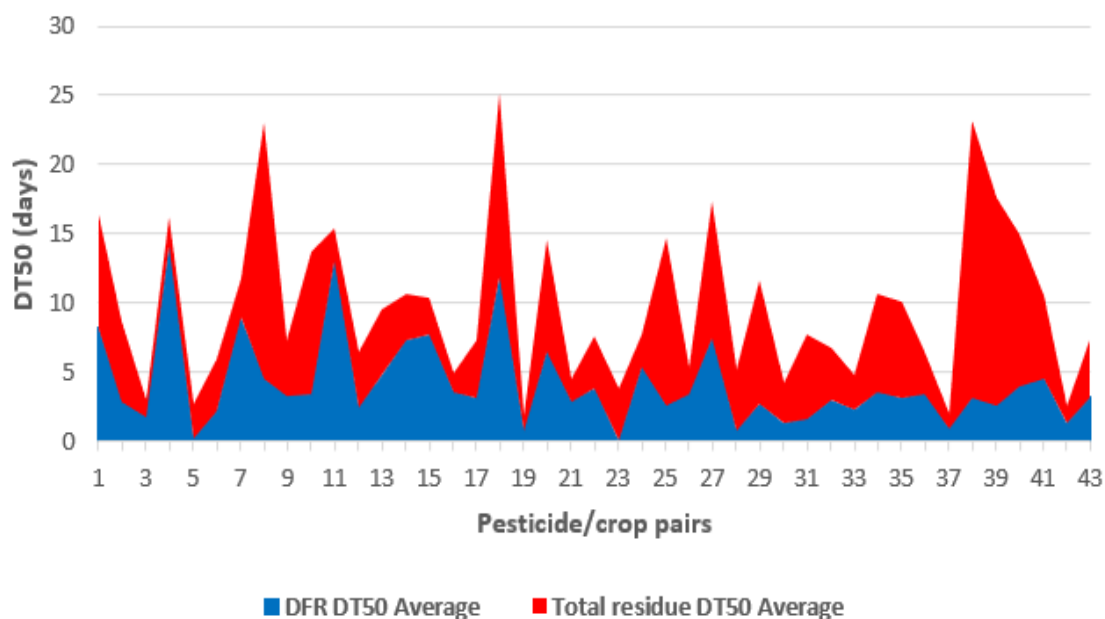
Figure 3.2 elucidates the DT50 of DFR and dietary residue studies extracted from the Pesticide Properties Database (PPDP) for matched crop pairs. 75% of the data showed an increase in the dietary DT50 compared to the DFR DT_{50}. The x-axis shows 43 pesticide/crop pairs, while the y-axis shows the DT50 or half-life in days.

Figure 3.2: DFR and total residue DT_{50} mean values among selected pesticide/crop pairs in the PPDB.

The half-lives for the back-transformed data proved to be statistically higher in the case of dietary residue compared to the decline in DFR when the non-parametric U- test was used for tested active substances. On the other hand, the significant difference using the parametric T-test was not achieved for difenoconazole, and the significance was on edge for Adepidyn™ or Pydiflumetofen. Nevertheless, the numerical back-transformed DT_{50} data for all

tested active substances proved to be higher in an average of 5 to 23% in the dietary studies than in the DFR studies.

Table 3.3: A comparison of the back-transformed DT$_{50}$ values for the tested active substances

Active substances (pesticides)	DT$_{50}$ (Days)		Statistical significance	
	Dietary studies	DFR studies	Parametric T-test P-value (2-tailed)	Non-parametric U-test P-value (2-tailed)
Lambda-cyhalothrin	20.5	4.9	*<.001	0.00002
Adepidyn™ or Pydiflumetofen	32.3	13.1	**0.05	*0.04
Cyantraniliprole	7.8	3.2	*<.001	*0.003
Cyprodinil	24.6	0.8	*<.001	*<.0001
Emamectin-benzoate	5.6	0.4	*<.001	*<.0005
Difenoconazole	22	14.2	**0.3	**0.8

*Table 3.3 elucidates the DT$_{50}$ values (days) for the tested active substances following back-transformation. This back transformation is from their Log10, or square root values used during the statistical analysis of the non-normal distributed data gathered. *P-value <0.05 indicates significance among values compared. **P-value ≥0.05 indicates non-significance among values compared.*

Table 3.4: PPDP selected pesticides on different crops in different geographical regions

Pesticide active substances	Trial crops	Countries
Acephate	Lemon, Tomatoes	USA (several states), China
Alpha-Endosulfan	Peppers (sweet)	USA (several states)
Azinphos-methyl	Pears	USA (California), Uruguay
Bifenthrin	Eggplant	India, West Africa
Carbendazim	Apple, lettuce	India, Finland, China
Carbophenothion	Oranges	USA (several states)
Carbosulfan	Oranges	USA (several states)
Cyhalothrin	Tea	China
Cypermethrin	Green beans, tomatoes, cabbage(spring), Chinese cabbage	Egypt, India, China, USA, Canda
Cyprodinil	Tomatoes	USA (several states), Italy, Poland
Deltamethrin	Eggplant	India, West Africa
Diafenthiuron	Chinese cabbage	Republic of Korea
Dialifos	Grape	USA (California)
Diazinon	Tomato	Venezuela, N/A
Dieldrin	Peach	USA (Illinois), N/A
Dimethoate	Cabbage, cucumber	USA (Maryland), Egypt, N/A
Endosulfan	Eggplant	India, West Africa
Ethion	Grape	USA (California), Canada, Iran
Imidacloprid	Grape	India, China, Chile
Lindane	Chickpea	India
Mancozeb	Grapes	Italy, China, Spain
Methamidophos	Tomato	USA, Brazil, Spain, Venezuela
Methomyl	Tomato, grapes	Egypt, Canada, Spain, N/A
Oxyfluorfen	Onion	Canada, India
Parathion	Apple, lettuce, orange, pear, peach	USA (several ststaes), N/A
Parathion methyl	Apple, grape	India, USA (several states), Italy
Phenthoate	Lemon, orange	USA (several states)
Phosphamidon	Orange	USA (California)
Propiconazole	Wheat	China
Quinalphos	Tea	China
Thiamethoxam	Tomato	China, India, Egypt

3.5   Discussion

Pesticide residues analysed from different crops were characterised by a high degree of variability. This variability was expected due to many factors, including physicochemical differences between the active substances, the pesticide formulation types, the plant matrix on which the residue was measured, foliage texture, and plant architecture. Differences in meteorological conditions and different methodological and residue quantification techniques may also contribute to this variability. Such variations resulted in non-normal distributions of residues for the active substances, which agrees with many findings in the open literature (Charistou et al., 2022; Farkas et al., 2015; Korpalski et al., 2005). Despite this variability, the field trial data were characterised as high quality as documented evidence was available showing compliance with GLP standards and the availability of several replicates from the selected active substance/crop pairs that allowed an accurate statistical analysis. This limitation was that few data pairs were available for these high-quality studies. In contrast, whilst a much larger number of data pairs were identified in the literature, the quality of the data was not verifiable. Residue level data at fixed time points tended not to be available; instead, just the calculated DT_{50} values were published with many other limitations in the meteorological conditions mentioned. This agrees with the latest EFSA guidance on reviewing the DT_{50} data extracted from the PPDP and their recommendation on avoiding using the open literature to judge the data by using high-quality GLP studies generated by the industry (Charistou et al., 2022)

Despite the positive medium correlation coefficient found between the two residue types using the field trial data, it was evident that the dissipation rate for all the active substances tested for the dietary residues was higher than that for DFR. Furthermore, the numerical back-transformed DT_{50} data for all tested active substances proved to be higher in an average of 5 to 23% in the dietary studies than in the DFR studies. Hence, accepting the limitation caused by the small number of studies, this work does provide some initial evidence that the dietary DT_{50} values are a reasonable surrogate for absent or limited foliage DFR DT_{50} values. The non-significance observed in the case of difenoconazole residue decline using both types of statistical tests for the DFR and dietary residue tested from Syngenta's trials could be due to the small sample size tested (n=7) on one crop (i.e., apple) unlike the other active substances tested where at least two crops were tested.

Moreover, in the case of adepidyn™ or (pydiflumetofen), a significant difference was only shown with one of the two statistical tests (U test). The conclusion is still that decline in DFR for these active ingredients is no slower than for dietary residue. On the other hand, there was no statistical correlation between 31 residue studies extracted from the PPDP on the 20 matching crops available for comparison purposes. That could be due to the enormous variabilities mentioned in the open literature studies gathered in the database(Lewis & Tzilivakis, 2017a).

For the literature data extracted, most of the data (75%, n=42) followed the same pattern of higher DT_{50} of total residue compared to the DFR DT_{50} mean values. That is in line with the latest EFSA guidance findings on the PPDP assessment, which clearly stated that "*The limited number of reliable data points (28) for DT_{50}, collected from 15 active substances (only six of them currently approved in the EU), could indicate that the current default value of 30 days for*

DT$_{50}$ is probably a conservative value;" that is despite that the assessment revealed some values exceeding the 30 days default values but with different uncertainties flagged in the data collected (Charistou et al., 2022).

There are, however, a few notable exceptions to this pattern, for example, a higher DT$_{50}$ value (14 days) for the DFR study of azinphos-methyl on pear compared to 2.1 days for the dietary DT$_{50}$. This low dietary DT$_{50}$ is believed to be because the study had been conducted during a rain shower, and much of the residue could have been washed off. Rain-rinse and other factors such as photolysis, hydrolysis, metabolism and growth dilution are critical degradation processes influencing the fate of pesticides in or on plants and consequently affect the dissipation rate (Gao et al., 2020). In addition, it is noted that azinphos-methyl is non-systemic, and the dissipation of surface residues due to wash-off would be reflected in the whole plant or commodity data. Another example is the active substance carbendazim on lettuce, where the DFR DT$_{50}$ (9 days) was higher than the dietary DT$_{50}$ (2.8 days). This high DFR DT$_{50}$ is believed to be due to the study being conducted undercover and not in an open field condition where residues are more exposed to weathering and wash-off. This explanation aligns with technical guidelines on data requirements for setting MRLs (European Commission, 2019b). The guidelines stated, "*The results of outdoor trials are normally not representative for indoor conditions/protected crops, because these structures offer varying degrees of protection from environmental conditions which influence the residue behaviour. Hence, if pesticide use under indoor conditions is envisaged, residue trials representative for these conditions need to be provided"* (European Commission, 2019b).

Considering the GLP field data and data gathered from the literature, the initial evidence suggests that a DT$_{50}$ value calculated from dietary residue studies could

potentially act as a convenient and typically conservative surrogate DT_{50} for DFR on a specific crop/active substance combination or even on different crop matrices. Such dietary dissipation values could be helpful if there is a lack of DFR data and there is no opportunity to generate any (perhaps due to regulatory timelines) or the dissipation behaviour of the active substance is not well known. In these instances, using dietary residue decline as a reference to determine the appropriate length for a DFR study or even under some circumstances to allow the limitation of the DFR study to measure only the dislodgeable fraction just after application (DFR0) could be possible. This could be used along with the residue dissipation curves to assess the risk of workers' re-entry into the treated field without needing long-tailed DFR studies to calculate the DT_{50}. Foliar dissipation curves have long been used as a risk mitigation tool for establishing safe re-entry intervals using first-order decay kinetics (Charistou et al., 2022; M. Dong & Beauvais, 2013; Korpalski et al., 2005; Willis & McDowell, 1987). This approach could save time and resources without carrying out long-tailed DFR studies while still adhering to the precautionary principle. In return, this would benefit the agrochemical industry and the regulatory bodies and ease the registration process accordingly.

3.6 Conclusion

As far as it is possible to tell, it is assumed that this is the first study to directly investigate the correlation between the dissipation of dietary residues and DFR for pesticides. Despite the limited data available from DFR pesticide residue studies, the field data showed that dietary residue DT_{50} was numerically higher and appeared sufficiently protective for the selected pesticide/crop pairs, along with most of the sourced literature data.

The EFSA default DT_{50} for all active substances has been decided by taking into consideration both dietary and DFR decline data. Therefore, this study suggests the use of dietary residue DT_{50s} as a suitable surrogate for DFRs, which is in line with the EFSA guidance. Although this study was limited to a side-by-side comparison of DT_{50} values for active substances applied to the same crop, where sufficient and reliable data are available that show good agreement with the current findings, it may even be possible to extrapolate to different crops if sufficient data exists.

It is hoped that this study, demonstrating that in most cases, currently approved pesticide active substances have DFR DT_{50} values much lower than the 30-day default value, will help re-ignite debate in this area. Risk assessments for PPPs must adhere to the precautionary principle, but recent work has demonstrated that there is considerable compounded conservatism in European re-entry worker risk assessments (Kluxen et al., 2021). Consequently, a more realistic estimate of DT_{50} for DFR is justified as a proposal in this context.

Chapter 4 : DFR Laboratory Method Development and Validation

4.1 Introduction

Pesticide use is determined by regulatory agencies worldwide to ensure pesticides' proper, safe, and consistent use. Accordingly, a pesticide risk assessment is considered an essential component of pesticide regulation in the most developed world (Krieger and Ross, 1993). In the European Union (EU), as published in the most recent European Food Safety Authority (EFSA) guidance for Regulation (EC) No 1107/2009, "*the risk assessment for plant protection products (PPPs) must be carried out for all exposure scenarios".* These scenarios involve operators, workers, residents, and bystanders that can be expected to occur because of the proposed use of PPPs. Most of these scenarios will fall into a category for which standardised first-tier exposure assessment should be applied using the guidance, where previously set default values exist for the applicant's use. For scenarios not covered in the first-tier assessment, the applicant may also use an ad hoc, higher-tier exposure assessment by generating experimental data based on actual exposure (Charistou et al., 2022; EFSA, 2014a).

The outcomes of the public consultations on EFSA guidance 2014 also identified various scenarios for which exposure estimates were least satisfactory due to data gaps. Consequently, recommendations were made for further research that would reduce current uncertainties (Charistou et al., 2022; EFSA, 2014a). From EFSA guidance 2014 and 2022, it is clear that the available data for worker exposure are not reliable enough due to the limited data set and statistical uncertainties that exist (Charistou et al., 2022; EFSA, 2014a). Hence, the *ad hoc* EFSA working group ("WoG") strongly recommended further collection and

production of data on specific Transfer Coefficients (TC) and dislodgeable foliar pesticide residue (DFR) values to produce more realistic exposure assessments (Charistou et al., 2022).

Historically, 49 years ago, the DFR determination method was first developed by Gunther et al.(1973) (Gunther et al., 1973). In 2009, the technique was then published by the USA Environmental Protection Agency (EPA) in the Occupational and Residential Exposure Test Guidelines (OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation) (EPA, 1996). The same method is also recommended in the DFR USA Agriculture Task Force (ARTF) draft protocol and has been broadly used in the open literature (Charistou et al., 2022; Kasiotis et al., 2017). The EPA Guidelines describe the technique and sampling methods used to quantify DFR, which is based on the definition, "*DFRs are the amount of chemical residues deposited onto the leaf surface that has not been absorbed into the leaf or dissipated from the surface, and that can be dislodged by shaking leaf samples in a detergent solution*" (Gunther et al., 1973). The guideline is intended to meet the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) testing requirements according to the EPA Test Guidelines for Pesticides and Toxic Substances Uses (EPA, 1996, 2009). The test guidelines (OPPTS 875.2100) are also referenced by the European Commission (EC) in a document for the authorization of plant protection products in Europe (European Commission, 2020) and mandate the study in Europe to follow the Good Laboratory Practice standards (GLP). Despite the OPPTS Guidelines 875.2000 and 875.2100, there is still no harmonised method for conducting DFR studies throughout the open literature, and most of the current studies follow the previously mentioned method with minor differences (Charistou et al., 2022).

The method validation process for any experiment is crucial for the method's reproducibility and accuracy. Several validations attempt for the DFR method has been made by (Bruce et al., 2006). These attempts studied the effect of different types of washing solutions, different washing volumes, wash duration, and shaking methods that were used in some of the DFR studies in the literature to compare them with the EPA-developed method (OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation) (EPA, 1996, 2009). This developed DFR method involved soaking 400 cm^2 of a total leaf surface area in two single washes of 100 ml of Aerosol OT 0.01% (w/v) (Bis(2-Ethylhexyl) sulfosuccinate, sodium salt Dioctyl sodium sulfosuccinate) for 10 min on a reciprocating shaker (EPA, 1996, 2009). Notwithstanding these validation attempts were on a very small scale and involved only two pesticides and two leafy crops (i.e., cabbage and lettuce), they did not reveal statistically different results between most of the employed techniques except for one technique when compared to the EPA method (Bruce et al., 2006).

However, the DFR definition considers that all pesticides that exist on the surface after the pesticides dry are dislodgeable (EFSA, 2014a). Therefore, ensuring that no pesticide residue is left on the leaf was crucial to conclude a precise and reproducible DFR from the current lab method. Moreover, according to the only available guideline for testing the DFR, "*the EPA Occupational And Residential Exposure Test Guidelines OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation [EPA 712–C–96–267]*" (EPA, 1996, 2009), DFRs represent chemical residues on the surfaces of treated foliage that are available for transfer to exposed populations (e.g., re-entry workers) during contact with those treated leaf surfaces. Therefore, from the EPA (2009) guidelines, the definition of DFR requires all residues found on the surface after the pesticide dry to be considered

dislodgeable and should be quantified during the dislodging procedures; extraction-to-exhaustion was crucial in the current laboratory method.

The initial guidelines of the current method did not include any validation of the wash-off solution used and its efficiency in dislodging all the residue from the plant surface. However, Gunther et al. (1973) DFR method guidelines emphasised the need for further validation requirements on the efficiency of the dislodging procedure (EPA, 1996; Gunther et al., 1973). This highlights the importance of validating the volume of wash-off solution needed for each crop or leaf type before conducting the DFR field studies required by the non-dietary risk assessment of PPPs.

Monitoring pesticide residue on plants is considered the gold standard for evaluating pesticide safety. Although generating enough data through conducting DFR field experiments is a robust way to derive a more realistic DFR default value for regulatory non-dietary risk assessment, it is not always achievable. This is not only because field experiments are generally expensive, seasonal, and time-consuming (BfR, 2020) but also because of the data's privacy and ownership across the biggest agrochemical companies in the industry. Hence, the study in this Chapter aimed to develop a new standardised laboratory method for quantifying DFR for research purposes with a description of the method validation. This newly introduced method could be vital in generating sufficient DFR data on many targeted crops under controlled and manageable environmental conditions. Furthermore, DFR-generated data from this method could be used in conjunction with the field experimental data for the regulatory authorities to set accurate and more reflective DFR default values for various crop groups and PPPs.

The proposed technique is relatively rapid. It would also allow the investigation of multiple factors that could influence DFR, potentially enabling further extrapolation between DFR studies if any correlation among the influencing factors is proven. Eventually, such a technique would save time, money, and resources for the industry and the registration authorities.

4.2 Aims and objectives

This chapter aims to develop and describe a newly developed laboratory method for quantifying DFR with comprehensive process validation. The method is deemed to be controllable, cost-efficient, time-saving and takes hours rather than days. In addition, the technique could be used to investigate factors that affect DFR and allow for the further generation of robust data.

4.3 Methodology and Method Validation

The description of the DFR analytical method below highlights the possible advantage of using it before conducting the DFR field experiment. The technique validation involved testing different leaves from different crops (i.e., French bean, tomato, soybean, oilseed rape, and wheat). All leaves were tested on 10% difenoconazole (DFZ) formulations. These DFZ formulations were formulated at Syngenta Jealott's Hill International Research Station for research purposes only. Additionally, these formulations are not registered nor considered at any registration stage for commercial use.

The formulations tested were two different Emulsifiable Concentrates (EC) with different solvents incorporated and a wettable powder (WP) formulation. The EC formulation is defined as *"a liquid, homogenous preparation to be applied as an emulsion after dilution in water"*, and the (WP) is *" a powder that is applied as a suspension after dispersion in water"* (OECD, 2001). The DFZ ECs were different

in the solvent used in the formulation, where the first EC had acetophenone solvent. In contrast, the other EC used had a solvent system which is a mixture of octanoic acids- decanoic acid-N, N-dimethyl amide, denoted as DFZ EC(x). The previously mentioned crops/leaves were selected to estimate and validate the new technique. They were chosen for their easy growing conditions and their variable foliar texture. The application of this method is not exclusively limited to the pesticide, nor the crop types mentioned above and could be used for testing any crop/pesticide combinations.

4.3.1 Plant growth and selection

Plants were grown in all-purpose commercially available compost. A Sanyo versatile environmental growth chamber model MLR-351 purchased from SANYO Electric Company, Sussex, the United Kingdom, was used for growing the plants, as shown in Figure 4.1 below. The Sanyo chamber was adjusted to provide optimum growing conditions for the uniform growth of each plant as per the optimum conditions mentioned in Table 4.1 below. All plants were watered uniformly through the capillary matting system (mats of 3mm thickness) underneath the pots in plastic trays to preserve the soil moisture. All plants were kept in the growing chamber throughout their growing period. Before treatment, plants with an approximately similar growth stage, height, and leaf size were selected to minimise the variabilities among the plants in the experiment and ensure that selected plants were free from any infestation and deformities.

Approximates of plant heights (stems) were measured using a ruler from the soil surface to the tip of the plant. Next, the approximate leaves' surface area was measured using the millimetre graph paper method by taking a leaf and tracing it over a graph paper. The grids covered by the leaf were counted to give the area and then multiplied by two to account for the double-sided surface area of the selected leaf (Fascella et al., 2009a).

Figure 4.1 shows plant pots placed on a capillary mat (3mm thickness) during seed germination to allow a proper and uniform watering pattern. This figure shows the Sanyo growth chamber Model MLR-351 used in the experiment.

Figure 4.1: Plant pots and Sanyo growing chamber used for growing the plants.

Table 4.1: *Plants' optimum growing conditions for the DFR laboratory method validation.*

Plant's type (Variety)	Sowing depth (cm)	Temperature (c°)		Light duration (h)		Targeted and selected leaf	*Plant height (cm)
		Day	Night	Day	Night		
Dwarf French bean "Phaseolus vulgaris" Variety: Tender green	3-4	25	20	16	8	Both lateral leaves of the first emerged trifoliate	13
Wheat "Triticum aestivum " Variety:	2-3	20	16	14	10	First and second emerged true leaf	12
Oilseed rape "Brassica napus" Variety: Charger	2-3	20	16	14	10	Second and third emerged leaves	7
Tomato "Solanum lycopersicum" Variety: Alicante	0.5-1	25	20	16	8	three leaves on the tip of the Second emerged branch	12
Edamame Soya Bean "Glycine max" Variety: Green Shell	3-4	25	20	16	8	Both lateral leaves of the second emerged triflate	17

Table 4.1 shows the optimum growing condition that were set for the plants to grow up to the required growth stage. *Plants heights were measured by ruller from the soil surface to help in the produceability of the same studies in the future.

4.3.2 Pesticide spray preparations and leaf washing solution.

The pesticide spray was prepared fresh on the treatment day by diluting the pesticide formulation in distilled water to match the field dilution according to the Good Agriculture Practice (GAP) standards for DFZ.

The method validation used two emulsion concentrate (EC) 10% with varying solvent systems and one wettable powder (WP). The first EC formulated included solvent naphtha, while the second EC, denoted as DFZ EC(X), had a mixture of octanoic acid-decanoic acid-N, N-dimethylamide. These formulations were supplied for the research purpose only and are not in the market or under any registration procedures for commercial uses.

The DFZ analytical standard with a purity of 98.8% was manufactured and supplied by Syngenta Crop Protection AG GLP testing facility WMU (Switzerland) for the analysis. The prepared concentration (approximately 0.625 mg mL^{-1}) was prepared, which corresponds to most tested plants' average field application rate (125 g DFZ 200 L^{-1} hectare^{-1}).

Following OPPTS Guidelines and prior reports (EPA, 2009; Iwata et al., 1977), Plant leaves were dislodged using an aqueous surfactant solution of Aerosol OT 0.01% (w/v) purchased from ThermoFisher Scientific, Stortford, UK. As the shelf life of the Aerosol OT 0.01% (w/v) at room temperature is 48 h according to the producer, a stock of 0.01% (w/v) concentration was prepared fresh on each treatment day from the concentrate.

4.3.3   Plant treatment and DFR lab technique.

Before treating the plants to quantify the residue on the surface (DFR), validating the appropriate and efficient amount of the wash-off solution was crucial to ensure the dislodgeable fraction of the residue was thoroughly rinsed off and recovered from the treated leaves. Therefore, five replicates of each targeted plant leaf mentioned in Table 4.1 above were selected for the validation process. Plants were chosen for their common uses and leaf texture differences with varying hairiness and waxy cuticle.

Treatment was performed by dispensing 40 uniform droplets (20 droplets per leaf) of 0.2 μL each onto the surface of the targeted leaves (2 leaves for each replicate). The plants were treated with DFZ 10% formulations prepared with a concentration of approximately 0.625 mg mL^{-1}. The Picus® Electronic Pipette, Single Channel model 735021 was used and purchased from Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany. The electronic micropipette was used to generate droplets of a 0.2 μL size, the smallest reproducible volume that any commercially available micropipette can achieve; to mimic the actual spray in the field as possible. Thus, the amount of DFZ that is expected to be on each tested replicate (2 leaves) is 5 μg. Plants were kept stable at room temperature (22 °C) for 3 h after the treatment allowing the sprayed pesticide to dry on the leaf surface before cutting the treated leaves using clean scissors and forceps. Leaves were then placed in clean and securely capped glass bottles before being washed in multiple, consecutive volumes of freshly prepared Aerosol OT 0.01% (w/v) to ensure a complete rinse off the residue from the plant-treated leaves (Table 4.2 below). The glass bottles were left rolling on an electronic roller for 15 min before the solution was removed and replaced with another fresh aliquot of the washing solution, as shown in Table 4.2 below. This

was repeated several times, such that every two leaves were washed at least for three intervals. Before the chromatographic analysis, decanted residue solutions for each wash were labelled and stored at (-4 °C) in a cold room. The approximate leaves' surface area was measured using the millimetre graph paper method by taking a leaf and tracing it over graph paper. The grids covered by the leaf were counted to give the area and then multiplied by two to estimate the double-sided surface area of the leaf (Fascella et al., 2009b). The residue analysis was done within a week of the storage. A descriptive summary of the method is illustrated in Figure 4.2 below.

Table 4.2: Aerosol OT 0.01% (w/v) required volume and number of washes to rinse off different plant leaves.

DFZ formulation	Crops/leaves	Number of washes	Total volume of AOT 0.01% required (mL)
DFZ EC 10%	French bean	1	30 mL
	Tomato	2	45 mL
	Oilseed rape	3	45 mL
	Wheat	2	45mL
	Soya bean	3	45mL
DFZ WP 10%	French bean	2	45 mL
	Tomato	1	30 mL
	Oilseed rape	3	45mL
DFZ EC(X) 10%	French bean	2	30mL
	Tomato	2	30mL
	Oilseed rape	3	45mL

Table 4.1 summarises the total volume of Aerosol OT 0.01 (w/v) required to wash- off all the DFZ residues from the leaf surface after the spray dry.
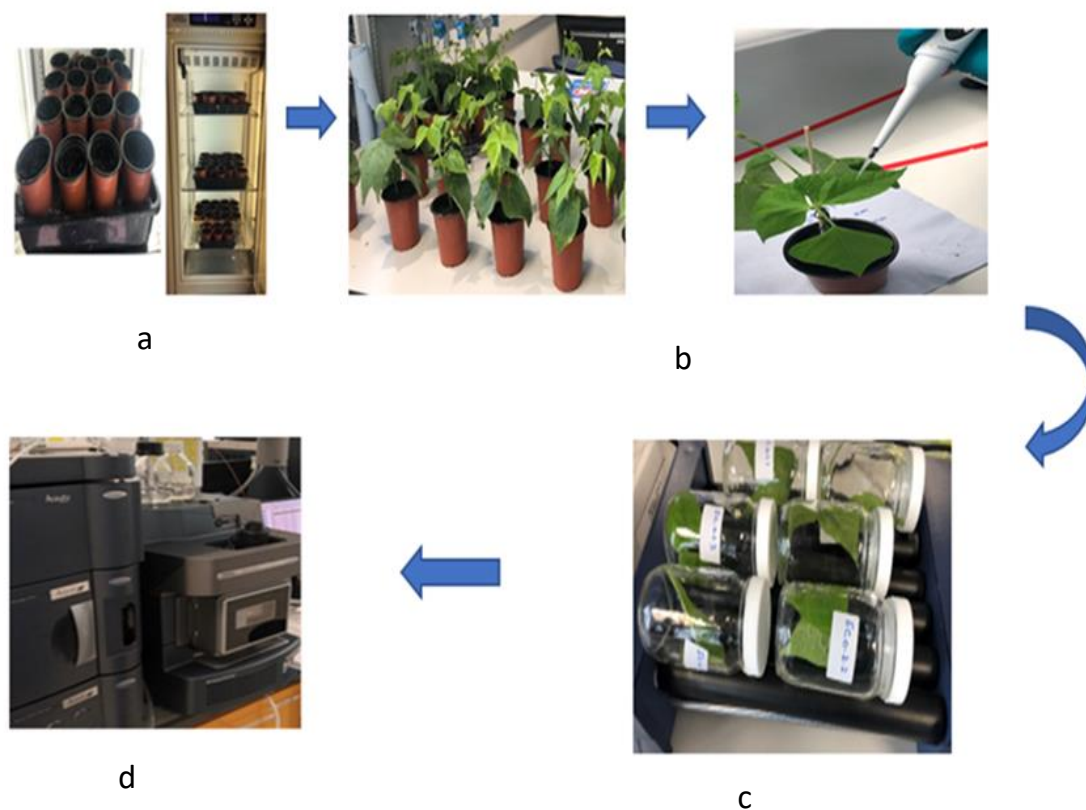
Figure 4.2: Descriptive summary of the DFR laboratory methodology

Figure 4.2 elucidates with pictures the new laboratory technique used to evaluate the dislodgeable foliar residue of pesticides.

a- Plants were sown in all-purpose compost in the Sanyo growth chamber.

b- Plant leaves were treated with an electronic micropipette generating a uniform droplet of 0.2 μl.

c- The glass bottles were left rolling on an electronic roller for a complete rinse off the residue from the plant surface using Aerosol OT 0.01% (w/v).

d- DFR Chromatographic analysis was carried out using a Liquid Chromatography-Mass Spectrophotometer (LCMS).

4.3.4 Uniformity test for DFZ WP 10% (w/v) formulation.

In addition, and due to the nature of the WP formulation that forms suspension on dilution, it was crucial to assess for any limitation of the technique in terms of variability of the leaf dosing and to ensure the efficacy of the method among the different formulations investigated. The effectiveness of the technique was explored by conducting two validation experiments to assess the uniformity of the DFZ WP 10% spray dosing using the controlled pipette method to despense droplets on targeted leaves that would be representative of the sprayed formulation. First, a concentration of (0.625 mg mL^{-1}) corresponding to recommended application rate (125 g DFZ 200 L^{-1} hectare^{-1}) in the (GAP) was prepared from the DFZ WP 10% (w/v) in a 100 mL volumetric flask. The prepared spray solution was poured into a 500 mL clean beaker and then stirred to ensure the utmost mixing of the suspension for 30 min on a magnetic stirrer. Next, the electronic micropipette was used for pipetting 4 µl of the prepared spray formulation (containing 2.5 µg DFZ) to reflect the volume drawn into the pipette to apply 20 droplets (0.2 µL each) to every leaf in the DFR methodology (Section 4.3.3). The drawn 4 µL were dispensed into clean 10 mL volumetric vials half-filled with Aerosol OT 0.01% (w/v), followed by completely filling the vials to the 10 mL mark. Mirroring the DFR lab method methodology, 0.5 mL from each volumetric flask was drawn into another 0.5 mL of Acetonitrile in 2 mL LCMS capped vials for the chromatographic analysis using LCMS. The DFZ nominal concentration in each vial is 0.125 mg L^{-1}. In total, 20 replicates were performed in order to assess the uniformity of dosing between leaves.

In a second experiment, using the same prepared DFZ WP 10% spray (0.625 mg mL^{-1}), a volume of 0.2 µL with a DFZ concentration of (0.125 mg L^{-1}) was drawn from the spray using the electronic micropipette, which was then dispensed in 1

mL of 50:50 of Aerosol OT 0.01%: Acetonitrile solution prepared in a 2 mL LCMS capped vials for the chromatographic analysis using LCMS. The nominal concentration of DFZ in each vial was 0.125 mg L^{-1}. In total, 20 replicates were performed in order to assess the uniformity of dosing between leaves. The concentration of DFZ of each replicate was then assayed by high-performance liquid chromatography, and both the average of the concentrations and RSD% were used to estimate the uniformity of the concentration in the droplets.

In addition, another experiment was performed to test the homogeneity of the concentration in the suspension formed from DFZ WP 10% (w/v) dilution. This is mainly because of the expected sedimentation of the active substance due to the gravity in the suspension formed. The same concentration of (0.625 mg mL^{-1}) corresponding to recommended application rate (125 g DFZ 200 L^{-1} $hectare^{-1}$) in the (GAP) was prepared from the DFZ WP 10% (w/v) in a 100 mL volumetric flask. The prepared spray solution was poured into a 500 mL clean beaker and shaken to ensure the utmost mixing of the suspension for 30 minutes on a magnetic stirrer. This was followed by diluting a 5 mL aliquot in 100 mL of Aerosol OT 0.01% (w/v), followed by another aliquot of 1 mL in 100 mL of Aerosol OT 0.01% (w/v). This step brought the concentration to 0.3125 mg L^{-1}. In the last step of the chromatographic analysis, 0.5 mL from the previous dilution was diluted in 0.5 mL of Acetonitrile in 2 mL LCMS capped vials. This last step halved the concentration to 0.1562 mg L^{-1}. 17 replicates only were used in this experiment due to foam formation at the last 3 vials prepared during the dilution of the suspension. The vials were shaken by hand for 30 seconds before each dilution step, and the micropipette drew the required volume from the top of the aliquots to avoid any formed bubbles due to the agitation.

4.4   Difenoconazole chromatographic analysis

A Waters Corp. (Manchester, United Kingdom) Xevo TQ-S tandem quadrupole mass spectrometer coupled to a Waters Acquity UPLC I-Class was used for Hydrophilic interaction liquid chromatography HILIC-MS/MS analysis. The chromatography was conducted using a 1.8 μm, 2.1× 50 mm Waters Acquity carbon 18UPLC column (Waters Limited, Wilmslow, UK). The equilibrium time for the column was 0.1 min, and the column was held at 40 °C (±5) while the sample injection volume was 1 μL. The running time was 7 min, and the retention time of the difenoconazole was at 1.14 min approximately.

The analysis included two transitions from the Q1 molecular ion (MH$^+$) 406.085 m/z to Q3 111.0396 m/z with confirmatory Q3 of 251.0702 m/z, ES+(difenoconazole) using the mass spectrometer for the primary and confirmatory detection of difenoconazole respectively. The mobile phase composition (A1) consisted of OPTIMA grade water with 0.2% formic acid, and a mobile phase (B1) was 100% Acetonitrile OPTIMA grade. The mobile phase gradient is described in Table 4.2 below, and the total mobile phases flow rate was 0.4 mL/min throughout the analysis.

LC-MS chromatogram of the blanks and sample solution validation was visually examined to ensure the integrity of the analysis and the existence of any signal interference. The Limit of Detection (LOD) and Limit of Quantification (LOQ) was set by assessing the precision and accuracy levels of a concentration gradient ranging from 0.0002 and up to 0.08 μg mL^{-1} along with the determination of minimum concentration with a noise-to-peak ratio greater than 10:1. Linearity was assessed as a part of the analytical method validation, where a linear response was observed for two sets of DFZ calibration standards at five concentration levels by dilution using 50:50 of 100% Acetonitrile and 0.01% (w/v)

Aerosol OT in the range between 0.004 - 0.04 µg mL^{-1}. For research purposes, the two sets' concentration gradient was prepared from the 98.8% DFZ analytical standard was manufactured and supplied by Syngenta Crop Protection AG GLP testing facility WMU (Switzerland) for the analysis.

Five fortification levels of DFZ equivalent to 0.2 µg mL^{-1}, 0.1 µg mL^{-1}, 0.08 µg mL^{-1}, 0.04 µg mL^{-1}, and 0.016 µg mL^{-1} (two replicates at each level) were analysed as part of the quality control procedure. The accuracy and precision of the validation method were performed according to the SANTE/2020/12830 Rev.1 methods (European Commission, 2021) as recommended by EFSA guidance 2022 (Charistou et al., 2022). A minimum of two fortification levels appropriate to the LOQ and the likely residue level or 10x LOQ should be assessed with an ideal mean recovery range from 80-110% (European Commission, 2021). For precision, the relative standard deviation (RSD%) should be ≤ 2% per fortification level. 7 replicates were used for each concentration level tested for accuracy and precision using the regression equation calculated from the regression function "y= bx+a" where b value is the slope coefficient, a is the intercept constant, Y is the dependent variable (plotted on the Y-axis), and X is the independent variable (plotted on the X-axis). Samples were prepared in a final volume of 1 ml by diluting 0.5 mL of the residue in the washing solution with 0.5 mL of Acetonitrile 100% LCMS grade.

The effect of different leaves on the analysis of difenoconazole EC 10% and WP 10% were examined, although different leaves' effects on DFZ EC (X) 10% were not tested due to the time limitation of the project and the remarkable similarity in both EC formulations. A concentration of 0.8 mg mL^{-1} of difenoconazole was prepared for that purpose. Then, 8 µl of this prepared concentration was dropped in a 30 mL Aerosol OT 0.01% freshly prepared in a volumetric beaker. This was

then followed by diluting 0.5 ml of the prepared solution into 0.5 mL of 50:50 Aerosol OT 0.01%: Acetonitrile 100% reaching a final concentration of 0.106 µg mL^{-1} for a all the three replicates. Another three replicates of the same concentration were prepared where the targeted leaves were immersed. This was then followed by a shaking procedure on the electronic roller for 15 min before starting the analytical procedures to quantify the difenoconazole concentration in both solutions prepared.

It is well known that the difenoconazole chosen for this experiment belongs to the triazole group of fungicides known to have high photochemical stability and low biodegradability, making it persistent in water bodies (Maurya et al., 2019). In addition, a standard stability test was performed to comply with the latest SANTE/2020/12830 Rev.1 methods, which included the guidance document on pesticide analytical methods for risk assessment and post-approval control and monitoring purposes for confirmation purposes (European Commission, 2021). The guidance indicated that standard solutions (stock, calibration etc.) should be stored in a fridge or freezer. The stability of an existing standard was then checked by preparing a new stock standard and comparing the residue degradation. The means from at least 5 replicate measurements for each solution should not differ by more than 10% according to the guidelines. In the stability test, the prepared standard was kept freezing in -80 ˚C for a month period. Each concentration level was injected 5 times in the LCMS, and the mean value was compared with the newly prepared standard.

According to the literature, the storage stability of difenoconazole in plants when stored under freezing conditions (-18°C) was proved to be stable for at least 12 months (Anastassiadou et al., 2021). Thus, no further investigation was carried

out to validate the stability of the DFZ residue samples when stored with plant wash-off extracts at -20 °C for less than a month period before the analysis.

Table 4.3: Difenoconazole mobile phase gradient.

Gradient	Time (min)	Flow (ml/min)	%A*	%B*
1	Initial	0.4	40.0	60.0
2	1.0	0.4	40.0	60.0
3	3.0	0.4	20.0	80.0
4	3.1	0.4	10.0	90.0
5	4.0	0.4	10.0	90.0
6	4.1	0.4	40.0	60.0
7	5.1	0.4	40.0	60.0
8	7.0	0.4	40.0	60.0

*Table 4.3 shows the mobile phase gradient and elution time. The analysis started with A1 (40 %): B1 (60 %) for 1 min followed by a continuous increase in the organic solvent flow to A1 (20 %): B1 (80%), A1 (10%): B1 (90%) at 3 and 3.10 and up to 4 min, respectively, before being returned to the starting condition of A1 (40%): B1(60%) until the end of the run. *A consisted of OPTIMA grade water with 0.2% formic acid. *B was 100% Acetonitrile OPTIMA grade.*

4.5   Results

4.5.1   Analytical analysis of difenoconazole

The difenoconazole analysis followed a precision and accuracy level higher than the levels recommended in the referenced method SANTE/2020/12830 Rev.1 methods (European Commission, 2021) by following an acceptable range of precision (RSD ≤2%) and accuracy (≥ 97.5%) The practical LOQ was set to 0.004 $\mu g\ mL^{-1}$. In contrast, the LOD level was set to 0.002 $\mu g\ mL^{-1}$ for the difenoconazole analysis as per the levels of precision and accuracy described in Table 4.4 below. The calibration curve of the DFZ analysis proved linearity with a correlation coefficient R > 0.999, and the regression equation generated was (Y= 2E+06X + 476.93), which was used to interpret all the concentrations of the analysis based on the LCMS response as shown in Figure 4.4 below. From the visual identification of the determined DFZ chromatographic peak at the LOQ, there was no interference from co-eluting peaks indicated at the retention time of DFZ, as shown in Figure 4.3 below. The mean recovery of each sample solution of selected five fortification levels of DFZ EC and WP 10% (0.21 $\mu g\ mL^{-1}$, 0.1 $\mu g\ mL^{-1}$, 0.08 $\mu g\ mL^{-1}$, 0.04 $\mu g\ mL^{-1}$, and 0.016 $\mu g\ mL^{-1}$) were all within the acceptable range of 98-102%. In addition, the accuracy and precision level (RSD%) for the five replicates prepared at LOQ level, 10x LOQ, and a concentration in between proved to be at least 97.5% and ≤ 2%, respectively. The accuracy of the prepared concentration gradients for the standard was measured by calculating the concentration of the test substance at least at 3 concentration levels using the calibration plot, and the results were expressed as a percentage using Equation 4.1 below :

$$\frac{MC}{TC} \times 100 \qquad\qquad \textbf{\textit{Equ 4.1}}$$

Where MC = Measured concentration using the calibration curve and

TC   =   Theoretical or nominal concentration based on the weight.

These were checked for all DFZ 10% (w/v) formulations tested (i.e., EC, WP, and EC (X)), as shown in Table 4.5 below.

DFZ standard stability proved to be stable under freezing conditions of -80 °C. The stability difference between the newly prepared standard and the old stored one is illustrated in Table 4.6 , with no value above 3.5%.
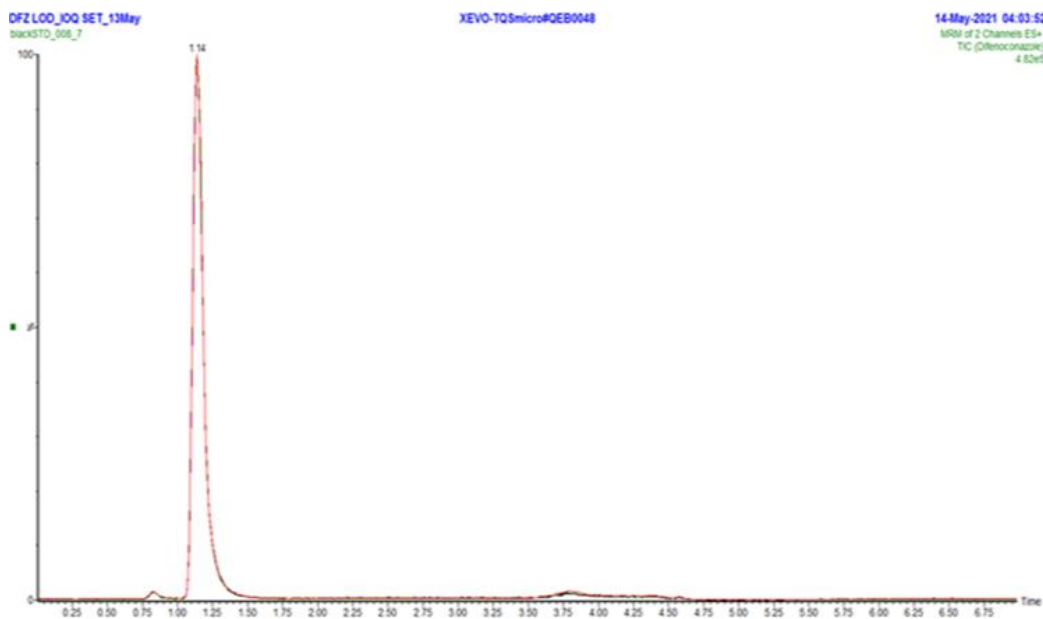
Figure 4.3 shows the difenoconazole peak at the LOQ level (0.004 μg mL-1) during the sample analysis. No interference with any peaks at that level was observed.

Figure 4.3: Difenoconazole chromatogram at the LOQ level (0.004 μg mL^{-1}).

Table 4.4: Precision and accuracy calculation of the concentration gradient used in the DFZ analysis.

Prepared Conc. (µg mL^{-1})	Average Response Area	Calculated Conc. Average (µg mL^{-1}) SD (+/-)	*Precision (RSD%)	**Accuracy%
0.004	9802	0.004 (± 0.00002)	0.6%	98.0%
0.005	12117	0.005 (± 0.00006)	1.4%	102.0%
0.006	14647	0.006 (± 0.00004)	0.7%	100.0%
0.008	19298	0.008 (± 0.00006)	0.8%	100.0%
0.01	24414	0.01 (± 0.00007)	0.8%	102.0%
0.02	46951	0.02 (± 0.00005)	0.3%	100.0%
0.04	93515	0.04 (± 0.0001)	0.04%	100.0%

*Table 4.4 shows the calculated precision and accuracy of the concentration gradient used in the difenoconazole analysis. Data represent the mean (±SD) of n = 7 determinations. SD± mean is the standard deviation. * RSD% is Relative Standard Deviation % while *STDV is sample standard deviation. ** Accuracy % of analytical determination of concentration is calculated by comparing the calculated concentration for each standard derived by back-calculation of the peak area's response using the calibration plot with the actual prepared concentration (see Equation 4.1 above).*

$$y = 2E+06x + 476.93$$
$$R^2 = 0.9997$$
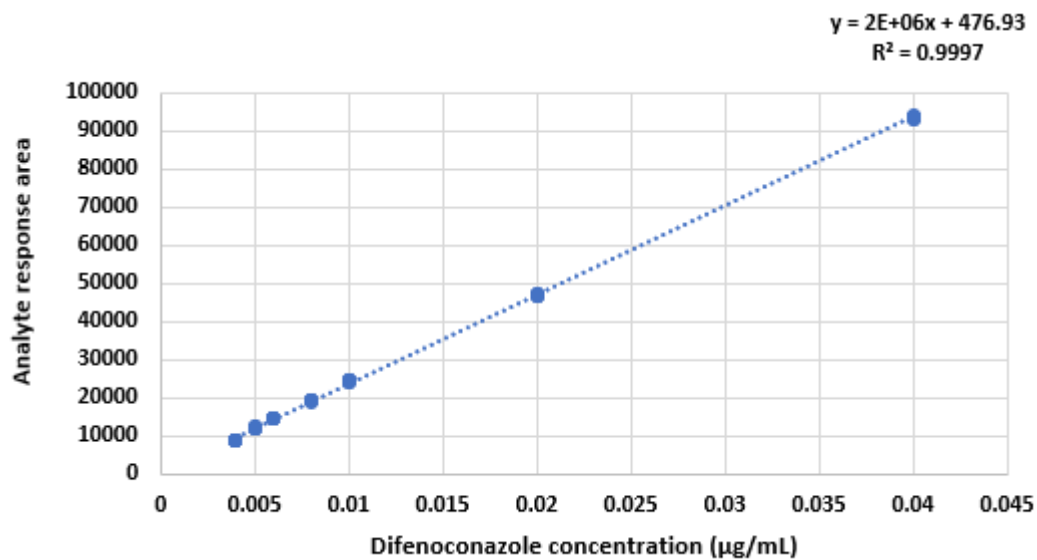
Figure 4.4 shows a difenoconazole calibration plot (range 0.004-0.04 µg mL^{-1}) prepared from the analytical standard (purity 99.8% (w/w)) followed by LC-MS/MS analysis; each point is an average of 5 injections at each concentration level.

Figure 4.4: Difenoconazole calibration plot (conc. range from 0.004 to 0.04 µg mL^{-1}).

Table 4.5: Precision, accuracy, and sample fortification data for difenoconazole (EC, WP, and EC (X) 10%).

Descriptive Statistics	Difenoconazole Nominal Concentration (µg mL⁻¹)								
	DFZ EC 10%			DFZ WP 10%			DFZ EC (X) 10%		
	0.004	0.01	0.04	0.004	0.01	0.04	0.004	0.01	0.04
Mean	0.004	0.01	0.04	0.004	0.001	0.04	0.004	0.01	0.04
Standard Deviation (±SD)	0.00004	0.0001	0.0002	0.00003	0.0001	0.0002	0.00006	0.0001	0.0001
*RSD% (Precision)	1.0	1.4	0.5	0.7	1.5	0.6	1.4	1.3	0.3
Accuracy %	97.0	97.0	99.0	99.0	97.0	98.0	99.0	101.0	100.0
N (replicate number)	5	5	5	5	5	5	5	5	5

*Table 4.5 shows the analysis of five samples at three fortification levels of difenoconazole 10% EC WP10% and EC (X) 10%. These levels are the LOQ (0.004 µg ml⁻¹), 10-fold the LOQ (0.4 µg mL⁻¹) and a concentration in between (0.01 µg mL⁻¹). *RSD % is the relative standard deviation percentage of each tested concentration level. (±SD) is the standard deviation of n = 5 determinations. Accuracy % of analytical determination of concentration is calculated by comparing the calculated concentration for each standard derived by back-calculation of the peak area's response using the calibration plot with the actual prepared concentration (see Equation 4.1 above).*

Table 4.6: Difenoconazole analytical standard stability test.

Nominal conc. (µg mL⁻¹)	Stored standard		Newly prepared standard		Stability difference (± %)
	Mean calculated conc. (±SD) (µg mL⁻¹)	Recovery %	Mean calculated conc. (±SD) (µg mL⁻¹)	Recovery %	
0.004	0.004 (±0.00002)	102%	0.004 (±0.00005)	99.%	±3.0%
0.006	0.006 (±0.0001)	102%	0.006 (±0.00005)	103%	±0.4%
0.008	0.008 (±0.00002)	98%	0.008 (±0.00005)	98%	±0.4%
0.04	0.04 (±0.0004)	99%	0.04 (±0.0001)	100%	±1.5%
0.08	0.08 (±0.001)	98%	0.08 (±0.0004)	98%	±0.0%

Table 4.6 elucidates the stability of the DFZ standard concentrations at 5 different levels for 30 days from the preparation when stored freezing at -80 °C. A recovery % was compared between the newly prepared standard and the stored standard concentrations to comply with the latest level (≤10%) accepted in the referenced method SANTE/2020/12830 Rev.1 methods (European Commission, 2021)

4.5.2 Validation of the plant matrices' effect on the analysis.

This step aimed to investigate the efficiency of using the whole wash-off process in the presence and absence of the plant material to detect any trial errors that may occur due to photolysis, or any other reasons related to the plant matrices. These studies involved spiking two replicates of 30 mL of the washing-off solution with the in-use known concentration of the spray solution for each formulation used (i.e., EC, WP) in the presence and absence of the plant matrices tested. This step was waived for the DFZ EC(X) 10% (w/v) due to the limitation and constraints of Covid 19 on the PhD project.

According to the latest European Commission guidelines, The mean recoveries of at least two prepared replicates should be within 90-110% for formulations of a 10% concentration (European Commission, 2019a). The results indicated that the recovery % was between 103-98%, showing no effect of the different plant matrices used on the analysis process and the quantification of the formulation used, as illustrated in Table 4.7 and Table 4.8 below.

Table 4.7: The effect of different plant leaves on the analysis of difenoconazole EC 10% (w/v).

Plant/crop leaf	Plant Matrix	Practical concentration (μg mL^{-1})	Nominal concentration (μg mL^{-1})	Practical concentration Average (μg mL^{-1})	Recovery (%)
Dwarf French bean	Absent	0.106 0.107 0.102	0.106	0.105	99.0%
	Present	0.108 0.108 0.109	0.106	0.108	102.0%
Wheat	Absent	0.098 0.113 0.115	0.106	0.11	104.0%
	Present	0.111 0.118 0.115	0.106	0.11	104.0%
Oilseed Rape	Absent	0.099 0.110 0.107	0.106	0.105	99.0%
	Present	0.101 0.110 0.108	0.106	0.106	100.0%
Tomato	Absent	0.106 0.106 0.106	0.106	0.106	100.0%
	Present	0.105 0.106 0.107	0.106	0.106	100.0%
Soya bean	Absent	0.107 0.105 0.106	0.106	0.106	100.0%
	Present	0.106 0.106 0.106	0.106	0.106	100.0%

Table 4.7 shows The effect of different plant leaves on the analysis of difenoconazole EC 10%. A final concentration of 0.106 μg mL^{-1} in three replicates was prepared. In addition, another three replicates of the same concentration were prepared where the targeted leaves were immersed. This has been followed by a shaking procedure on the electronic roller for 15 minutes before starting the analytical procedures. A recovery% of approximately 99-103% was observed.

Table 4.8: The effect of different leaves on the analysis of difenoconazole WP 10% (w/v).

Plant/crop leaf	Plant Matrix	Practical concentration (μg mL^{-1})	Nominal concentration (μg mL^{-1})	Actual concentration Average (μg mL^{-1})	Recovery (%)
Dwarf French bean	Absent	0.108 0.108 0.105	0.106	0.107	101.0
	Present	0.110 0.113 0.105	0.106	0.109	103.0
Tomato	Absent	0.106 0.105 0.105	0.106	0.105	99.0
	Present	0.107 0.105 0.105	0.106	0.106	100.0
Oilseed rape	Absent	0.105 0.106 0.105	0.106	0.105	99.0
	Present	0.106 0.105 0.105	0.106	0.106	100.0

Table 4.8 shows The effect of different plant leaves on the analysis of difenoconazole WP 10%. First, a final concentration of 0.106 μg mL^{-1} in three replicates was prepared. Then, another three replicates of the same concentration were prepared, where the targeted leaves were immersed. This has been followed by a shaking procedure on the electronic roller for 15 minutes before starting the analytical procedures. A recovery% of approximately 97-100% was observed.

4.5.3 Validation of the accurate wash-off volume for efficient DFR

These studies aimed to determine the appropriate, and efficient wash-off volume required to dislodge all the DFR for each active substance/ plant material combination studied. In addition, these studies aim to ensure that the volume of the wash-off solvent or detergent in use and the duration of the washing process are efficient in recovering all the DFR that exists on the plant leaves.

All plant leaves tested were treated with the same concentration of DFZ EC%, WP 10% or EC(X) 10% (0.625 mg mL^{-1}), showing different Aerosol OT 0.01% (w/v) volumes required to dislodge all the residue from the leaf surface completely. Therefore, a consecutive number of washes and different volumes of the wash-off solution (Aerosol OT 0.01% (w/v) were required to rinse off all the difenoconazole residue from the leaf surfaces even though all plants were treated with the same concentration of any DFZ formulation tested. A summary of the wash-off solvent required for each DFZ formulation/crop combination, along with the amount calculated for each cm^2 foliage treated, is shown in Table 4.9 below.

Table 4.9: Summary of AOT 0.01% (w/v) required volume and number of washes for each DFZ formulation /crop combination tested.

Pesticide	Crops/leaves	Number of washes	Total amount of AOT 0.01% required	Surface area (two leaves double-sided) (cm2)	AOT 0.01% volume required per cm^2 leaf
DFZ EC 10%	French bean	1	30 mL	45	0.7
	Tomato	2	45 mL	58	0.8
	Oilseed rape	3	45 mL	132	0.3
	Wheat	2	45mL	28	1.6
	Soya bean	3	45mL	65	0.7
DFZ WP 10%	French bean	2	45 mL	55	0.8
	Tomato	1	30 mL	53	0.6
	Oilseed rape	3	45mL	146	0.3
DFZ EC(X) 10%	French bean	2	30mL	52	0.6
	Tomato	2	30mL	45	0.7
	Oilseed rape	3	45mL	140	0.3

Table 4.9 summarise the required volume and number of washes of AOT 0.01% (w/v) for each DFZ formulation/ crop combination tested. Table 4.9 also shows the amount of wash required for every 1 cm^2 tested leaves.

Table 4.10: Validation of the wash-off solution (Aerosol OT 0.01%) volume required to dislodge DFZ EC 10% (w/v) on different plant leaves.

Crops/ Variety	Number and volume of washes	A total volume of wash needed (mL)	Washing interval (min)	Wash Concentration Mean (+/-SD) ($\mu g\ mL^{-1}$)	Total washes mean (+/-SD) ($\mu g\ mL^{-1}$)	Total washes RSD %	Surface area (two leaves double-sided) (cm^2)
Dwarf French bean "*Phaseolus vulgaris*" Variety: Tendergreen	First wash (30 mL)	30	15 min	0.02 (± 0.0008)	0.02 (± 0.0008)	4.0	45
	Second wash (15 mL)		15 min	<LOQ			
Wheat leaves "*Triticum aestivum*" Variety: Skyfall	First wash (30 mL)	45	15 min	0.17 (± 0.010)	0.2 (±0.01)	6.0	28
	Second wash (15 mL)		15 min	0.02 (± 0.002)			
	Third wash (15 mL)		15 min	<LOQ*			
Oilseed rape "*Brassica napus*" Variety: Charger	First wash (15 mL)	45	15 min	0.01 (± 0.001)	0.04 (±0.002)	5.0	132
	Second wash (15 mL)		15 min	0.01 (± 0.001)			
	Third wash (15 mL)		15 min	0.01 (± 0.001)			
	Fourth wash (15 mL)		15 min	<LOQ*			
Tomato "*Solanum lycopersicum*" Variety: Alicante	First wash (30 mL)	45	15 min	0.1 (± 0.009)	0.1 (±0.009)	9.0	58
	Second wash (15 mL)		15 min	0.013 (± 0.001)			
	Third wash (15 mL)		15 min	<LOQ*			
Soybean "*Glycine max*" Variety: Green shell	First wash (15 mL)	45	15 min	0.12 (± 0.02)	0.2 (±0.021)	11.0	65
	Second wash (15 mL)		15 min	0.04 (± 0.004)			
	Third wash (15 mL)		15 min	0.02 (± 0.003)			
	Fourth wash (15 mL)		15 min	<LOQ*			

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Table 4.10 illustrate the validation of different volume of the wash-off solution needed for different plants/crops when all sprayed with difenoconazole (DFZ) EC 10%. Data represent the mean (±SD) of n = 5 determinations. SD± mean is the standard deviation. RSD% is calculated from the mean, and the standard deviation of total washes for each crop/leaf tested is multiplied by 100.

RSD % is the percentage of relative standard deviation between replicates of each wash. *Values below the Limit of Quantification (LOQ ≤ 0.004) were considered zero.

Table 4.11: Validation of the wash-off solution (Aerosol OT 0.01%) volume required to dislodge DFZ WP 10% (w/v) on different plant leaves.

Crops/ variety	Number and volume of washes	A total volume of wash needed (mL)	Washing interval (min)	Wash Concentration Mean (+/-SD) (μg mL^{-1})	Total washes mean (+/-SD) (μg mL^{-1})	Total washes RSD %	Surface area (two leaves double-sided) (cm^2)
Dwarf French bean	First wash (30 mL)		15 min	0.084 (± 0.01)			
"Phaseolus vulgaris" Variety: Tendergreen	Second wash (15 mL)	45	15 min	0.03 (± 0.005	0.1 (± 0.016)	14.0	55
	Third wash (15 mL)		15 min	<LOQ*			
Oilseed rape	First wash (15 mL)		15 min	0.08 (± 0.01)			
	Second wash (15 mL)	45	15 min	0.03 (± 0.004)	0.1 (±0.015)	13.0	146
"Brassica napus" Variety: Charger	Third wash (15 mL)		15 min	0.01 (± 0.002)			
	Fourth wash (15 mL)		15 min	<LOQ*			
Tomato	First wash (30 mL)		15 min	0.08 (± 0.01)	0.1 (± 0.013)		
"Solanum lycopersicum" Variety: Alicante	Second wash (15 mL)	30	15 min	<LOQ*		16.0	53

Table 4.11 illustrate the validation of different volume of the wash-off solution needed for different plants/crops when all sprayed with difenoconazole (DFZ) WP 10%. Data represent the mean (±SD) of n = 5 determinations. SD± mean is the standard deviation. RSD% is calculated from the mean, and the standard deviation of total washes for each crop/leaf tested is multiplied by 100.

*RSD % is the percentage of relative standard deviation between replicates of each wash. *Values below the Limit of Quantification (LOQ ≤ 0.004) were considered zero.*

Table 4.12: Validation of the wash-off solution (Aerosol OT 0.01%) volume needed to dislodge DFZ (X) 10% on different plant leaves.

Crops/ Variety	Number and volume of washes	A total volume of wash needed (mL)	Washing interval (min)	Wash Concentration Mean (+/-SD) ($\mu g\ mL^{-1}$)	Total washes mean (+/-SD) ($\mu g\ mL^{-1}$)	Total washes RSD %	Surface area (two leaves double-sided) (cm^2)
Dwarf French bean "*Phaseolus vulgaris*" Variety: Tendergreen	First wash (15 mL)		15 min	0.11 (± 0.01)	0.1 (± 0.011)	9.0	52
	Second wash (15 mL)	30	15 min	0.013 (± 0.002)			
	Third wash (15 mL)		15 min	<LOQ*			
Oilseed rape "Brassica napus" Variety: Charger	First wash (15 mL)		15 min	0.13 (± 0.01)	0.2 (± 0.011)	7.0	140
	Second wash (15 mL)		15 min	0.03 (± 0.002)			
	Third wash (15 mL)	45	15 min	0.009 (± 0.001)			
	Fourth wash (15 mL)		15 min	<LOQ*			
Tomato "Solanum lycopersicum" Variety: Alicante	First wash (30 mL)		15 min	0.05 (± 0.003)	0.1 (± 0.006)	6.0	55
	Second wash (15 mL)	30	15 min	0.0045 (± 0.0004)			
	Third wash (15 mL)		15 min	<LOQ*			

Table 4.12 illustrates the validation of different volumes of the wash-off solution needed for different plants when sprayed with difenoconazole (DFZ) 10% EC(X) (w/v). Data represent the mean (±SD) of n = 5 determinations. SD± mean is the standard deviation. RSD% is calculated from the mean, and the standard deviation of total washes for each crop/leaf tested is multiplied by 100. *Values below the Limit of Quantification (LOQ ≤ 0.004) were considered zero

4.5.4 Uniformity test

As illustrated in Table 4.13 below, the DFZ WP 10% (w/v) content of the droplets applied to the leaf demonstrated poor uniformity in the applied amount of DFZ in individual droplets dosed. The RSD% was 55% when aliquots of 4 µL were dispensed, showing the variability of the volume removed from the spray formulation before applying the 20 droplets to a leaf. In the case of the 0.2 µL droplets, the RSD% approximately doubled to 105%, demonstrating the potential for highly variable spot dosing onto the leaves. The average recovered concentration from each of the 0.5 mL aliquots drawn from the last 100 mL vial from the spray dilution (100 mL vial) for the 17 replicates analysed was significantly higher than the nominal concentration (676%), with fewer variabilities (RSD 10%) between all tested replicates (n=17).

Table 4.13: The uniformity of the DFZ WP% in the dispensed droplet and the stock suspension.

Pesticide formulation	4 µL droplet volume (0.625 mg L^{-1})		0.2 µL droplet volume (0.625 mg L^{-1})		Stock Conc. (0.625 mg L^{-1})	
	Average accuracy of recovery (+/-SD) %	RSD%	Average accuracy of recovery (+/-SD) %	RSD%	Average accuracy of recovery (+/-SD) %	RSD%
DFZ Wp 10% (w/v)	35.0 % (±19)	55.0%	66.0% (±68)	105.0%	676.0% (±77)	10.0%

Table 4.13 shows the accuracy % of the dispensed droplets from the micropipette and the difference in the concentration of the DFZ WP 10% in the prepared suspension for the analysis. The data above was generated from 20 replicates for the droplets (4 µL and 0.2 µL) and 17 replicates for the stock concentration analysis.

4.6 Discussion

The DFR analytical method implemented proved to be accurate and precise following the OCDE/GD(97)184 guidance for the conduct of studies of occupational exposure to pesticides during agriculture application (OECD, 1997). While the guidelines recommended an accuracy value between 70-120%, the method's accuracy was between 98- 102%. For the analytical laboratory's capability to perform accurate and precise analysis, the precision value less than or equal to 20% (Relative Standard Deviation RSD%) is recommended; however, the current precision of the developed method is less than or equal to 2%. Furthermore, according to the European Commission's guidance for generating and reporting methods of plant protection products, the acceptable mean recovery range is between 90-110% for formulations of a 10% concentration (European Commission, 2019a). Nevertheless, the mean recovery of each sample solution of the five fortification levels of all tested formulations (EC 10%, WP 10% and EC(X) 10%) exceeded the above level in the presence and absence of the plant matrices, indicating the method's suitability.

Furthermore, this method has been applied in the laboratory under relatively controlled conditions compared to field studies. Thus, maintaining a higher precision and accuracy was recommended for quality assurance purposes. Moreover, The precision and accuracy of the three fortification levels tested have been confirmed to comply with the quality assurance criteria of the SANTE/2020/12830 Rev.1 methods (European Commission, 2021), indicating a suitable range of precision and accuracy used in the analysis as elucidated in Table 4.4.

The sample dislodging procedures described in the DFR initial methodology involves washing the whole leaves or leaf punches of a specific surface area (400 cm^2 double-sided) in two 100 mL aliquots of aqueous surfactant solution (i.e. Aerosol OT 0.01%

(w/v)), shaking the leaves in the aqueous surfactant solution for 10 min before retaining the residue for the analysis (EPA, 2009). Nevertheless, the method's guidelines indicated the need for a further technique to validate the efficacy of the washing-off solution to rinse all the residue from the treated leaves (EPA, 2009; Gunther et al., 1973).

From the data generated in this study, different leaves or crops were proved to require different wash-off solution volumes to rinse off all the DFZ residue from the leaf surface when tested in the laboratory under controlled conditions, as shown in Tables 4.10, 4.11 and 4.12 above. That indicates that different formulations of the same active substance may require a different amount of the wash-off solution to dislodge all DFR from targeted leaves. This highlights the importance of this step in any DFR laboratory study.

In contrast, dislodging all types of leaves in the same volume of the wash-off solution regardless of the existing differences between theses leaves could underestimate the pesticide's dislodged fraction, leading to misleading quantification of the DFR and consequently poor comparison between different DFR estimations. Such a gap in the literature could lead to an inaccurate estimate of the non-dietary risk associated with PPPs use if the experiment is conducted in the laboratory. Moreover, the three DFZ formulations (i.e., EC, EC (X) and WP) required different wash-off volumes to rinse off all the pesticide residue, highlighting the importance of the validation step before concluding any laboratory DFR experiment.

For further illustration of the importance of such validation, a simple calculation of the wash-off solution being used in the current methodology and the initial guideline for testing the DFR "the EPA Occupational And Residential Exposure Test Guidelines OPPTS 875.2100 Foliar Dislodgeable Residue Dissipation [EPA 712–C–96–267]" (EPA, 2009)

was performed. In the initial DFR method, 1 cm^2 of leaf surface area required 0.5 mL of the washing solution. This estimation is based on the fact that the initial DFR method recommends using 200 mL (two equal aliquots) of the wash-off solution to rinse 400 cm^2 of leaves' surface area. By applying the same calculation on the tested leaves (i.e., dwarf French bean, wheat, oilseed rape, tomato, and soya bean) with their required volume of the wash-off solution proved in the validation studies above, one could notice that they required more than 0.5 mL from the wash off solution. However, this was not the case with oilseed rape, requiring 0.3 mL per cm^2 from the washing-off solution when tested with all DFZ formulations.

This illustrated the importance of the validation step in the DFR laboratory study to accurately and adequately quantify the entire fraction of the pesticide residue left on the surface after the spray drying. The same concept is applicable when the leaves mentioned were treated with DFZ (X) EC 10% and DFZ WP 10%. Possibly the data generated from testing the method on the dislodgeability of DFZ could be used to investigate many factors separately or in conjugations that could affect the magnitude of the DFR.

In addition, the method validation included experiments to explore the reason for the higher RSD% of DFRs for leaves treated with the DFZ WP 10% (w/v) formulation compared to the emulsion formed from the DFZ EC 10% (w/v) formulations during the validation of the wash-off solution needed for each crop/leaf tested. Although the RSD% was around (13.0-16.0%) for the DFZ WP 10% (w/v) formulation, it was still higher than the RSD% from the DFZ EC 10% experiments on the same crops/leaves (RSD% around 4.0-11.0%). An investigation of the uniformity of the generated droplet and homogeneity of the tank concentration was conducted. These experiments showed a non-uniformity in the DFZ WP 10% content of individual aliquots drawn into

the micropipette. Furthermore, the concentration of the DFZ WP 10% spray was non-homogeneous, with a recovered concentration corresponding to (676 ±77) % of the nominal concentration (0.625 mg mL^{-1}), as shown in Table 4.13.

However, there is a shortage of studies addressing the chemical concentration accuracy and spray mixture uniformity discharged from variable-rate spray equipment (Côté et al., 2012). The poor uniformity in the applied amount of DFZ WP 10% formulation in individual droplets dosed, along with the high concentration recovered in the spray suspension, could consequently reflect on the RSD% in the DFR laboratory studies using the previously mentioned technique (Chapter 4, Section 4.3.3). This would indicate some uncertainty in the DFR results when assessing the factors that could affect DFR with dry formulations such as WP. Nevertheless, research showed that precise measurements of the spray concentration in application tanks also showed variations in the spray concentrations during the field application (O'Connell et al., 1993). It was then recommended by some of the USA pesticide regulatory authorities (California state) to consider tank mix samples qualitatively rather than quantitively (O'Connell et al., 1993). This by ensuring the accurate mixing of the active substance according to the label has been achieved prior to the experiments.

In contrast, the application effectiveness in the field is significantly influenced by how uniformly the pesticide active ingredients are discharged from a sprayer throughout the application period, especially for dry formulations (i.e., WP and WG). Also, without sufficient and continuous agitation in sprayer tanks, most of these dry formulations have tendencies either to float on the surface or get deposited at the bottom of the tank. In either case, pesticides will not be applied at uniform rates, and the concentration could increase or decrease significantly (Ucar et al., 2000). As a result, variations from recommended pesticide concentrations and dosage may considerably

occur and affect the successful application of the pesticides (Abbasi et al., 1997; Gonzalez et al., 1996).

In this validation experiment, the increased recovery % observed in the spray replicates could be due to the location of the sampling within the vial, which was from the top surface of the suspension, where an accumulation of the active substance in the creaming layer may exist. The recovery of the WP DFZ concentration in a smaller droplet of 0.2 μL was better (66 ± 68%) than those from the 4 μL volume (35 ± 19%); this could be due bigger volume could involve drawing more undissolved particles in the suspension that could alter the concentration compared to smaller volume drawn by the pipette. As a result of such variability, excessive amounts of the active substances could present in some droplets than others, which could affect the amount of residue deposited on the surface, especially if that could lead to consequent variabilities in the drying time over the targeted surfaces.

Field DFR experiments do not mandate specific spray equipment to be used in the experiment in terms of the specification related to the pressure or the agitation requirement of the spray. Hence, this tank concentration variability could occur in the field as per the limited research in this area (Ucar et al., 2000). In general, there is little research in the open literature on the uniformity of the pesticide distribution in the sprayer tanks during the application (Akesson et al., 1948; French, 1942; Ozkan & Ackerman, 1999; Ucar et al., 2000). However, many factors that affect the uniformity and homogeneity of such formulations, such as tank size and shape, agitator design and location, sprayer operation, etc., were also proposed. Thus, even with this well-known variation of types of equipment, tanks and operation systems in field application which could result in different effects of the WP formulation homogeneity, this current DFR lab method could not be exactly accurate in providing a precise

conclusion on DFR fraction with RSD% approximately (13 - 16%) (see Table 4.11). Furthermore, it could be challenging to mimic the in-field application of WP formulations due to the sophisticated agitation possibilities and different tank sizes, shapes, and operations currently present in field operations.

Despite this level of variability in the DFR laboratory experiment in terms of testing the WP formulations, and due to the acknowledged variations, that also exist in the field (especially with tank concentrations), the current method could still indicate the residue trend and allow generating more DFR data that could be compared with the in-field DFR data. Thus, a correction factor could be estimated to merge and extrapolate between both.

Data on the dislodgeable foliar residue of pesticides are scarce in the literature (Badawy et al., 2021). EFSA has acknowledged the same in all guidance on assessing the exposure of operators, workers, residents, and bystanders in risk assessment for plant protection products (Charistou et al., 2022; EFSA, 2014a). The report clearly stated the need to generate and collect further DFR data to reflect realistic default values. Moreover, the public consultation report on the same guidance also emphasised the lack of correlation data between factors that affect DFR. Such a gap in the literature could be due to the high cost of the DFR field studies. Besides, this data remains confidential and is only used for registration purposes.

Studying these factors that could influence the degree of pesticide dislodgeability could be a key solution in facilitating the registration and allowing the extrapolation between studies. This must occur based on a scientific finding using fast, affordable, reproducible methods. Therefore, the same could easily be performed using the laboratory-developed method. Except for WP formulations, this is mainly because the developed technique provides a high level of analytical accuracy, precision and more

confidence using rigours validation steps to estimate the DFR in the lab. Although the method could not be accurate with WP formulation compared to ECs, it could still help generate more DFR data

This laboratory-developed method could be a step in identifying the expected residue in the field before employing more extensive and expensive field studies. In addition, this method would allow future researchers to identify factors that could affect DFR using different formulations, co- formulas, metrological conditions, and many other factors solely or in conjugations. Ultimately, that would add more data of the same context in the literature that permits further analysis.

4.7   Conclusion

The newly DFR-developed laboratory method was validated and proved to be a fast, easy, and cost-effective method to predict the dislodgeable residue on plant leaves. The predicted residue could be normalised and extrapolated to values that best describe the field conditions in the future when field data become available to the degree that allows statistical analysis and comparisons. The method is also controllable and could be managed and operated in different desirable environmental conditions or seasons that best describe the DFR field conditions. Besides, the method's described validation adds an extra piece of certainty to the generated data and allows better prediction of the residue level. The lab method recapitulated the available field DFR methodology. Still, it involved the controlled application of droplets to leaves and validation of the wash-off solution used to rinse off all the residue from the leaf surface before the analytical quantification of such residue. From the verification of the wash-off solution used in the case of DFZ ECs 10% or WP10%, one should notice that different formulations of the same pesticide active substance could require a different volume of the same wash-off solution to reach complete rinse of the residue from the same

leaf surface. This step highlighted the importance of validating the washing solution volume required to rinse the residue from the plant surface thoroughly. In addition, the importance of the validation process revealed the limitation of the laboratory DFR method in the generation of DFR data from WP formulations. This was due to the nature of the WP formulation and the physical stability of the suspension prepared in the laboratory, which requires continuous agitation and pressure to maintain the stability and homogeneity of the spray. The experimental error in the WP formulations was noticed to be around 16% compared to less than 11% in the case of the EC 10% formulation. Nevertheless, generating DFR laboratory data for dry formulations using this method and comparing it with the in-field data would be recommended to estimate the real difference and indicate the possibility of using the lab method if dry formulations were involved.

The method would enable the generation of more publicly available data points to allow further extrapolation between the laboratory and in-field experiments. As the proposed technique is relatively rapid and could be performed within hours rather than days, this could also allow investigating multiple factors that may influence DFR, which eventually qualify for a better understanding of such residue to save time, money, and resources for both the industry and the pesticide registration authorities.

Chapter 5 : Formulation Type, Additives and Co-formulants as Factors That May Affect Dislodgeable Foliar Residue (DFR).

5.1   Introduction

Preparation of the pesticide-active substances in a form suitable for use is referred to as *"formulation"*. Pesticides come in many different formulations due to variations in the active ingredient's solubility, ability to control the pest, and ease of handling and transport (Sarwar, 2016). The active substance is the formulation's portion responsible for the killing, repelling, or controlling targeted pests. Despite the importance of such active substances in the formulation, there are other vital components of the formulation; these components are known as *"inert ingredients"* and may range from 0% to 99.99% of the total formulation ingredient (Graham et al., 2014).

Different types of agrochemical formulations can be identified depending on the application, customer acceptability, and regional market requirements. At present, most agrochemical companies attempt to formulate a product in a form that can be applied globally. Table 5.1 below summarises the various major formulation types used for agrochemicals. The first three classes are considered 'classical' formulation types, while the latter has been introduced more recently. However, there has been a dramatic shift from wettable powder (WP) formulations to wettable granules (WG), from emulsion concentrate (EC) to the emulsion in water formulations (EW). In addition to the increased uses of suspension concentrate (SC) formulations due to their environmental advantages, being water-based, and their ease of application (spontaneous dispersion on dilution into water) (Hazra et al., 2017). Nevertheless, formulations based on older technologies such as EC or WP are still available and

represent the most significant volume of plant protection products applied to crops (Knowles, 2008).

Table 5.1 Agrochemical formulations.

Formulation Type	Abbreviation	Description
Emulsifiable Concentrate	EC	Oil suspension of active substances plus emulsifiers
Wettable powder	WP	Solid active substance plus fillers plus dispersing/wetting agent
Solution	SL	Solution of the active substances, mostly in water
Suspension concentrate	SC	Solid/liquid dispersion (suspension)
Emulsion in water (concentrated)	EW	Oil-in-water emulsion
Suspoemulsion	SE	A mixture of suspension and emulsion
Granule	GR	Active substance absorbed on a filler
Water dispersed granule	WDG	Active substance plus filler plus dispersing agent that is readily dispersed in water

Table 5.1 elucidates the most common formulations used in the agrochemical industry for crop protection and their abbreviations.

The success of the formulation is governed by many factors summarized as follows (Knowles, 2008),

- Physicochemical properties.
- Biological activity and mode of action.
- Method of application.
- Safety in use.
- Formulation costs.
- Market preference.

The formulation contains other (or inert) ingredients that may aid in applying the active substance. Inert ingredients could be solvents, carriers, catalysts, synergists, adjuvants, or other compounds. They are added to improve the formulation efficacy and applicability (Cox & Surgan, 2006).

EC formulations have been very popular for many years and represent the most significant volume of pesticide formulations demanded worldwide. They are made from oily or low melting waxy active ingredients, which are soluble in non-polar hydrocarbon solvents such as xylene, C9-C10 solvents, solvent naphtha, and odourless kerosene, or many other hydrocarbon solvents (Koul, 2019). In addition, surfactant emulsifiers are often added to the EC formulations to ensure spontaneous and continuous emulsification with good emulsion stability properties in the spray tanks, especially with different water hardness and climatic conditions (Knowles, 2008).

Comparably, wettable powders are a historical formulation that has been in use for many years and tends to be made from solid active ingredients with a high melting point suitable for grinding. The grinding could be through a mechanical grinder or air milling; air milling usually gives much finer particles (5-10 microns) than mechanical milling (20-40 microns). In addition, WP formulations usually contain dry surfactants as powder wetting and dispersing agent with inert carriers and fillers such as silica particles which prevent the active substance particles from fusing and aggregating together (Knowles, 2008).

In the literature, formulation type and formulation ingredients (co-formulants) which are solvents, carriers, inert material, wetting agents, etc., have been clearly reported by Gunther *et al. (*1995) to affect DFR in his statement, "*The formulation ingredient associated with the active ingredient including the solid components of WP formulations may mediate the full migration of the pesticide into the waxy and other*

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subsurface layers of the foliage by strong sorptive action and as a result of that, the pesticides left on the surfaces for a longer time is accounted to be residues rather than deposits" (Gunther & Blinn, 1955a). Moreover, Whitmyre *et al.* (2004) and the California Department of Pesticide Regulation (CDPR) conducted three studies in which DFR was measured following Endosulfan EC or WP applications. In all the studies, the WP formulation resulted in higher DFR (Beauvais et al., 2010; Whitmyre et al., 2004). As a result, a historical concept of the WP formulation as the worst-case end-product became popular on regulatory platforms (i.e., EPA). Consequently, the EPA recommended considering the range of formulations available for the active substance during the assessed registration. Thus, data may be required for each formulation type. Therefore, the WP end product formulation on its maximum application rate with minimum dilution is recommended for plant protection product registration (EPA, 2017).

In general, results from the literature are not conclusive concerning the effect of different formulations in residue deposits and the behaviour of pesticides. This could be due to the difficulty in isolating other factors that may affect the residues, such as different species and varieties used, different adjuvants, types and concentrations of co-formulants, plant growth and many other factors (Buzzetti, 2017; X. M. Xu et al., 2008).

The ever-increasing costs of pesticides during the last decade, coupled with concerns over minimising their persistence in the environment, have created a strong need to improve the performance of pesticides by optimizing the formulation and application method (Kalyabina et al., 2021). To achieve such a goal, understanding the nature of pesticide residues on or in plants is crucial in ascertaining that the uptake by the target organism is adequately achieved. In other words, not just to increase the total deposits

on plants but also to optimise the ratio of surface (dislodgeable) to subsurface (penetrated) residues to achieve an effective pest control strategy (Westlake et al., 1973). This ratio's magnitude depends on the desired activity of the pesticide in question.

For that reason, adjuvants and surfactants are materials added to spray solutions to improve the performance of crop protection compounds (*i*.e., herbicides) by enhancing the solubility or the compatibility of the active substances. Adjuvants are "*an ingredient in the pesticide prescription, which aids or modifies the action of the principal ingredient*"(Krogh et al., 2003). Published information indicates that adjuvants play a significant role in droplet size spectra, deposit patterns, foliar residues (both dislodgeable and penetrated), and persistence characteristics of pesticides (A. Sundaram et al., 1985; K. Sundaram & Sundaram, 1987).

The adjuvant is a broad term describing any additive to a spray tank that enhances pesticide activity. The composition of adjuvants depends on the active substances' physicochemical properties and the formulation types (emulsifiable concentrate, wettable powder, solution, granules, etc.) (Mesnage & Antoniou, 2018). Besides, much evidence suggests that adjuvants may even increase pesticide toxicity (Chen et al., 2018; Kucharski & Sadowski, 2011; Mesnage et al., 2013; Mesnage & Antoniou, 2018). Therefore, adjuvants can be divided into two general types: (1) formulation adjuvants and (2) spray adjuvants.

The first type consists of adjuvants, which are part of the formulation. In contrast, the second type of adjuvants is added along with the formulated product to the water in the tank of the spray equipment before application on the fields. Spray adjuvants are sometimes called tank mixing additives or just adjuvants, whereas formulation adjuvants are called additives or inert. Examples of adjuvants are surfactants, spreader

stickers, crop oils, antifoaming materials, buffering agents, and compatibility agents (Hochberg, 1996).

Kirkwood (1993) defined adjuvants based on their mode of action; thus, the effect of adjuvants on herbicides may involve effects on "(1) surface phenomena, (2) penetration through the cuticle or stomata, and (3) tissue absorption and systemicity"(Kirkwood, 1993). Later, other researchers classified adjuvants into utility and activator adjuvants (Tu et al., 2001). While utility adjuvants improve the physical properties of the spray (known as spray modifiers) (Penner, 2000), activators may enhance the biological effectiveness of the active substances (McMullan, 2000). Utility adjuvants are usually used for pH adjustment and buffering, as compatibility agents, foaming and antifoaming agents, dyes, hygroscopic substances, wetting agents (spreaders), solubility agents, water conditioners, drift controllers and retention aids (stickers) (Tu et al., 2001). The most common activator is the surfactant. The term surfactant is derived from "surface active agent" and should not be confused with "adjuvant" since adjuvants are not limited to surfactants (Penner, 2000).

The primary function of surfactants is to reduce the surface tension within the external surface layers of water. The lower the surface tension in a pesticide solution, the better the pesticide coverage, allowing more pesticides to reach their target (Arand et al., 2018b). The ability of the adjuvants to adsorb at surfaces or interfaces is based on their chemical structure. Still, the consequence of this adsorption is the surface or interfacial tension reduction of the spray, which is usually measurable by tensiometers (Arand et al., 2018a). Moreover, surfactants also play a significant role in pesticide formulations' performance, microstructure, and physicochemical stability. For example, the suspensibility of a powder-in-liquid suspension or the emulsification of multiple-phase formulations.

An emulsion is a thermodynamically unstable system of two kinds of incompatible liquids. According to the differences in the continuous phase, it can be classified as oil-in-water (o/w) emulsion, water-in-oil (w/o) emulsion and multiple emulsions (Zheng et al., 2020). The difference between oil in water and water in oil emulsion is that o/w emulsions are comprised of oil droplets suspended in an aqueous phase. In contrast, w/o emulsions are the opposite- water droplets suspended in a continuous oil phase (Madhu, 2018; Zheng et al., 2020).

The emulsion promotes the stability of the dispersion of the droplets of the two phases by the emulsifier action when dissolved in the respective phases. By adding a surfactant, the stability of the emulsion can be enhanced (steric stabilization) through complete coverage of the droplet interface by the reasonably thick adsorbed layer of the surfactant. On the other hand, the stability is reduced when insufficient surfactant concentration prevents dispersed droplets from bridging (particularly at high dispersed phase concentration) and leads to rupturing the droplet-continuous phase film boundary. Thus, this observation clearly shows that in the absence of a stable surfactant, the emulsified water droplets are unstable, and coalescence occurs when two interfaces make close contact (Zheng et al., 2020).

Following application to the plant surfaces as liquid droplets, the efficient emulsifiers present a strong ability to lower the interfacial tension, short characteristic times of adsorption and high coverage degree of the water-oil interfaces (e.g., on a leaf surface). In addition, the equilibrium and dynamic adsorption properties of the various surfactant species used to stabilise emulsions contribute to determining the mechanical properties of liquid-liquid interfaces and also these films formed following the application of the formulation to leaf surfaces (Tcholakova et al., 2004; Zheng et al., 2020). Therefore, the diversity and complexity of EC formulations, regardless of

their active substance effect, could be a determining factor that affects DFR based on the emulsion stability, emulsifier and/or the type of surfactant present in these formulations.

Water surface tension is high because water molecules are equally attracted to each other inside the droplets in all directions. Surfactant molecules, on the contrary, have both water and oil properties, one end of its molecules is water-loving (hydrophilic), and the other is oil-loving (lipophilic) (Hall et al., 1999). Therefore, when mixed with water molecules, it replaces water's very cohesive bonds with less cohesive bonds between water and surfactant. As a result, the internal forces are reduced, and less energy is required to deform the droplet; consequently, more droplet spreading will result (Hall et al., 1999). Theoretically, this will eventually lead to more absorption and less DFR on the surface. If the droplet sets on a waxy texture, the surfactant lipophilic part will also stick to the leaf surface, and the hydrophilic part will keep the bond with the water, increasing this leaf's wettability (Hall et al., 1999).

The surfactant molecules must be evenly dispersed in the liquid, with their hydrophilic and lipophilic parts evenly aligned with the water molecules, thereby minimising the surface tension. At this stage, "critical micelle concentration" (CMC) is formed (Moroi, 1992). In the case of pesticide formulations, most adjuvant CMC is achieved at a low concentration, about 0.1% by spray volume (Hall et al., 1999). This CMC is often well below the typically formulated concentration of the surfactant, such that sufficient surfactant molecules are present in the formulation to adsorb at either solid-liquid or dispersed droplet-continuous phase interfaces in the spray formulation (Shi et al., 2011).

There are four different types of surfactants: *anionic surfactant*, which is negatively charged to enhance foaming, and other spreading characteristics, and *cationic*

surfactant, which are positively charged and often very toxic to plants*. The third type is amphoteric surfactants*, which are very specific and, depending on the water's pH, form a positive or negative charge. The fourth type is the *non-ionic surfactants* that do not have a charge in the solution, and they are the most commonly used in the horticulture industry (Czarnota & Thomas, 2013). Thus adjuvants are selective and, when used correctly, do not harm plants, remain stable, and breakwater surfaces (Czarnota & Thomas, 2013). The principal class of non-ionic surfactants are alkylphenol ethoxylates, long-chain alkanol ethoxylates, long-chain alkylamine ethoxylates, and sorbitan esters and their ethoxylates such as Tweens and Spans. In general, partial esters such as span products are lipophilic, while Tween products are hydrophilic. Whilst alkylphenol ethoxylates are among the first commercially available non-ionic surfactants and have enjoyed widespread use in agrochemicals, Some others, such as the nonylphenol ethoxylates, have been banned due to their metabolite toxicity (De Ruiter et al., 2003).

In the literature, two fundamental routes of solute penetration across the plant cuticle are extensively discussed: the *"lipophilic route"*(Baur, 1998; Niederl et al., 1998; Jörg Schönherr & Luber, 2001) and the *"hydrophilic route"* (Jörg Schönherr & Luber, 2001; Schreiber, 2005). Furthermore, cuticular permeability has also been studied extensively due to the vital role of foliar application in crop protection. Most PPPs are lipophilic; as a result, the lipophilic route has been researched extensively (Schreiber, 2005).

The mobility of active substances proved to be accelerated via the lipophilic pathway by substances added to the agrochemical solution known as *"plasticisers"* or *"accelerators"*. They are known to enhance the pesticide active ingredient intake into the plant by reversibly changing the structural properties of the plant cuticle

(Schreiber, 2005). It is hypothesised that these substances decrease the crystalline platelets' size and enhance the amorphous phase's fluidity and consequently decrease impermeable crystals in the cuticle, leading to improving the diffusion coefficient. An example of a plasticising molecule is the organophosphate tris (2-ethylhexyl) phosphate (short: TEHP) (Arand et al., 2018b). These phenomena affect the total absorption and retention of an active substance on the plant leaves (D. Singh & Singh, 2008).

Generally, practitioners usually apply herbicides with various commercially available adjuvants and surfactants to increase the efficacy of the used herbicides (Chow, 2017). However, a study reported an investigation of a non-ionic surfactant combination with 2,4D herbicide effect on DFR. The results, in conclusion, showed that the surfactant inclusion might slightly reduce the DFR and, accordingly, reduce the potential human dermal exposure (Maxwell et al., 2018). On the contrary, the DFR of chlorothalonil and chlorpyrifos on a cranberry bog increased DFR in the presence of a spreader-sticker adjuvant (Putnam et al., 2003). The addition of this adjuvant was meant to extend the foliar pesticide residue during the infestation peak time. Nevertheless, residues should be kept in balance with those remaining on fruits and leaves during harvest or maintenance of the crop to maintain safety and proper use.

Also, the influence of different adjuvants inclusion in a fenitrothion formulation, for example, has been studied by Sundaram and Sundaram (1987); the polymeric adjuvant addition to the formulation reported significantly larger spray droplets during the treatment and higher DFR in balsam fir needles than those containing surfactants and co-surfactants (non-ionic/anionic). In addition, the exact reported formulation was reported to provide a higher ratio of DFR to the penetrated residue and a slower dissipation rate than other formulation combinations (K. Sundaram & Sundaram, 1987)

Selecting a solvent capable of dissolving these active ingredients is vital in the formulation. A suitable solvent that can dissolve the active ingredients and allow further dilution of the dissolved active substance with water is desirable to improve the performance of the active substances therein. Therefore, an effective surfactant system plays a significant role in the solvent combination (Wong Hung et al., 2013). In the same context, these solvents range from organic non-biodegradable to natural ingredients, differentiating between agrochemical products in the market. For example, organic solvents such as xylene, mineral oil, kerosene, isophorone, diethoxol, cyclohexane or n-butanol are commonly used as the key solvent in the EC. Also, they are miscible with a wide range of liquid formulations (Wong Hung et al., 2013). Evidence from the literature suggests that using different solvents may lead to the different responses of the plant leaves and consequently might lead to different DFR of the same active substance if formulated using different solvents from such a wide range of selection (Wong Hung et al., 2013).

5.2   Aims and Objectives

The studies reported in this Chapter aimed to investigate the effect of the formulation types, adjuvants, and additives (different solvent systems) as factors that were identified as potentially having a crucial impact on DFR. Three different studies were conducted on selected plants using difenoconazole EC 10%, WP 10%, and another EC 10% with different solvent systems (denoted hereafter as DFZ(X). Difenoconazole is a broad-spectrum systemic triazole fungicide that controls various fungal diseases in several vegetable and ornamental crops. It acts as a seed treatment, foliar spray and systemic fungicide(FAO, 2007; Ncbi, n.d.). Different difenoconazole formulations were chosen and combined with and without different adjuvants to investigate the effect of the various constituents on the degree of DFR. Also, the impact of the formulation's

solvent system on difenoconazole was investigated. In addition, an analysis of the formulation's dynamic surface tension (DST) measurements was performed in relation to the DFR magnitude of each formulation.

5.3 Materials and Methods

Three different formulation of Difenoconazole 10% (w/v) were formulated and supplied by Syngenta (United Kingdom); two emulsion concentrate (EC) 10% with varying systems of solvent and one wettable powder (WP) formulation. The first EC formulated included solvent Naphtha, while the second EC, denoted as DFZ(X) 10%, had a mixture of octanoic acid-decanoic acid-N, N-dimethylamide; These formulations were supplied for the research purpose only and are not on the market or under any registration procedures for commercial uses. However, the solvents used in the EC formulations are commonly incorporated in many PPPs. Difenoconazole analytical standard (purity of 98.8% (w/v)) was manufactured and supplied by Syngenta Crop Protection AG GLP testing facility WMU (Switzerland) for the analysis purposes.

The adjuvant, Tween 20 surfactant, under the tradename "TWEEN 20-LQ-(CQ) was purchased from Croda, France SAS. In addition, the surfactant Tris(2-Ethylhexyl) phosphate under the tradename "DISFLAMOLL TOF", denoted hereafter as (TEHP), was purchased from LANXESS Deutschland GmbH, Industrial & Environmental Affairs (Germany). Finally, acetonitrile and highly purified water used in the LC-MS analysis were OPTIMA grade and purchased from TheromFisher Scientific, United Kingdom.

All research experiments in this Chapter followed the same method and the validation procedures detailed in Chapter 4. This included the description of the DFR analytical method, method validation, plant growth, treatment, and chromatographic analysis of difenoconazole. In addition, different crops, namely French bean, tomato, and oilseed

rape, were all grown according to the growing conditions mentioned in section 4.2.1 in Chapter 4.

Table 5.2: Summary of the DFZ formulations and crop combinations used.

Formualtion	Plants/crop leaves
DFZ EC 10% (w/v)	French bean, tomato and oilseed rape
DFZ EC10% +Tween-20 0.1% (w/v)	French bean
DFZ EC 10% + TEHP 0.1% (w/v)	French bean
DFZ EC 10% + TEHP 0.3% (w/v)	French bean
DFZ WP 10% (w/v)	French bean, tomato and oilseed rape
DFZ WP 10% +Tween-20 0.1%(w/v)	French bean
DFZ EC(X) 10% (w/v)	French bean, tomato and oilseed rape

Table 5.2 is a summary table of all the formulations and crop combinations tested in Chapter 5.

This Chapter investigated the effect of different difenoconazole 10% (w/v) formulation types (i.e., EC, WP and another EC formulation formed in another solvent system (EC (X)) on the French bean, tomato and oilseed rape. Also, the effect of in-tank mixing of DFZ 10% WP and EC with different adjuvants were explored. These adjuvants are Tween-20 0.1% (w/v) and TEHP 0.1% (w/v) and 0.3% (w/v) on French bean. The last experiment of this Chapter investigated the effect of different solvents incorporated in the EC formulation on the degree of the DFR. This study used the DFZ EC(X) 10% (w/v) formulation to investigate the DFR recovery % on the same tested crops, as summarised in Table 5.2 above.

All sprays were prepared fresh on the treatment day according to section 4.3.2 in Chapter 4. All DFZ spray concentrations were approximately (0.625 mg mL^{-1}) across all the formulations used to allow a proper comparison, corresponding to the average field application rate of (125 g DFZ 200 L^{-1} ha$^{-1)}$). The DFR laboratory method used in this section is the same method detailed and validated as described in Chapter 4 and summarised in Figure 4.2, "*Descriptive summary of the DFR laboratory methodology*".

Each treatment group comprised ten replicates of selected plants besides three replicates for the control group. The control group involved no pesticide application to detect any residue level that may exist, which could interfere with the interpretation of the obtained results. All plants were treated in the lab under a constant temperature of 21C°.

Dynamic surface tension (DST) measurements for all the formulations were investigated using the bubble pressure tensiometer model BP100 from Kruss GMBH (Hamburg, Germany). The maximum bubble pressure method is easy and convenient for measuring surface tension. In this method, the capillary is immersed in the solution, and a gas bubble is created inside the liquid using gas with controllable pressure (Rapp, 2017). As the pressure increases, the size of the bubble increases until its diameter is identical to the diameter of the capillary (hemispherical bubble). The bubble's pressure is measured at several time points, and the instrument then calculates the surface tension at any given surface time (the measurement of the bubble surface tension at any specific time). The surface age is defined as the time interval between the minimal measured pressure, identified with the bubble formation, and the maximum pressure, which marks the onset of the spontaneous bubble detachment. The corresponding value of surface tension belongs to the latter moment (Christov et al., 2006). The changing surface tension or the dynamic surface tension can then be conveniently measured at the changes in the bubble size over time if the pressure is kept constant (Rapp, 2017).

The Young-Laplace equation below allows for the determination of the surface tension. It establishes the relation between the internal pressure (IP) of a spherical bubble, the radius of curvature (r) and the surface tension (σ).

$$IP = \frac{2\sigma}{r} \qquad\qquad \textbf{\textit{Equ 5.1}}$$

The most significant curvature of the gas bubble is expected at the point when the maximum pressure is measured. At that time, the capillary radius will equal the radius of the formed bubble. During this, a pressure maximum (P max) is measured. The hydrostatic pressure ($P°$) is given by the depth of the immersed capillary and the liquid density (Rapp, 2017).

$$\sigma = \frac{(P\ max - P°) * r}{2} \qquad\qquad \textbf{\textit{Equ 5.2}}$$

The resultant surface tension corresponds to the specific value of certain surface age (ms). This time dependence is the main difference for measuring the static surface tension, as the surface age is the time from the start of the bubble formation to the occurrence of the P maximum (Rapp, 2017).

Using the tensiometer, 40 mL of DFZ formulations and water were sampled in the measurement chamber. Single surface tension values (mN m^{-1}) were measured over a specific time period, ranging from 10 ms to 100000 ms surface age for all tested formulations. In addition, water measurements were tested up to 53000 ms surface age due to the early observed confidence in the equilibrium surface tension. Therefore, results can be interpreted from the decreasing DST curves as a function of time, as different surfactants diffuse at differential rates, determined by individual surfactant and formulation microstructure, to the surface of the forming droplet. This surface

ageing process contributes to the response of the droplet with respect to spreading on a leaf surface after application and the extent of its subsequent liquid film formation.

5.3.1 Statistical analysis

All the raw data were analysed using SPSS, IBM version 27.0 (BM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). All collected residue data were tested for normality using the Shapiro-Wilk test. A significance value (P-value) $\geq$ 0.05 of the test indicated the normality besides ensuring that the skewness and Kurtosis values are in the acceptable range of the normal distribution (-1,1) and (-3,3), respectively (Shapiro & Wilk, 1965).

The One-way ANOVA (Analysis of Variance) was used to analyse the difference between the means of tested groups (more than 2 independent groups). Tukey's HSD was used to detect the post hoc significance between the groups in case of any significance was noticed. This test was used to compare all the possible pairs of means.

Significant differences among group means were calculated using the F statistic, which is the ratio of the mean sum of squares (the variance explained by the independent variable) to the mean square error (the variance leftover). If the F statistic was higher than the critical value (the value of F that corresponds with alpha value (P), usually 0.05), then the difference among groups was deemed to be statistically significant (T. Kim, 2017). In addition, the means' 95% confidence interval (CI), the standard deviation (SD), and the relative standard deviation RSD were calculated during the statistical analysis.

An Independent T-test was used to compare the DFR mean values for 2 independent treatment groups after confirming the data normality using SPSS, IBM version 27.0.

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SPSS considers any data value to be an outlier if it lies outside the 3rd quartile + 1.5 * interquartile range and 1st quartile − 1.5 * interquartile range. In addition, SPSS considers any data value to be an extreme outlier if it lies outside the following ranges: 3rd quartile + 3 * interquartile range and 1st quartile − 3 * interquartile range. Outliers are displayed as tiny circles or astricts in the plot with an associated number indicating which observation in the dataset is the outlier.

Determining the effect of these outliers was investigated. Therefore, the statistical tests were conducted twice, with outliers (i.e., the entire sample) and without outliers. If the presence of the outliers did not statistically change the results and their presence did not affect the normality of the data, the results were reported without omitting these outliers.

5.4   Results

In this first experiment, the effect of different difenoconazole formulations (i.e., EC 10% and WP 10%) on DFR recovery %, as shown in Table 5.3, revealed no significant difference between both formulations on French bean and oilseed rape. The significance value of both crops was above the significance level of the independent T-test (P = 0.1, 0.1), respectively, when equal variances are not assumed as tested by the levant test of equal variances (Levant test P value ≤ 0.05 indicates that equal variances are not assumed). However, statistical significance was achieved in the case of tomato with a P-value ≤ 0.0005. The mean DFR recovery% (±SD) in the case of the DFZ EC 10% (w/v) on tomato leaves was higher (60.0 ± 1.2%) than the WP 10% (w/v) (39.0 ± 5.0%), while both DFR mean recovery % were at the same intensity with the other tested crops (i.e., French bean and oilseed rape). The normality of the data was tested using the Shapiro-Wilk normality test in SPSS. All the data sets for each treatment group proved to be normally distributed, with no value below the significance level of 0.05.

This normality was confirmed by looking at a quantile-quantile (Q-Q) plot for each group, where data observations were approximately around the straight line.

Figure 5.1 below is a boxplot chart that compares the DFR median recovery (μg) of the two tested DFZ formulations (EC 10% and WP 10%) on different crops/leaves (i.e., French bean, tomato, and oilseed rape). The significance is evident in the case of tomato between both formulations tested. Lower and upper box boundaries are 25th and 75th percentiles, respectively, while the line inside the box is the median, and the error bars represent the confidence interval (CI).

Table 5.3: Comparison between DFR recovery of different DFZ formulations (EC 10% and WP 10%) on French bean, tomato, and oilseed rape leaves.

Descriptive statistics	French bean		Tomato		Oilseed rape	
	EC 10%	WP 10%	EC 10%	WP 10%	EC 10%	WP 10%
N (Replicates)	10	10	10	10	10	10
Mean µg (±SD)	4.1 (±0.1)	3.7 (±0.7)	3.0 (±0.1)	2.0 (±0.2)	1.6 (±0.2)	1.8 (±0.3)
Median (µg)	4.1	3.6	3.0	2.0	1.6	2.0
RSD%	4.0 %	17.0%	2.0 %	12.0 %	11.0%	16.0%
DFR Recovery% Mean (±SD)	82.0% (±3.0)	74.0% (±13.1)	60.0% (±1.2)	39.0% (±5.0)	31.0% (±3.3)	37.0% (±6.1)
Shapiro-Wilk Normality test (P-value) (Significance P≤ 0.05)	0.08**	0.3**	0.4**	0.9**	0.4**	0.1**
T-test (significance 2-tailed)	0.1		≤ 0.0005 *		0.1	

*Table 5.3 shows the descriptive statistics comparing two different formulations of the DFZ 10% (i.e., EC and WP) on French bean, tomato, and Oilseed rape. *Independent T-test assuming non-equal variance between the groups has been used and showed a significant difference between tested formulations on tomato leaves only (p ≤0.0005). **Difenoconazole DFR residue from both formulations on all cops/leaves proved to be normally distributed when tested by the Shapiro-Wilk test using SPSS* where P≥0.05. RSD % is the percentage of relative standard deviation among samples of each tested concentration level, while the SD± is the standard deviation for the 10 determinations (n=10).*

Figure 5.1 is a boxplot chart for the DFR median recovery of different DFZ formulations (EC 10%, WP 10%) on French bean, tomato, and oilseed rape leaves. Lower and upper box boundaries are 25th and 75th percentiles, respectively, while the line inside the box is median, and the error bars represent the 95% confidence interval (CI). The significance was observed in the comparison between both formulations on tomato only. Circles or asterisks on either end of the box plot indicate potential outliers in the data, which were checked and proved not to affect the statistical results.
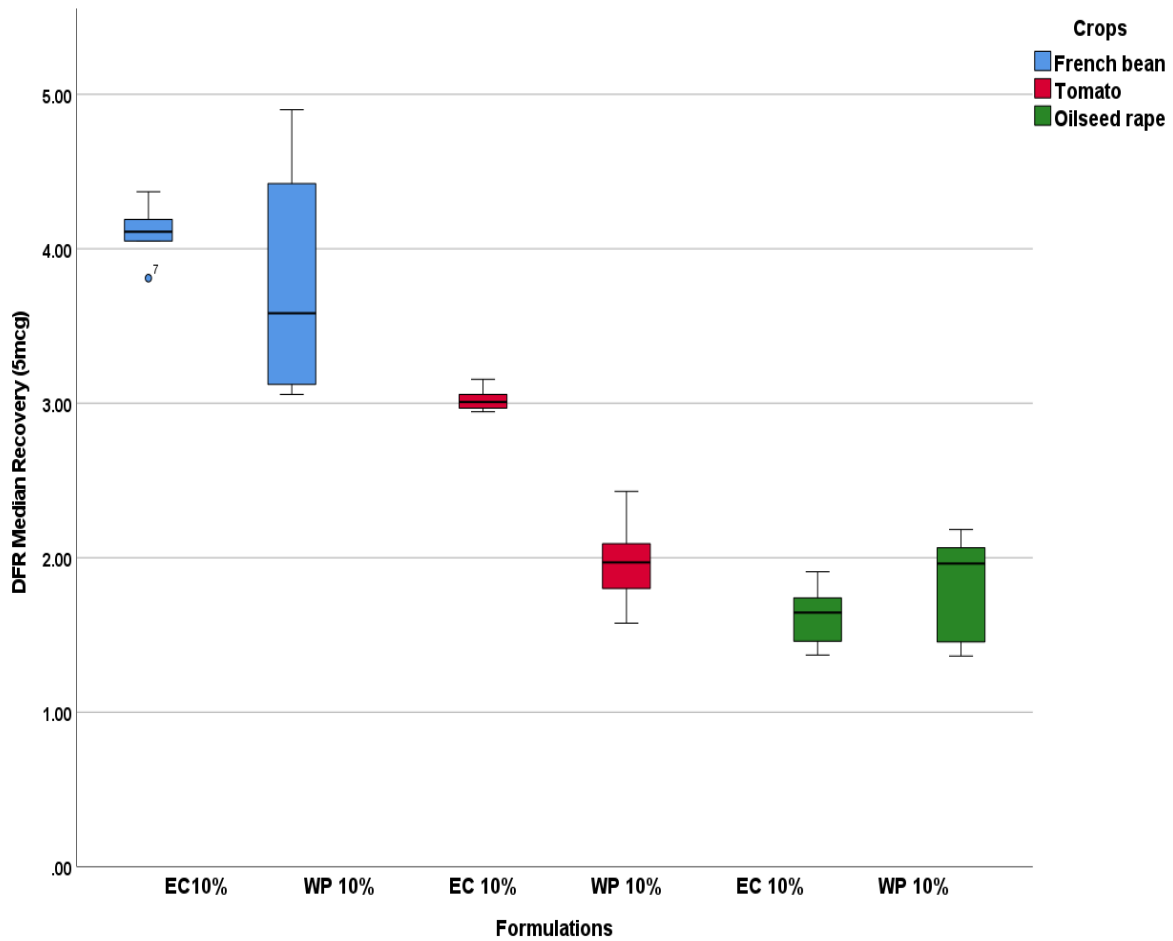
Figure 5.1: Boxplot chart for DFR recovery of different DFZ formulations (EC 10%, WP 10%) on French bean, tomato, and oilseed rape leaves.

In the second experiment of this Chapter, The DFZ EC 10% (w/v) formulation was mixed with Tween-20 0.1% (w/v) and TEHP at two different concentrations (0.1% and 0.3% w/v). The one-way analysis of variance showed that the effect of adjuvant addition was significant at least in one treatment group (p = 0.006). As shown in Table 5.5. The post hoc analyses using the Tukey HD post hoc test for multiple comparisons indicated that the significance was in the formulation containing TEHP 0.1% (w/v) when compared to EC 10% (w/v) only, with the latter resulting in more DFR in comparison.

Generally, the effect of the adjuvant addition on the degree of DFR was positive. It slightly decreased the percentage of DFZ DFR recovery in all the treated groups compared to the DFZ EC 10% without any adjuvant, as shown in Table 5.4. However, the effect of the adjuvant addition was statistically significant (p= 0.003) only with TEHP at the concentration of 0.1% (w/v), as shown by the Tukey HSD test in Table 5.5. The DFR mean recovery was the highest with the EC 10% formulation (82.0 ± 4.0%), followed by the TEHP at 0.3% and Tween-20 at 0.1% (w/v), leaving the DFR mean recovery% of (78.0 ± 4.1%), (77.0 ± 6.3%), respectively. On the other hand, the DFR mean recovery was the lowest (74 ± 4%), with the formulation containing TEHP adjuvant at a concentration of 0.1% (w/v). This result is well presented in the boxplot of Figure 5.2, showing the median DFR recovery % for each formulation tested on French bean leaves. The box plot shows the lower and upper box boundaries, which are the 25th and 75th percentiles of the data. In contrast, the line inside the box is the median, and the error bars represent the data's 95% confidence interval (CI). In addition, the normality of the data was tested using the Shapiro-Wilk normality test in SPSS. All the data sets for each formulation tested proved to be normally distributed with no value below the significant level of 0.05.

Due to the detection of outliers in the data set of EC 10% (w/v) and EC 10% with TEHP 0.1 % (w/v), as shown in Figure 5.2 below (circles or asterisks on either end of the box plot), determining the effect of these outliers was checked. For that reason, the statistical test was conducted twice, with outliers (i.e., the entire sample) and without outliers. The presence of the outliers did not statistically change the results, and their presence did not affect the normality of the data.

Table 5.4: The effect of different adjuvant addition on DFZ EC 10% DFR on French bean.

DFZ EC 10% formulation groups				
Descriptive statistics	DFZ EC 10% (w/v)	EC 10% +Tween-20 (0.1% w/v)	EC 10% +TEHP (0.1% w/v)	EC 10% + TEHP (0.3% w/v)
N(Replicates)	10	10	10	10
Mean μg (±SD)	4.1 (±0.2)	3.9 (±0.3)	[†]3.7 (±0.2)	3.9 (±0.2)
Median	4.1	3.9	3.7	3.9
(RSD%)	5.0%	8.0%	6 %	6%
DFR Recovery% Mean (±SD)	82.0 % (±4.1)	77.0 % (±6.3)	74.0 % (±4.0)	78.0% (±4.1)
Shapiro-Wilk Normality test (P value) (Significance P=0.05)	0.1**	0.8**	0.1**	0.6**
ANOVA Significance	P= 0.006			

*Table 5.4 elucidates the descriptive statistics of difenoconazole EC 10% with and without different adjuvants on French bean with different concentration (i.e., Tween-20 0.1% (w/v) and TEHP with both 0.1% and 0.3% (w/v)). †The mean difference is significant at the 0.05 level (p ≤ 0.05). Tukey's HSD test for multiple comparisons was tested with ANOVA, which showed a significant effect from adding TEHP 0.1% (w/v) only. The data above represent the mean (±SD) of (n=10) determinations. RSD % is the percentage of relative standard deviation among samples of each tested group. **Data proved to be normally distributed when tested by the Shapiro-Wilk test using SPSS* where P ≥0.05.*
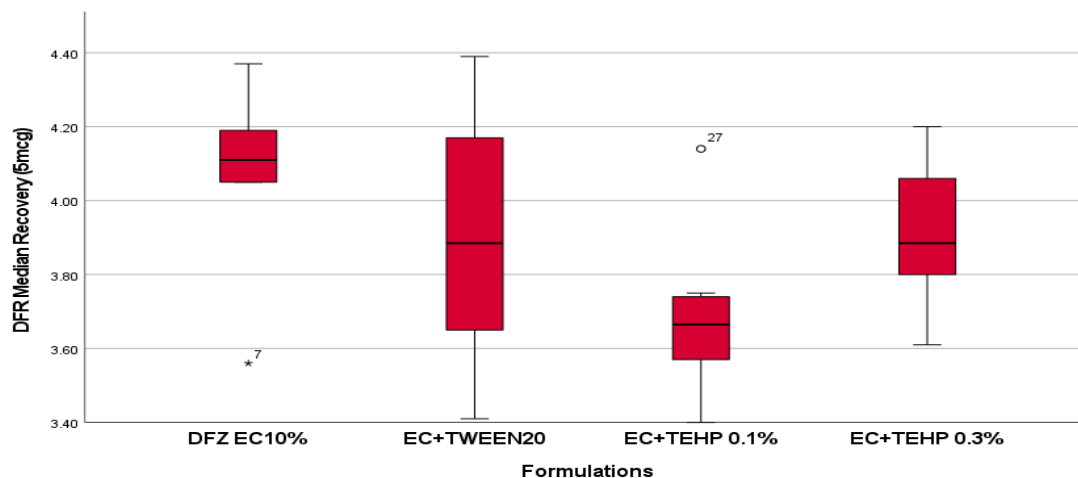
Figure 5.2 is a boxplot showing the DFR median recovery from different DFZ EC 10% w/v with and without the addition of different adjuvants (i.e., Tween-20 (0.1% w/v), TEHP 0.1% and 0.3% (w/v)). Lower and upper box boundaries are 25th and 75th percentiles, respectively, while the line inside the box is median, and the error bars represent the 95% confidence interval (CI). Circles or asterisks on either end of the box plot indicated potential outliers in the data, which were checked and proved not to affect the statistical results.

Figure 5.2: Box plot for difenoconazole EC 10% DFR median recoveries with and without adjuvants on French bean.

Table 5.5: Tukey HSD test for difenoconazole EC 10% DFR mean differences.

Tukey HSD test for multiple comparisons

(I) Formulations	(J) Formulations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
DFZ EC10%	EC+TWEEN20	.19500	.10547	.268	-.0891	.4791
	EC+TEHP 0.1%	.40400*	.10547	.003	.1199	.6881
	EC+TEHP 0.3%	.17900	.10547	.340	-.1051	.4631
EC+TWEEN20	DFZ EC10%	-.19500	.10547	.268	-.4791	.0891
	EC+TEHP 0.1%	.20900	.10547	.214	-.0751	.4931
	EC+TEHP 0.3%	-.01600	.10547	.999	-.3001	.2681
EC+TEHP 0.1%	DFZ EC10%	-.40400*	.10547	.003	-.6881	-.1199
	EC+TWEEN20	-.20900	.10547	.214	-.4931	.0751
	EC+TEHP 0.3%	-.22500	.10547	.162	-.5091	.0591
EC+TEHP 0.3%	DFZ EC10%	-.17900	.10547	.340	-.4631	.1051
	EC+TWEEN20	.01600	.10547	.999	-.2681	.3001
	EC+TEHP 0.1%	.22500	.10547	.162	-.0591	.5091

*Table 5.5 shows comparisons between all tested DFZ groups. * The mean difference is significant at the 0.05 level. Tukey HSD test for multiple comparisons showed a significant difference between DFZ EC 10% (w/v) and DFZ with TEHP 0.1% (w/v) added. The lower and upper bounds at the 95% confidence intervals were stated for every comparison.*

In the third experiment of this Chapter, The DFZ WP 10% (w/v) formulation was mixed with Tween-20 0.1% (w/v) or TEHP 0.1% (w/v). The one-way analysis of variance showed no significant effect of adjuvants addition (p = 0.044). The post hoc analysis using the Tukey HD post hoc test for multiple comparison significance was unnecessary in that case. The mean DFR recovery% of WP 10% (w/v) was similar with the addition of Tween-20 0.1% (w/v) (74.0 ± 13.0%) and (74.0 ± 12.0%) respectively. Despite the insignificant difference between WP 10% (w/v) formulation and the WP 10% +TEHP 0.1% (w/v) the latter DFR recovery% was slightly higher (78.0 ± 15.0%). In addition, the normality of the data was tested using the Shapiro-Wilk normality test in SPSS. All the data sets for each WP formulations tested proved to be normally distributed with no value below the significant level of 0.05. in conclusion, the DFR mean recoveries % for WP formulations were relatively close among all tested groups, with a recovery%

ranging from (74 to 78%). The RSD% for all WP formulations ranged from 12- 15%, reflecting the poor uniformity of the formulation, which caused an increase in the RSD% for the DFR experiment when tested with DFZ WP 10% (w/v). The DFZ WP uniformity experiment in Chapter 4, Sections 4.3.4 and 4.5.4, provides a detailed explanation of this observation.

Table 5.6: Different effects of adjuvant addition on DFZ WP 10% (w/v) DFR on French bean

DFZ WP 10% (w/v) formulation groups			
Descriptive statistics	**DFZ WP 10% (w/v)**	**WP 10%+Tween-20 0.1% (w/v)**	**WP 10%+TEHP 0.1% (w/v)**
N (Replicates)	10	10	10
Mean µg (±SD)	3.7 (±0.7)	3.7 (±0.6)	4.0 (±0.7)
Median	3.6	3.9	3.9
Variance (RSD%)	18.0%	16.0%	18.0
DFR Recovery% Mean (±SD)	74.0 % (±13.0)	74.0 % (±12.0)	78.0 % (±15.0)
Shapiro-Wilk Normality test (P value) (Significance P=0.05)	0.08**	0.1**	0.3**
ANOVA significance	P= 0.4[†]		

*Table 5.6 elucidates the descriptive statistics of difenoconazole WP 10% (w/v) with and without different adjuvants (i.e., Tween-20 0.1% (w/v) and TEHP 0.1% (w/v). † The mean difference is insignificant at the 0.05 level. When tested with ANOVA, there was no significant effect from adding either Tween-20 0.1% (w/v) or TEHP 0.1% (w/v) to the DFZ WP10% formulation (P=0.44). The data above represent the mean (±SD) of (n=10) determinations. RSD % is the percentage of relative standard deviation among samples of each tested group. **Data proved to be normally distributed when tested by the Shapiro-Wilk test using SPSS* where P≥0.05.*
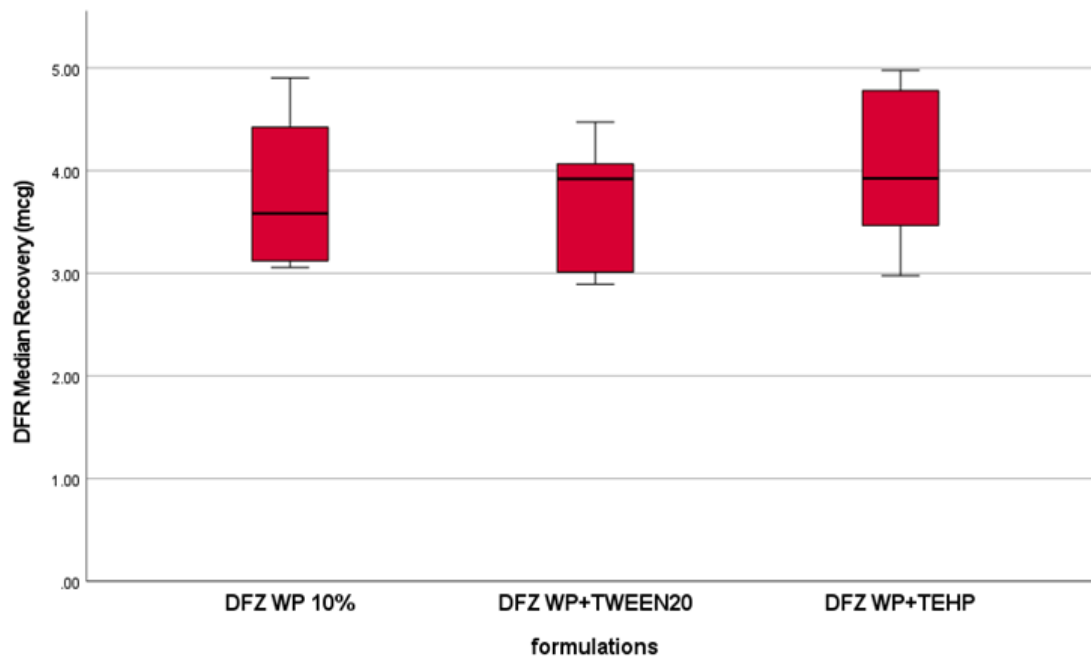
Figure 5.3 is a boxplot showing the DFR median recovery from DFZ WP 10% (w/v) with and without adding different adjuvants (i.e., Tween-20 0.1% (w/v), TEHP 0.1% (w/v). The error bars represent a confidence interval of 95%.

Figure 5.3: Box plot for difenoconazole WP 10% (w/v) DFR median recoveries with and without adjuvants on French bean.

Table 5.7: Comparison between DFR recovery of different DFZ formulations (EC 10%, EC(X) 10%) on French bean, tomato, and oilseed rape leaves

Descriptive statistics	French bean		Tomato		Oilseed rape	
	EC 10%	EC(X) 10%	EC 10%	EC(X) 10%	EC 10%	EC(X) 10%
N (Replicates)	10	10	10	10	10	9
Mean µg (±SD)	4.1 (±0.1)	4.1 (±0.2)	3.0 (±0.1)	3.3 (±0.3)	1.6 (±0.2)	1.9 (±0.1)
Median (µg)	4.1	4.1	3.0	3.3	1.64	1.8
RSD%	4.0 %	5.0%	2.0 %	9.0%	11.0%	4.0%
DFR Recovery% Mean (±SD)	82.0% (±3.0)	82.0% (±4.1)	60.0 % (±1.2)	65.0% (±6.0)	31.0% (±3.3)	37.0% (±1.7)
Shapiro-Wilk Normality test (P-value) (Significance P≤ 0.05)	0.1**	0.2**	0.4**	0.1**	0.4**	0.2**
Independent Sample T-test (significance 2-tailed)	0.8		0.3		0.001	

Table 5.7 shows the descriptive statistics comparing two different formulations of the DFZ 10% (i.e., EC 10% and EC(X) 10%) on French bean, tomato, and oilseed rape. *Independent sample T-test assuming non-equal variance between the groups has been used and showed a non-significant difference between tested formulations on tomato and French bean leaves only (P≥0.0005). **Difenoconazole DFR residue from both formulations on all cops/leaves proved to be normally distributed when tested by the Shapiro-Wilk test using SPSS* where P≥ 0.05. RSD % is the percentage of relative standard deviation among samples of each tested concentration level. The SD± is the standard deviation for the 10 determinations (n=10) except in the case of oilseed rape with 9 determinations with EC(X) 10% (w/v) treatment.

The DFR mean recovery (µg ± SD) of the DFZ EC(X) 10% (w/v) on tested crops (i.e., French bean, tomato and oilseed rape) followed the same trend and residue magnitude as that of the EC 10% DFR mean recovery on matched crops. The mean recovery was the highest on French bean leaves (4.1 ± 0.2), followed by tomato (3.3 ± 0.3), with the lowest DFR recovery recorded on oilseed rape (1.9 ± 0.1).

The DFR recovery (% ± SD) of DFZ EC(X) 10% on French bean, tomato and oilseed rape were all at the same residue magnitude with approximately similar DFR mean% when

compared with DFZ EC 10%. In other words, the mean DFR% for both EC formulations (i.e., EC 10% and EC(X) 10%) on French bean was (82.0 ± 3.0%) (82.0 ± 4.1%), while for tomato was (60.0 ± 1.2%), (65.0 ± 6.0%) and on oilseed rape was (31.0 ± 3.3%), (37.0 ± 1.7%) respectively. Nevertheless, there was no significant difference between all tested crops using the two EC formulations except for oilseed rape when tested using the independent T-test with P values above 0.05 except for the oilseed rape P=0.001 indicating the significance as shown in Table 5.7 above. Nevertheless, both DFR residue mean values on oilseed rape from both EC formulations were the lowest recorded value compared to other crops. In addition, and like other crops tested, the residue magnitude on oilseed rape was comparably close (31.0 ± 3.3%) and (37.0 ± 1.7%) for EC 10% (w/v) and EC(X) 10% (w/v), respectively, as shown in Figure 5.4 below. Circles or asterisks on either end of the box plot indicate potential outliers in the data, which were checked and proved not to affect the statistical results.

Figure 5.4 is a boxplot chart for the DFR median recovery of different DFZ EC formulations (EC 10%, EC(X) 10%) on French bean, tomato, and oilseed rape leaves. Lower and upper box boundaries are 25th and 75th percentiles, respectively, while the line inside the box is median, and the error bars represent the 95% confidence interval (CI). The non-significance was observed in comparing both formulations on French bean and tomato leaves. Circles or asterisks on either end of the box plot indicate potential outliers in the data, which were checked and proved not to affect the statistical results.
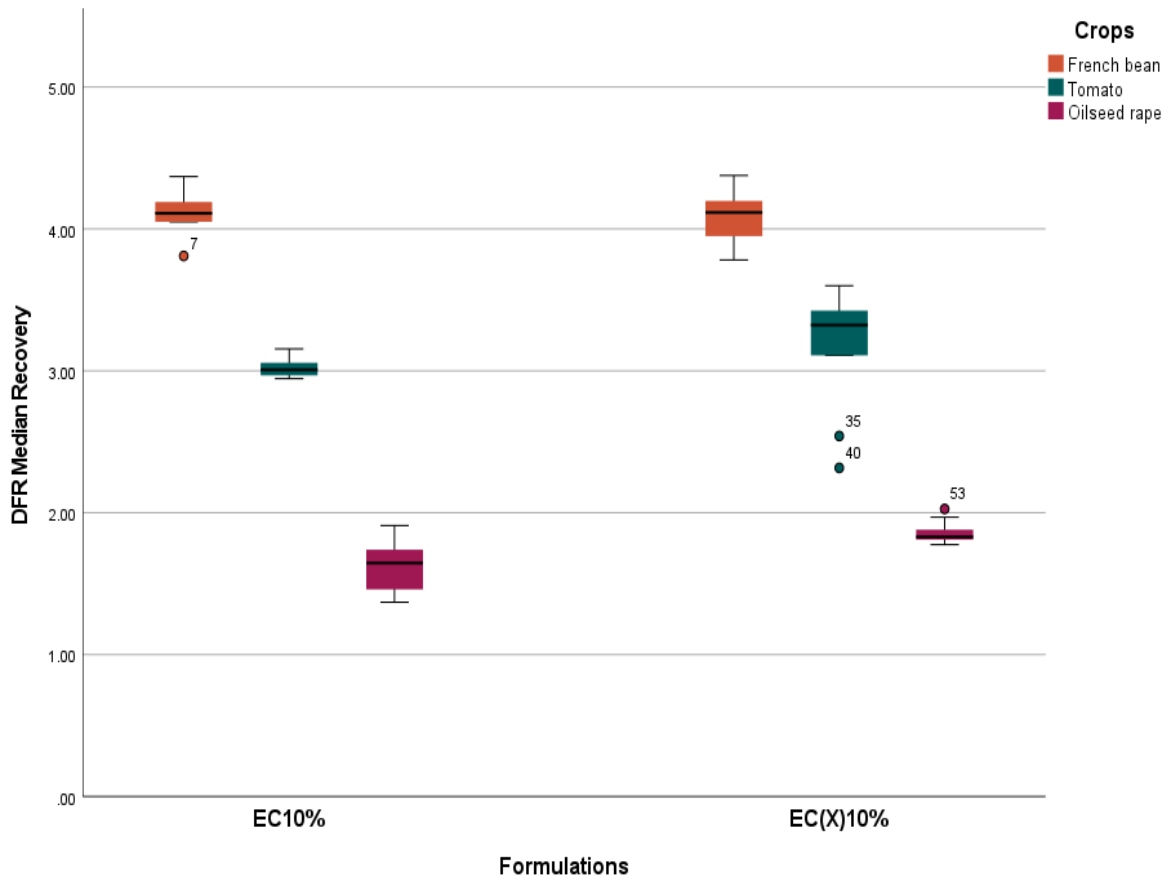
Figure 5.4: Boxplot chart for DFR recovery of different DFZ EC formulations (EC 10%, EC(X)

10%) on French bean, tomato, and oilseed rape leaves.

Figures 5.5 and 5.6 below show the dynamic surface tension curves as a function of time ranging from 10 to 100,000 ms. Pure distilled water was used to dilute all the formulations; thus, the same was used for the control experiment. As shown in the yellow dotted line, there was no decrease in the water surface tension during the first 53000 ms of the measurements. The DST of water remained constant at approximately 72 mNm^{-1}. In all DFZ formulations tested except DFZ EC(X) 10% (w/v), the starting point or initial DST was slightly lower or equal to the water DST, which declined over time until the equilibrium surface tension was reached. In contrast, the initial DST for DFZ EC(X) 10% was 40 mNm^{-1}. The DST of WP formulations declined more steeply than EC formulations, reaching DST below 40 mNm^{-1}, approximately at 10000 ms surface age. However, WP +Tween-20 0.1% (w/v) initial DST was the lowest compared to other WP formulations (around 66 mNm^{-1}); its DST decline rate was comparably slower than other DFZ WP formulations and reached approximately DST below 40 at the same surface age (5000 ms).

On the other hand, the EC(X) 10% (w/v) initial DST was far lower than all tested formulations (around 40 mNm^{-1}); still, its decline rate was slower than any other tested formulation reaching the equilibrium of approximately 30 mNm^{-1} by 1000 ms surface age. Nevertheless, the DFZ EC(X) 10% (w/v) recorded the lowest DST among other tested formulations, and its equilibrium DST at the end of the curve (100000 ms) was 6 degrees less than the other ECs tested formulations which were recorded around 36 mNm^{-1}.
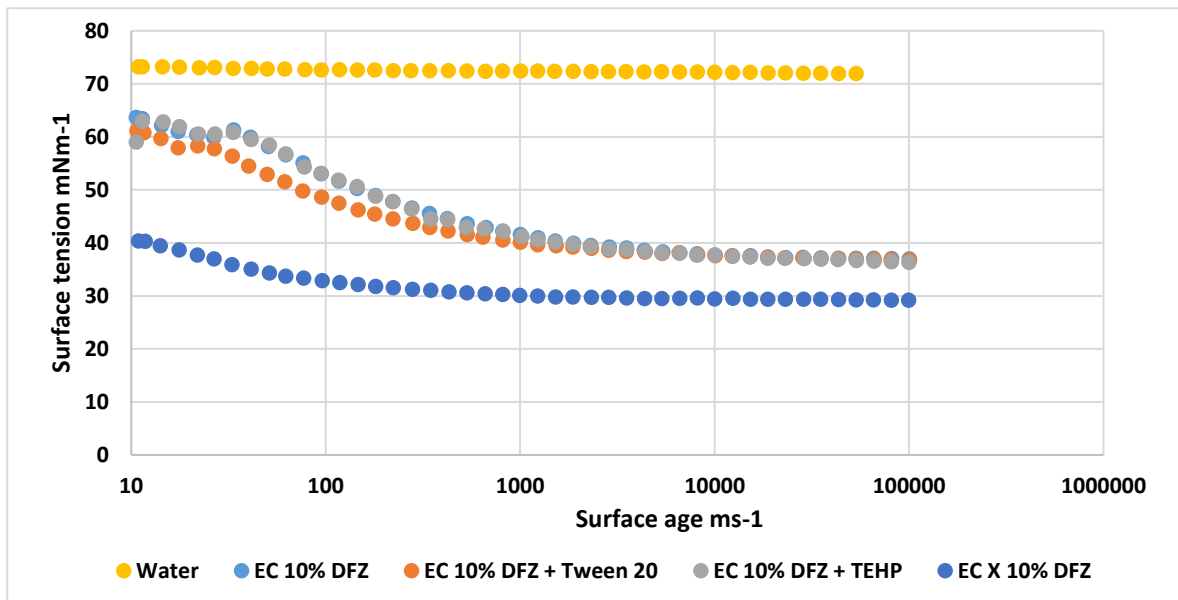
Figure 5.5 shows the results of DST measurement expressed as a relation between the surface age (ms) and surface tension (mNm⁻¹). The surface age is defined as the time interval between the minimal measured pressure, identified with the bubble formation, and the maximum pressure, which marks the onset of the spontaneous bubble detachment. The surface tension value on the Y- axis corresponds to the latter moment. All DFZ EC formulations were diluted in water achieving a concentration of DFZ (0.625 mg mL⁻¹). Adjuvants (Tween 20 and TEHP) were added to the formulation with a concentration of 0.1% (w/v).

Figure 5.5: Different Dynamic surface tension measurements for DFZ EC 10% (w/v) formulations.
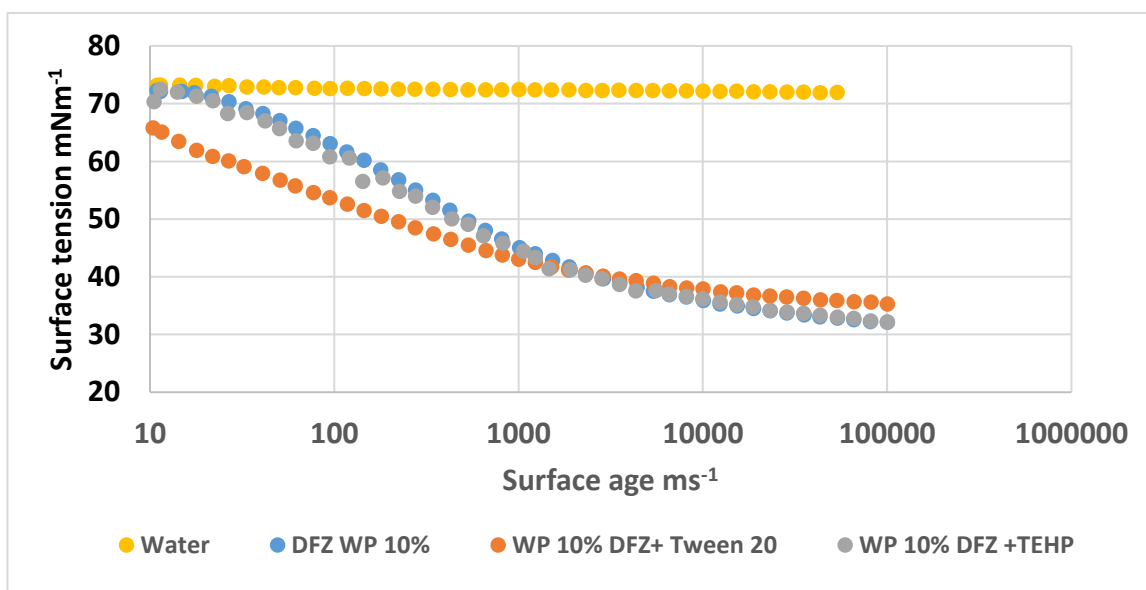
Figure 5.6 shows the results of DST measurement for different DFZ WP formulations expressed as a relation between the surface age (ms) and surface tension (mNm^{-1}). All DFZ WP formulations were diluted in water achieving a concentration of DFZ (0.625 mg mL^{-1}). Adjuvants (Tween-20 0.1% (w/v) and TEHP 0.1% (w/v)) were added to the formulation with a concentration of 0.1% (w/v).

Figure 5.6: Different Dynamic surface tension measurements for DFZ WP 10% (w/v) formulations.

Table 5.8 below shows the initial DST values for all tested formulations along with the DST values at 5360 ms surface age. Approximately 5000 ms was the estimated time for the droplet to develop from the pipette tip and touch the leaf surface using the controlled pipetting method in the developed DFR laboratory method implemented.

Table 5.8: Dynamic surface tension initial and contact time for all tested formulations.

Formulations tested	Initial DST (mNm^{-1}) at 10 surface age (ms)	DST at impaction time (mNm^{-1}) 5360 surface age (ms)
Water	73.0	72.0
WP 10% (w/v)	72.3	37.5
WP 10% (w/v) + Tween 20 (0.1% w/v)	66.0	39.0
WP 10% (w/v) +TEHP (0.1% w/v)	71.0	37.7
EC 10% (w/v)	64.0	38.0
EC 10% (w/v) + Tween 20 (0.1% w.v)	61.0	38.0
EC 10% (w/v) +TEHP(0.1% w/v)	60.0	38.0
EC(X) 10% (w/v)	40.0	30.0

Table 5.8 shows all tested formulations' initial and contact DST values using bubble pressure tensiometer BP100 from Kruss GMBH (Hamburg, Germany). The DST at approximately 5000 ms is the DST at droplet contact time with the targeted leaves using the electronic micropipette in the DFR developed method.

5.5   Discussion

A new formulation is usually a term used to present the pesticide for sale in the market, which generally includes, in addition to the active ingredient(s), different adjuvant(s), and other formulants combined to render the product valuable and effective for the purpose claimed. The difficulty in isolating all these factors has been identified as the cause that the effect of different formulations on the residue pattern is not conclusive in the literature (Buzzetti, 2017; X. M. Xu et al., 2008).

In the present studies, there were no significant differences between the DFZ WP 10% and EC 10% formulations on oilseed rape and French bean; this could be due to the non-ionic surfactant present in the WP tested formulation. The existence of the non-ionic surfactant in a WP formulation is not an old practice as the major inert ingredients of wettable granules (WG), and (WP) formulations are used to be wetting agents, dispersants, and diluents; other inert may include anti-foaming agents, binders, and disintegration agents (De Schampheleire et al., 2009). On the other hand, the presence of an efficient emulsifier which is usually a surfactant in an EC formulation is crucial. Therefore, Syngenta's WP formulation could be benefited from adding the non-anionic surfactant (poly (oxy-1,2-ethanediyl), alpha isotridecyl-omega-hydroxy)(IMAP, 2020). The addition of such surfactant could result in equating to the EC formulation's effect. As a result, the active ingredient in DFZ WP formulation became more miscible in water than any other WP formulation lacking this non-ionic surfactant. This could delay the aggregation and precipitation of the AI in the formulation.   Despite the statistical insignificance between both formulations on French bean, the DFR recovery % was slightly higher in the EC 10% (82.0 ± 3.0%) than the WP 10% (74.0 ± 13.1%). One should note here that the 18% RSD associated with the WP formulation compared with the

RSD% in EC 10% formulation tested on French bean is due to the poor uniformity of dosing of the DFZ WP 10% formulation as illustrated in Section 4.5.4.

In general, spray deposition, adhesion, droplet coverage, and leaf retention were enhanced when non-ionic surfactants were incorporated into the formulation (Basu et al., 2002; Yu et al., 2009b). in addition, many researchers found that when surfactants are used, the foliar uptake of pesticide from sprayed droplets and biological efficacy of the active substances were improved (Holloway et al., 1992; Uhlig & Wissemeier, 2000; Yu et al., 2009b; Zabkiewicz, 2000). This result also agrees with the findings of many researchers where the EC formulations of the different pesticides provided better retention leaving more residue than the WP-tested formulations (Buzzetti, 2017; Yao et al., 2014). In contrast, the data gathered by the USA ARTF proved the proximity of DFR from EC and WP formulations on different leaves. In this data, 75 studies involving WP pesticide spray left an average less normalised DFR of 0.906 $\mu g\ cm^{-2}$ Ib-ai $acre^{-1}$ compared to 1.1 μg from the 95 studies that involved EC sprays (Bruce & Korpalski, 2008).

On the contrary, the DFR was significantly different (P=0.006) between both formulations (i.e., EC 10% and WP 10%) on tomatoes. The DFR mean Recovery % (±SD) on tomato with DFZ WP 10% was (39.0 ± 5.0%) compared to (60.0 ± 1.2%) with the EC 10%. Besides the explanation above on the non-anionic surfactant's role, it is hypothesised that tomato leaves, unlike the other leaves, bear trichomes that puncture the spray droplets into smaller droplets. These smaller droplets could reach the epidermis easier and enhance the absorption before the pesticide dries (Franceschi & Giaquinta, 1983; Li et al., 2018). In addition, tomato trichomes are known to be hydrophilic and increase the affinity of the pesticide spray to the epidermis (Kasiotis et

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al., 2017). Ultimately, the absorption of the WP formulation increased with the aid of the non-ionic surfactant initially incorporated, leaving less DFR on the surface.

Furthermore, the suspension formed from the WP spray may initially benefit from the several surfactants incorporated in the formulation to lower the surface tension of the generated spray droplets. These incorporated surfactants (non-ionic and polymeric) namely poly(oxy-1,2-ethanediyl), alpha isotridecyl-omega-hydroxy/ sodium dibutylnaphthalenesulphonate and 1-Butenedioic acid (2Z), sodium salt (1:2) enhance the miscibility and reduces the droplet size during the spray. As a result, this could increase pesticide absorption. Despite that, EC formulation also incorporated surfactant that helped in the deposition and absorption of the AI from the formulation, but the suspension nature of the WP spray may be benefited more from the existence of these types of adjuvants in the formulation. In general, surfactants in agrochemicals were initially used to aid the dissolution of low solubility substances such as DFZ in the spray solution (Faheem, 2012). The dissolution of DFZ in WP formulation on French bean leaves was probably enhanced by incorporating these surfactants and consequently leads to fast absorption into the leaf epidermis. As a result, this might be why the mean WP 10% DFR recovery % of DFZ was less (74.0 ± 13.0%) than EC 10% recovery on French bean leaves (82.0 ± 3.0%), as presented in Tables 5.6 and 5.7 above. This could also be evident by the steep decline of the DST in the DFZ WP 10% formulation. The surface tension of the WP formulation nearly halved from 71.0 to 37.5 (m Nm^{-1}) at 5360 ms (estimated impaction time), reaching the equilibrium. In comparison, this fast rate of decline in the DST was not observed in the case of EC, where DST declined from 64.0 to 38.0 (m Nm^{-1}) in the same period. The differences in the surface tension-surface age profiles between the formulations reflect differences in the rate at which the droplets will be able to spread and form a film on the leaf

surface, as well as affect the rate of agent dissolution and absorption into the leaf tissue (Faheem, 2012).

The effect of the spray depends on both the characteristics of the spray and the texture of the targeted leaves. Thus, the presence of suitable leaf texture/Crop (i.e., tomato) magnified the non-anionic surfactant effect present in the DFZ WP 10% and resulted in a 20% lower DFR (39.0 ± 4.7%) than those treated with EC 10% formulation (60 ± 1.2%).

The effect of different adjuvant addition on the DFZ WP 10% DFR revealed no statistical effect on the French bean leaves when tested with ANOVA (P=0.4). On the contrary, one-way ANOVA revealed that there was a statistically significant difference in the DFR intensity between at least two groups in the case of DFZ EC 10% when mixed with adjuvants (Tween-20 (0.1% w/v), TEHP (0.1% w/v), and TEHP (0.3% w/v). In the latter, the Tukey's HSD test for multiple comparisons was necessary which indicated the significant different was between DFZ EC 10% group and DFZ EC 10%+TEHP 0.1% (w/v) where p = 0.003, 95% C. I= [0.119, 0.688]).

The reason the DFZ WP 10% showed no significant decrease in the DFR intensity when mixed with all adjuvants tested could be due to the nature of the WP formulations. Therefore, further addition of other adjuvants in the presence of the non-ionic surfactant initially in the WP did not impact the DST of the formulations at the impaction time. This could be due to having employed the optimum concentration of the predominant surfactants in the formulation prior to the addition of the test adjuvants, which achieves the utmost mix possible between the active substance and the adjuvant. Thus, the potentiation effect of any additional adjuvant is minimal. The literature shows that the stronger the bond between the active substance (AS) and the adjuvant in the formulation, the better the adjuvant's biological effect (Faers &

Pontzen, 2008). WP formulations form a suspension after dispersion in water where the active ingredient is less miscible, and the active substance tends to aggregate participate, particularly during the period of solvent evaporation after application to the leaf, compared to the EC formulations. In the latter, there is more chance for the active substance (DFZ) particles to dissolve better, for example, in the residual oil- and surfactant-rich film after the evaporation of the continuous phase in the formulation film after spreading to the leaf. This is due to emulsion formation in the case of ECs, which implies a homogenous concentration of the active ingredient (OECD, 2002). After spray application and dry-down on the leaf surface, a deposit containing the AI particles and the adjuvant will remain on the surface and will be regarded as DFR. Therefore, for an adjuvant to enhance the uptake of a lipophilic particulate of the AI, it is hypothesised that a closer association between the adjuvants and the AI is required. This is also illustrated in Figure 5.4, where:

(a) shows the AI on the leaf surface without any adjuvant and

(b) the AI and adjuvant present with no association, resulting in low uptake.

On the contrary, (c) and (d) show a different level of AI association with the adjuvants, resulting in a comparably higher uptake of the pesticide into the leaf and consequently lowered DFR (Faers & Pontzen, 2008).
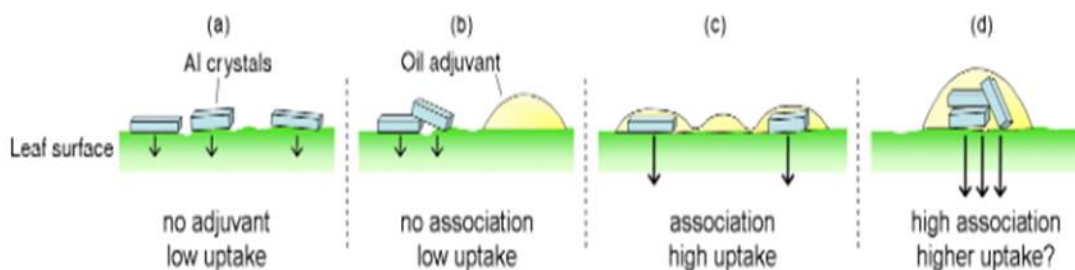
Figure 5.4 illustrated different association levels between the active pesticide ingredient (AI) and an adjuvant, (a) and (b) showing low AI uptake into the leaf with a weak association between both elements, while (c) and (d) showed an enhanced uptake due to better association between both the adjuvant and the AI particles. The Figure was adopted from (Faers & Pontzen, 2008).

Figure 5.7: Adjuvant and active ingredient association and its impact on foliar uptake.

A higher association between the active substance and the surfactants in the formulation spray will result in a better and faster uptake, as illustrated in Figure 5.7 (Faers & Pontzen, 2008).

The low DST (40.0 m Nm^{-1}) of DFZ EC (X) 10% at the initial surface time of 10 ms indicates a better association between the active substance and the surfactant present in this formulation than in all tested formulations. Furthermore, the low DST combined with the rapid surface ageing behaviour (Figures 5.5 and 5.6) also leads to an enhanced ability of the droplet to spread on the leaf surface, as the molecular mobility of the surfactants facilitates a more dynamic interface-interface interaction. This behaviour is demonstrated graphically in Figures 5.7 (c) and (d). In addition, such a proposed association could be the reason for the faster rate at which this formulation reached the equilibrium (30 m Nm^{-1}) at 1000 ms surface age compared to others.

On the other hand, other EC formulations showed a slower rate for reaching the equilibrium at around 1000 ms surface age from the surface tension of (64.0 – 60.0 m Nm^{-1}) to (38.0 m Nm^{-1}) compared to the DFZ WP formulation from (72.0 – 66.0 m Nm^{-1}) to (37.5 – 39.0 m Nm^{-1}) (Table 5.8). This relatively rapid DST decrease in the case of WP formulations indicated better incorporation of the adjuvants in the WP formulations compared to the EC formulations, which enabled the WP formulation to perform relatively similar in terms of the DFR % recovered despite the better-known characteristics of the emulsion formed from the EC dilution. Better spray uniformity may be achieved from the DFZ EC 10% formulations due to the uniformity and enhanced solubility of the active substance in the emulsion, but better absorption of the active substance into the leaf could be achieved from the better association of the adjuvant with the AI in the WP formulation.

The DFR effect of the rate at which the equilibrium is achieved was not captured in this research due to the longer time required to form the spray droplet using the micropipette in the lab (5000 ms), at which all the formulations tested have already reached equilibrium (Figures 5.5 and 5.6). However, the effect of this rate could better be reflected in the field condition using a spray method that generates droplets in a shorter time. Furthermore, exploring the same in the lab with different spray methods could be challenging in providing a controlled deposition of the residue but still applicable.

Both adjuvants used in this experiment were meant to act differently to reduce the DFR recovery% when mixed with the DFZ WP or EC formulation. Tween-20 is a surfactant known to reduce the surface tension of the spray droplets, increasing the wettability and the spreading of the pesticide on the leaves. In comparison, the TEHP is a plasticiser, sometimes called a penetrator, that is known to enhance the uptake of

the AI into the plant by reversibly changing the structural properties of the plant cuticle. It is hypothesised that this kind of adjuvant decreases the size of the crystalline platelets in the cuticle and enhances the pesticide fluidity by improving the diffusion coefficient and consequently enhancing the spreading on the surface (Arand et al., 2018). From the above, the less miscibility of the AI in the WP 10% formulation and the expected fast dryness of the AI on the leaf could be the leading cause of the poor functionality of both adjuvants tested (i.e., Tween-20 and TEHP). Further addition of other adjuvants in the presence of the non-ionic surfactant initially in the WP did not result in further conjugation between the AI and these adjuvants. This could be due to the presence of the initial non-ionic surfactant, which already achieved the utmost mix possible between the active substance and the adjuvant. Thus, the potentiation effect of any additional adjuvant (i.e TEHP and Tween-20) was minimal. This was observed from the close mean DFR recovery% on French bean (74.0 ± 13.0%. 74.0 ± 12.0% and 78.0 ± 15.0%) in WP 10%, WP 10% +Tween-20 0.1% (w/v) and WP 10% + TEHP 0.1% (w/v) treatment groups respectively.

In addition, the size of suspended particles (DFZ) could be another factor limiting the functionality of the TEHP when mixed with the WP formulation. This is because the smaller the particle size in the formulation, consequently the easier the penetration(De Ruiter et al., 2003; R. Singh & Arora, 2016). If compared with the ECs, WPs dispersing particles are generally larger; WPs are finely ground formulations with a particle size of about 5.0 μm and applied to the field after suspending in water compared to a range of dispersed phase particles of (0.1- 5.0 μm) in the EC formulations.

On the other hand, the good and homogenous miscibility of the AI in the emulsion formed from the DFZ EC 10% was found to enhance the absorption of the DFZ into the French bean leaves when the DFZ EC 10% was mixed with TEHP (0.1% w/v). This result

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was noticed from the reduction in the mean DFR recovery% (78.0 ± 4.1%) observed in the presence of the TEHP 0.1% (w/v) compared to the DFZ EC 10% alone (82.0 ± 4.1%) on the French bean leaves. It was also noticed that increasing the concentration of the TEHP from 0.1% (w/v) to 0.3% (w/v) did not decrease the DFR recovery % or increase the absorption and penetration of the DFZ into French bean leaves. These findings align with the concept that an accelerator's efficacy depends on the type of plasticizer and its concentration (Buchholz, 2006; Jörg Schönherr et al., 2001).

The results showed that adding Tween-20 0.1% (w/v) to DFZ EC 10% (w/v) had no significant improvement in the DFR recovery % on French beans. Still, a slight (not statistical) improvement from (82 ± 4.1) to (77.0 ± 6.3%) was observed when Tween 20 was added to the EC formulation. It is evident from other research that non-ionic surfactants have low functionality with lower soluble AIs (Buchholz, 2006). According to the PPDP, DFZ is classified as a low soluble pesticide (15 mg L^{-1} at 20 °C) (Lewis et al., 2016), therefore, Tween 20 had no significant effect on the DFR recovery%.

The DST of both EC formulations (i.e., EC 10% (w/v), and EC 10% + Tween-20 0.1% (w/v)) was similar (38.0 mNm^{-1}) at the impaction time (5000 ms) despite the slight improvement of the DST in the case of EC 10% + Tween-20 0.1% (w/v) at the initial formation of the droplet (61 mNm^{-1}) compared to the EC (64 mNm^{-1}) as illustrated in Table 5.8. The slight improvement of the DFR % in EC + Tween-20 could be due to more dissolution and association of the DFZ with the aqueous phase of the formed emulsion compared to the EC 10% formulation alone, where there is more possibility of a lipophilic AI (DFZ) to be incorporated in the oil phase of the spray emulsion than the aqueous phase. As a result, this could increase the DFR by making the AI present in an oily film over the plant surface after the evaporation of the aqueous phase. However, the AI's absorption, evaporation or breaking down could be based on other

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overlapping parameters. Also another possible explanation could be related to the evaporation and volatilization time of the sprayed film, which could be delayed due to the presence of adjuvants in a good mix with the EC formulation that permitted adequate time for the absorption (De Ruiter et al., 1990). Hence a slight decrease in the DFR % recovery was observed ($77.0 \pm 6.3\%$) with the addition of the Tween-20 0.1% compared to the DFZ EC formulation alone (82 ± 4.1).

Unlike in the WP formulation, the presence of the emulsifier in the EC formulation, which bonds the lipophilic particles with water, ultimately caused a slight improvement in the surface tension and the absorption into the French bean leaves and consequently lowered the DFR.

Despite the pressing argument that the most significant effect on leaf wettability is the surface structures, some physical tests were also considered crucial (i.e., dynamic surface tensions, contact angles etc.) to understand the spray behaviour (Taylor, 2011). Therefore, studying the dynamic surface tension of multiple spray solutions would give an idea of the expected retention and consequently could be compared to the DFR magnitude.

In this Chapter, the DST experiment was performed to analyse the difference in the DFZ tested formulation and reflect on each DST at a specific time (when the droplets touch the plant surface). It is hypothesised that during foliar application in the field, the majority of spray droplets impact the leaf surface after about 50 to 400 ms (Wirth et al., 1991), which was then averaged to 100 ms (De Ruiter et al., 1990). The interface saturation and the surface tension lowering are required to be achieved in such a small time frame for good retention achievement; alternatively, the droplets will shatter and bounce off the target (De Ruiter et al., 1990).

This estimated time in the field (100 ms) was estimated when mobile spray equipment was used with high velocity and pressure to induce the droplet travel from the nozzle to the leaf, unlike in the current experiment. The present investigation was performed in the lab, and the droplets were generated by pressing on the micropipette and waiting for the droplet of 0.2 µL to fully develop on the pipette's tip before placing it on the leaf surface. This process was estimated to take 5 seconds (5000 ms). The specific DST value at 5360 ms was selected for further assumption from the bubble pressure measurements.

Taylor, 2011 proposed a critical DST value below 55 to 60 mNm^{-1}, which would allow a full spreading and annulus formation of the droplet over targeted leaves (Taylor, 2011). From the generated data, water DST was constant at around 72 mNm^{-1} with no change, defined as the normal surface tension of water in the literature (Vargaftik et al., 1983). These results confirm no contamination in the water source used, which served as a diluent for all other formulations tested.

In the current laboratory experiment, the dynamic effect of the surface tension was not related to the generated results due to the late impaction time of the droplet with the plant surface (5000 ms) compared to (100 ms) in the field studies. However, one should note that all DFZ EC formulations reached their equilibrium surface tension at 1000 ms compared to WP formulation equilibrium at approximately 5000 ms. This could reflect the ability of the EC formulation to spread and diffuse faster compared to the WP formulation due to enhanced molecular mobility of the surfactants within the droplet. However, this would also depend on many other overlapping factors such as the texture of the targeted surface, droplet size, environmental conditions etc.

The DST measurements at the impaction time for the WP formulations (i.e., WP 10% (w/v), WP 10% (w/v) + Tween-20 0.1% (w/v) and WP 10% (w/v) +TEHP (0.1% w/v))

proved to have very close DST of 37.5, 39.0, 37.7 mNm^{-1} respectively as shown in table 5.8. The DST at the impaction time were also very close in most of the EC formulations tested (EC 10% (w/v), EC 10% (w/v) +Tween-20 0.1% (w/v) and EC 10% (w/v) + TEHP 0.1%) except for the EC(X) 10% (w/v). The DST values were all 38 mNm^{-1} except for DFZ EC(X) 10% (w/v) was 30 mNm^{-1}.

The close DST value for the ECs formulation and WPs formulation around 37-38 mNm^{-1} were reflected in the DFR % recovery comparison between both formulations on French bean and oilseed rape. In contrast, the DFR recovery % was different in the case of tomato leaves. This difference was discussed earlier due to the nature of the leaf and the strong affinity of the formulation to the hydrophilic trichomes that exist on tomato leaf surfaces. This adds another piece of evidence that the effect of WP formulations was equated to the EC formulation effect with an opportunity for the WP formulation to have improved performance, depending on the structure of the treated leaf/crop (i.e., tomato).

The DST did not change when adjuvants were mixed in the spray solution, and that has also been reflected in the DFR recovery % for both EC and WP formulations on French beans. On the other hand, the significant effect of TEHP 0.1% (w/v) addition to the DFZ EC was not related to the surface tension reduction but to the different activity of the TEHP as a plasticiser that reversibly changes the structural properties of the plant cuticle, which will improve the diffusion coefficient and consequently enhancing the spreading of the DFZ in the spray film on the surface (Arand et al., 2018b). Thus, the increased intrinsic solubility of the emulsion may enhance the functionality of this adjuvant in the EC spray compared to the suspension in the WP spray.

On the other hand, the EC(X) 10% DST was 8 degrees lower than all tested formulations recording 30 mNm^{-1} at the impaction time, possibly due to the intrinsic characteristics

of its components. It was expected to observe lower DFR recovery % than other EC formulations due to the reduced surface tension observed and the more possibility of DFZ penetration, but that was not the case. Overall, EC(X) 10% (w/v) DFR mean recovery % on French bean, tomato, and oilseed rape followed the same pattern as the EC 10% (w/v) DFR on the same crops. The residue pattern of French beans with the highest DFR (82.0 ± 4.1%) and oilseed rape with the lowest (37.3 ± 1.7%), with tomato DFR recovery% of (65.0 ± 6.0%) in between was very similar to the DFR% of EC 10% (w/v) formulation.

Despite the resemblance of both EC 10% and EC(X) 10% (w/v) recovery % on the same crops, there was a statistical difference observed between both formulation's mean DFR % on oilseed rape (P=0.001), with the EC(X) 10% (w/v) leaving a slightly higher residue (37.3 ± 1.7%) than the EC 10% (w/v) (31.0 ± 3.3). The slight elevation of the DFR in the oilseed rape compared to the EC 10% (w/v) could be due to the different composition of the formulation with a different solvent system used in both formulations. The existence of other solvents in the EC(X) 10% (w/v) (i.e., a mixture of octanoic acid-decanoic acid-N, N-dimethyl amide) could change the spray's physicochemical composition, leading to rapid evaporation of the aqueous part from waxy surfaces compared to hairy leaves. This could result in more DFZ (X) accumulation on the leaf surface and a delay in evaporation from the oily film formed.

5.6 Conclusion

The findings of this Chapter shed light on the importance of the PPP formulation as a factor that may affect DFR. From the results. It was well observed that the DFZ WP 10% (w/v) formulation was not the worst compared to the DFZ EC formulation. That gives an indication that WP formulation is not the worst formulation and tends to result in more DFR, as described in the literature. It was observed that the DFR magnitude from

both DFZ WP and EC formulations was comparable on French bean and oilseed rape leaves. However, the DFR % of DFZ WP 10% (w/v) on tomato was lower compared to the EC 10% (w/v) DFR. The reason for such an equal response from WP 10% was mainly the formulation composition. Besides that, this response could even magnify in the presence of suitable leaf textures such as tomato leaves and their hairy hydrophilic surface. Even though there is an intrinsic difference between DFZ EC and WP formulations, especially with WP formulations forming suspension on dilution, the innovation in the formulation science has succeeded in formulating DFZ WP formulation with effective adjuvants incorporated that enhanced its retention/absorption to the degree that equated the EC efficacy and beyond. That was also obvious from the DST values recorded at the initial and impaction time of the prepared spray of the two formulations.

From the results of this Chapter, it was well observed that mixing Tween-20 0.1% (w/v) or TEHP 0.3% (w/v) adjuvants with DFZ EC 10% (w/v) did not enhance the formulation's retention or absorption significantly. This also was observed from the no change in the DST between the DFZ EC 10% and those ECs mixed with adjuvants. On the contrary, a slight change in the DST was observed when Tween-20 0.1% (w/v) was mixed with the WP formulation at the beginning of the droplet formation but ended up with comparable DST to other WP formulations at the impaction time of the droplet on the leaf surface. The effect of adding Tween-20 0.1% (w/v) on the DFZ EC 10% formulation did not imply any DST change at the initial droplet formation or the impaction time with the leaf. This indicates that the DFZ formulations did not benefit from the in-tank mixing of surfactants like Tween-20 0.1% (w/v). On the contrary, the DFZ 10% EC formulation benefited from adding the accelerator TEHP with the appropriate typical concentration of 0.1% (w/v) and not 0.3% (w/v), which agrees with many findings that

suggest adjuvant critical micelle concentration (CMC) formation is usually at 0.1% (w/v) of the spray volume (Hall et al., 1999).

Different DFZ EC 10% (w/v) formulations, such as DFZ(X) 10% (w/v) with varying solvent components, did not considerably impact the DFR recovery % among crops tested. The solvents involved in both EC formulations were the most common solvents used in the agrochemical industry. In conclusion, the DFR recovery trend was the same with French bean > tomato > oilseed rape, with the lowest DFZ DFR recovery %. The residue trend was the same as the EC 10% on these matched crops despite the difference in DST between both formulations at the impact time (38 mNm^{-1} for EC 10% and 30 mNm-1 for DFZ(X) 10% (w/v)).

These findings indicate the importance of the formulation, co-formulants and adjuvants as factors that could affect DFR. It shows the importance of further research on the same area using the laboratory DFR-developed method to generate more data on the formulation effect, allowing further extrapolation and anticipation of the DFR differences. Understanding these significant differences between formulations in the laboratory using the controlled described method would allow possible extrapolation based on the statistical analysis between the generated lab and field data when available. For instance, more data on the DFR recovery resemblance of the EC and WP on different formulations and crop combinations would allow limiting the field studies to one of these formulations for regulatory purposes. Given that these laboratories generated data will match the limited in-field studies data. This could save time and resources and ease the registration process of PPPs. The same concept applies to the co-formulants and adjuvant composition and their impact on DFR

Chapter 6 : The Effect of Different Leaf Textures on the Dislodgeable Foliar Residues (DFR).

6.1   Introduction

Pesticides have a crucial role in assuring food security for an increasing worldwide population (Bonner & Alavanja, 2017). In the European Union, despite the importance of good agriculture practice (GAP) in mitigating the risk of pesticide exposure, risk assessments must be carried out for all exposure scenarios following regulation (EC) NO. 1107/2009 (Charistou et al., 2022). This exposure risk could affect not only the consumer through dietary intake but also workers, residents, and bystanders who may be present during agriculture operations (EFSA, 2014a). Such exposure is covered by the non-dietary risk assessment of plant protection products (PPPs) (Charistou et al., 2022; EFSA, 2014a). Some exposure scenarios involve exposure to PPPs through dislodgeable foliar residue (DFR), which is "the amount of residues present on leaves that can be washed from the leaf surface" (Iwata et al., 1977). Dermal exposure from contact with residues on foliage can be estimated as the product of the (DFR), the transfer coefficient (TC), and the task duration (T). Whilst monitoring of residues on plants is considered the gold standard to evaluate pesticide safety, DFR experiments are generally expensive, seasonal, and time-consuming (BfR, 2020). The European Food Safety Authority (EFSA) has acknowledged the scarcity of good quality and sufficiently reliable DFR data and recommended generating more good quality DFR and TC experimental data to identify and conclude the possibility of extrapolating results between crops and formulations (Charistou et al., 2022). In response, and as described in Chapter 4, a new laboratory method has been developed to study factors that could

affect DFR in controlled conditions and consequently allow for more data generation (Badawy et al., 2022).

Leaves are one of the most important plant parts in terms of the application and effect of plant protection products (PPPs). Leaves are crucial for gathering energy, respiration, and protection and provide the surface area for applying and absorbing PPPs into the plant tissue systems (Danowitz, 2012). Typically, plants have unique leaves that differ from one another based on several characteristics, such as shape, colour, texture, and margin (Massinon et al., 2017). Therefore, leaf identification helps in the classification of the plants and the plant families. Different leaf type classifications are exemplified by their form, shape, and other characteristics (Chaki et al., 2015; Zhao et al., 2015). In particular, it is possible to differentiate between extreme leaf texture differences, such as hairy and waxy leaves.

The plant cuticle is an essential component of the plant leaf, playing numerous roles in plant development, physiology, and interactions with the abiotic environment and other organisms (Puig et al., 2012). The cuticle is an extracellular hydrophobic layer covering all land plants' aerial epidermis, protecting against desiccation and external environmental stresses (Puig et al., 2012). The plant cuticle consists of a thin, continuous cutin layer, polysaccharides, and non-polar solvent-soluble waxes (Staiger et al., 2019). Waxes are essential in defending plants against abiotic and biotic stress. In the case of abiotic stress, the strongly hydrophobic waxes limit nonstomatal water loss (Riedel et al., 2009). In the case of biotic stress, waxes form part of the pre-formed plant defence system against organisms such as insects, bacteria, and fungi. Also, the crystalline structure of the wax is involved in reflecting the UV light to protect the plants (Gülz et al., 1991; Marcell & Beattie, 2002).

The leaf cuticle is the primary entry route for foliar pesticides, and it determines the efficiency of the sprayed pesticide in controlling targeted pests. Recent research in 2019 showed that the permeability of organic solutes, such as those in the pesticide product composition, could vary by up to four orders of magnitudes among different plant species (Staiger et al., 2019). Furthermore, this permeability increased by several orders of magnitudes after the cuticular wax extraction (Kerler & Schönherr, 1988; J. Schönherr, 1976; Staiger et al., 2019). This cuticular barrier to diffusion of organic solutes derives from the composition of waxy cuticles with different percentages of very-long-chain aliphatic (VLCA) and the cyclic compounds present like pentacyclic triterpenoids (Staiger et al., 2019). Therefore, plant cuticles could be a critical limiting barrier for the pesticide uptake from the foliar sprayed deposit, especially for foliar-applied pesticides (Bergman et al., 1991; Mcwhorter, 1993). Thus, understanding the properties of the cuticular permeation barrier to pesticides is essential in the agrochemical industry.

On the other hand, the barrier of pesticide uptake is not usually leading to more foliar residue regardless of the leaf texture. Spray droplets must adhere to the leaf and not bead up and roll off (L. Xu et al., 2011). In general, the amount of wax and the spray droplet coverage is inversely related (Yu et al., 2009b). In addition, waxy leaves are considered difficult to wet leaves due to the possibility of droplet rebounding that often scatters or rolls off the leaf surfaces leading to less DFR on their surfaces (L. Xu et al., 2011). Little research has been performed to explore how long the spray droplet could persist on different leaf surfaces, i.e., the evaporation time of droplets directly influences the absorption of the active ingredient. Increasing the lifetime of the spray droplets on the surface increases the absorption and consequently reduces the spray residue on leaf canopies (Yu et al., 2009a). It has been suggested that once the spray

droplet evaporates, leaves may stop absorbing chemicals that could crystalise and aggregate on the surfaces (Ramsey et al., 2005).

Regardless of the leaf texture, the efficiency of pesticide application decreases when there is a minimal interface between the droplet and the leaf surface (L. Xu et al., 2011). This could enable less absorption and more residue retention on the surfaces. In waxy leaves, the epicuticular wax, which is a mixture of hydrophobic lipids, covers the surface of the leaves. This wax is hydrophobic and usually makes it difficult to obtain good wetting of leaf surfaces with water sprays due to its small surface tension. As a result, the pesticide droplets may spread poorly on waxy leaves unless the surface tension of the spray solution is reduced to the critical surface tension of the leaf surface (Mcwhorter, 1993; Samuels et al., 2008). The hydrophilic or hydrophobic properties of leaf surfaces are usually characterised by the contact angle of the surface (θ) with a water droplet, which indicates the leaf wettability. Leaves could be termed as "super hydrophilic" if $\theta < 40°$, "highly wettable" if $\theta < 90°$, and "wettable" if $\theta < 110°$. If $\theta > 110°$, the leaves are classified as being non-wettable, while $\theta > 130°$ for highly non-wettable and $\theta > 150°$ for super-hydrophobic based on the contact angle of water droplets on a surface (Wang et al., 2015).

Thus, the extent of droplet spreading over a surface is determined by the nature of the chemical sprayed, the degree of surface roughness and the presence or absence of an air film below the droplet (Fogg, 1948). In contrast, the composition of surface waxes differs between species and varieties of the same species (Fernandes et al., 1964). Some fractions of the waxy material may have more influence on water repellency than others. For example, the presence of triterpenoid compounds such as ursolic acid may make the plant cuticle more challenging to wet (Fernandes et al., 1964). In other words,

it is crucial to note that not all waxy leaves behave the same regarding water repellence.

In addition to the above, external environmental factors (i.e., temperature, humidity, moisture, etc.) can also influence wettability by affecting the structure and composition of the surface (Rolland et al., 2022). These factors depend on the type and amount of leaf wax, surface energy, roughness, and surface cleanliness (Eyring, 1968; Yu et al., 2009b). Also, leaf wax and trichomes, with other factors, govern the wettability of the leaf and, thus, affect the intensity of DFR. Plants differ in their wax content and composition as well as their trichome density and types (Wang et al., 2015)

The accumulation of wax or the wax concentration per unit area differs between the plant leaves of different ages, as younger leaves possess higher wax concentration compared to older leaves, and even within the same leaf areas, there might be a variation in the wax formation (Ahmad et al., 2015). Therefore, different dislodgeable foliar pesticide residue concentrations could be expected from various plants as a response to the different concentrations of the epicuticular wax present on their surfaces (Lusa et al., 2015a).

On the other hand, many plants possess trichomes (hair-like structures) on their surfaces, and plant trichomes could be the leading cause of leaf roughness. Trichomes are single or multicellular epidermal appendages on the aerial parts of the plants. They have many functions, such as helping the plants protect themselves against herbivores, UV radiation, and water loss, and trichome density and morphology exhibit many structural adaptations and changes (Hülskamp, 2019). The density of trichomes has been found to have a more significant influence on the applied droplet coverage than the trichome length because closely spaced trichomes appear to produce air pockets beneath the droplets, which prevent leaf surface contact (Yu et al., 2009b). On the

other hand, the presence of a large number of glandular trichomes on the surfaces of hairy leaves increases the micro-roughness of the surface and hence, the spreading of the pesticide droplets on the surface (Mcwhorter, 1993). It is evident that there are three types of interaction between the leaf surface and the trichome. First, some trichomes do not influence the spread of the spray. Second, some types have a "segregating pattern" where trichomes appear to circular the surface moisture into patches, and the third type of interaction is known to have a "lifting strategy". In the latter, trichomes appear to hold water droplets (depending on the droplet diameter) on the tip of the trichomes, which could eventually affect the intensity of foliar residue (Wang et al., 2014, 2015).

In the same context, different types, and densities of trichomes have been identified on the same leaf. This has been identified on the leaf surfaces of each cultivar of the common beans (*Phaseolus vulgaris L.*) (Dahlin, 1992). Thus, the spreading efficacy of pesticide droplets on the hairy leaf surfaces may differ according to the density and the trichome type available on the surface, especially with the presence of hydrophilic trichomes. Examples of these hydrophilic trichomes are those present on tomato leaves which increase the affinity of the pesticide to the surface (increasing the DFR), unlike the common hydrophobic type of trichomes (Lusa et al., 2015a).

There have been several attempts in the published literature to study the effect of leaf types on the degree of pesticide DFR. For instance, Kasiotis *et al.* (2017) investigated the impact of the leaf texture on the degree of DFR by studying the crop type as a crucial factor. In the latter study, the crop type proved to be a critical parameter that profoundly affects DFR values for the fungicide, Bupirimate. The average DFR value in a pepper crop was one order of magnitude higher than on a tomato crop. The authors related this finding to the high-density trichomes found on tomato leaves, considered

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hydrophilic. These trichomes might interact with the pesticide and increase its affinity to tomato and, consequently, its absorption compared to pepper leaves which lack such a morphological defensive characteristic (Kasiotis et al., 2017). Interestingly, although both crops belong to the same *Solanaceae* family, they exhibit different morphological and physiological characteristics, which enabled them to react differently to chemicals (i.e., fungicides). Thus, there is strong evidence that the crop type or leaf texture may influence the intensity of DFR.

Another study was conducted by the USA Agriculture Task Force (ARTF), which grouped the leaf types statistically based on their texture into three categories: namely smooth, waxy, and hairy. The degree of DFR was reported to follow a hairy > smooth > waxy leaves pattern. In other words, hairy leaves yielded higher DFR values than smooth and waxy leaves providing that all other factors were constant. However, the report does not mention the variability of different leaves within the same texture category in responding to the residues (Bruce & Korpalski, 2008). Also, the study failed to consider the presence of different types of trichomes on the same leaf, nor did it define the method of classifying the leaves into every named group. These variabilities in the trichomes could result in the plants acting differently in response to the pesticide deposition and, consequently, affect the degree of DFR.

As part of the non-dietary risk assessment in the regulatory landscape, there is a continuous need to assess the risk associated with the residue on the plant leaves after the spray has dried (DFR). Due to the DFR default value (3 µg cm^{-2} per kg ha^{-1}) proposed in the EFSA guidance on assessing the exposure for operators, workers, residents, and bystanders, a public consultation report has flagged several questions and proposals for changes. Some of these public consultation's comments were about the leaf effect and its morphological characteristics on the DFR intensity (EFSA, 2014c). EFSA's

response to these comments emphasized the DFR data scarcity, which led to no conclusion on the impact of leaf texture on the DFR and disagreed on any possible extrapolation based on no available data (Charistou et al., 2022; EFSA, 2014c). To that end, the effect of leaf type/ structure on the degree of DFR appears to require more investigation and represents a gap in residue science. To help fill this knowledge gap, this Chapter included two experiments that investigated the effect of different leaf textures on the degree of DFR using two application methods. Understanding this factor could have a significant beneficial impact on the agrochemical industry and the regulatory bodies.

6.2   Aims And Objectives

This chapter includes two laboratory experiments that were set for investigating different leaves (crops) with varying degrees of hairy and waxy textures using the fungicide difenoconazole EC 10% (w/v). In the first experiment, plants with varying levels of trichomes on their surface were chosen, which are French beans *"Phaseolus vulgaris",* tomato *"Solanum Lycopersicum",* and soya bean plant *"Glycine max"*. Two waxy leaves with varying levels of wax deposition on the leaf surface were chosen, such as oilseed rape *"Brassica napus"* and wheat *"Triticum aestivum"*. The fungicide application was performed using the electronic micropipette as described in Chapter 4. On the other hand, in the second experiment, three types of leaves/crops were investigated (i.e., French bean, tomato and oilseed rape) but sprayed with a track sprayer. All plants were treated using difenoconazole EC 10% (w/v) to investigate the effect of different leaf textures on the degree of DFR.

6.3 Materials and Methods

The two experiments in this Chapter followed the same laboratory method and the validation procedures detailed in Chapter 4. This included the description of the DFR analytical method, method validation, selected plant growth stages, and chromatographic analysis of the residue. As shown in Figure 6.1, different crops, namely French bean, tomato, soybean, oilseed rape, and wheat, were grown according to the process given in Section 4.3.1 in Chapter 4. The DFR laboratory method used in this Chapter was summarised in Figure 4.2 of Chapter 4. "*Descriptive summary of the DFR laboratory methodology*". In both experiments, the targeted DFZ concentration was approximately (0.625 mg mL^{-1}), corresponding to the average field application rate of (125 g DFZ 200 L^{-1} hectare^{-1}). A slight deviation in the application rate was observed due to the track sprayer speed variation (137.5 g DFZ 220 L^{-1} hectare^{-1}). Nevertheless, the same targeted concentration was achieved (0.625 mg mL^{-1}).

The first experiment involved 10 replicates of the 5 selected plants alongside three of each for the control group. These 5 different leaves/ crops were French bean, tomato, soya bean, wheat and oilseed rape. All the treated plants were located in the laboratory under a constant temperature of 21 °C. In addition, this experiment involved a controlled application of DFZ on the targeted leaves using an electronic micropipette, as described in Chapter 4.

On the other hand, the second experiment of this Chapter involved three crops/leaves (i.e., French bean, tomato, and oilseed rape). The application of DFZ in this experiment was performed using a track sprayer, and this experiment was carried out at Syngenta International Research Station, Jealott's Hill, UK. The track sprayer method was used to mimic the pesticide application conditions that may exist in the field, which implies

variations due to the angle and speed of the application on the treated leaves beside the smaller droplets generated from the spray jet.

The track sprayer was equipped with a Teejet 8001EVS nozzle. The nozzle height was adjusted to be 35 cm from the targeted leaf in all the sprayed plants. Seven pots of each plant were placed on the treatment shelf in one row and sprayed simultaneously, as shown in Figure 6.4. The track sprayer was calibrated to deliver the same application rate approximately (137.5 g DFZ 220 L^{-1} hectare^{-1}) as in the first experiment. The calibration of the track spray involved weighing Petri dishes placed on the treatment shelf before and after water spray at different speeds and nozzles for many rounds. Calculating the desired application rate from the Petri dishes weight difference, nozzle type, height, and the track sprayer's required velocity to be used.

Due to the expected variation of the spray on the targeted leaves using the track spray method, 2 replicates (called T-0 hours) out of the 7 were used to indicate the DFR recovery directly after the application and before the spray dries. In contrast, the 5 remaining replicates (called T-3 hours) were left in their position for 3 h to allow the spray to dry before washing off the leaf using Aerosol OT 0.01% (w/v). The drying time for this experiment (3 hours) is the same as that applied in the DFR lab method described in Chapter 4. In addition to the explained methodology in the DFR technique, the approximate leaf surface area was measured using the millimetre graph paper by taking the targeted leaves and tracing them over a graph paper. The grids covered by the leaf were counted to give the area and then multiplied by two to account for the double-sided surface area of the selected leaf (Fascella et al., 2009a). All the treated plants were treated in the laboratory under a constant temperature of 21 °C. In both experiments, a control group involving no pesticide application was included to detect

any residue that may exist, which could interfere with the interpretation of the obtained results.

Contact angle measurements of 2 µL spray droplet over 3 leaf types (i.e. French bean, tomato and oilseed rape) using either DFZ EC 10% (w/v) or DFZ WP 10% (w/v) with a concentration of (0.625 mg mL^{-1}) were performed. Measurements on other leaves (wheat and soya bean) were not possible due to Covid-19 constraints on the project and the limited availability of the instruments involved. Since 0.2 µL droplets were too small to image clearly and accurately using the instrument, the imaging was performed by dispensing 2 µL droplet size in 3 replicates. Using a manual syringe, the 2 µL spray droplets were suspended over the leaves. The contact angle measurements and the droplet images were obtained and processed using the DataPhysics instrument – Model OCA 25-Contact Angle Measuring and Contour Analysis System purchased from DataPhysics Instruments GMBH, Germany and available at Syngenta International Research Station, UK. Specimen slides were prepared by placing a double-sided adhesive tape (Tesa Double face, Germany); leaves were cut using clean scissors and forceps with extra caution to avoid touching the leaf surface and then carefully placed on the adhesive tape for imaging. Each tested leaf was imaged with either a DFZ EC 10% (w/v) droplet or DFZ WP 10% (w/v). The dispensed droplets were filmed using a high-definition camera for 150 s for analysis. The contact angle measurements were then averaged between the right and left angles using the software from the impaction time of the droplet and up to 120 s. The contact angles for both sides of the droplet were estimated and averaged to one contact angle value for comparisons using the drop shape analysis (DSA) software. The growing conditions and the selected leave were the same as described in Chapter 4, Section 4.3.1.

Figure 6.1 shows different plants with different leaf textures chosen for the treatment. The plants described above were selected for their leaf texture. Three different plants with varying degrees of hair roughness have been selected (i.e., French bean, tomato, and soybean) in addition to 2 other plants with varying degrees of wax deposition (i.e., wheat and oilseed rape).

Figure 6.1: shows the different treated leaves, namely French bean, soya bean, tomato, and oilseed rape.

6.3.1 Statistical Analysis

All the raw data were analysed using SPSS, IBM version 27.0 (BM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). All collected residue data were tested using Shapiro-Wilk for normality. A significance value (P-value ≥ 0.05) of the test indicated the normality besides ensuring that the skewness and Kurtosis values are in the acceptable range of the normal distribution (-1,1) and (-3,3), respectively (Shapiro & Wilk, 1965).

One-way ANOVA (Analysis of Variance) was used to analyse the difference between the means of tested groups. Tukey's HSD was used to detect the post hoc significance between the groups in case any significance was noticed. This test was used to compare all the possible pairs of means.

Significant differences among group means were calculated using the F statistic, which is the ratio of the mean sum of squares (the variance explained by the independent variable) to the mean square error (the variance leftover). If the F statistic was higher than the critical value (the value of F that corresponds with alpha value (P), usually 0.05), then the difference among groups was deemed to be statistically significant (T. Kim, 2017). In addition, the means 95% confidence interval (CI), the standard deviation (SD), and the relative standard deviation RSD were calculated.

6.4   Results

6.4.1   The degree of DFR from different leaves treated with difenoconazole EC 10% (w/v) using the micropipette method.

DFR recovery % (±SD) of the DFZ EC 10 % (w/v) on the five tested crops/leaves are shown in Table 6.1 and Figure 6.1. The DFR on wheat leaves proved to be the highest with a percentage of (89.0 ± 4.0%) followed by the French bean (82.0 ± 2.9%), tomato (60.0 ± 1.2%), soya bean (52.2 ± 3.6%), and oilseed rape (31.0 ± 3.4%).

The micropipette method's relative standard deviation (RSD%) in all sample/leaf washes was below 11%. Control groups of each leaf/crop tested had no detectable residue, all of which were below the LOD level (0.002 µg mL^{-1}). The mean DFR of the wheat plant was the highest with a value of (4.7 µg ± 0.2), while tomato and soya bean showed relatively close DFR means (3.0 ± 0.1, 2.6 ± 0.2 µg), respectively. On the other hand, oilseed rape had the lowest value of DFR mean (1.6 ± 0.2 µg) compared to the other four mentioned crops.

The DFR of DFZ EC 10% (w/v) in all plants proved to be normally distributed when tested by the Shapiro-Wilk test using SPSS with no P-value below 0.05, as shown in Table 6.1. This result was also confirmed by looking at the plotted normal quantile-quantile(Q-Q) plots, where all data points lay around the mean of each tested group. Data points falling along a straight line in the Q-Q plot provided evidence that the data came from a uniform distribution with no skewness or kurtosis out of the normal range detected. In addition, skewness and Kurtosis values were within the acceptable range of the normal distribution (-1,1) and (-3,3), respectively. The comparisons between all tested groups have been performed using Tukey's HSD.

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In the case of DFR recovery from DFZ EC 10% (w/v) using the micropipette application method, all tested groups showed significant differences compared to each other at a significant level of P ≤ 0.05. In other words, the one-way ANOVA, along with Tukey's HSD test, showed a statistically significant difference in DFR mean values between all treated groups (P≤ 0.005).

Table 6.1: The effect of different leaf textures/crops on the DFZ EC 10% w/v DFR using the Micropipette method.

Descriptive statistics	French bean	Tomato	Soya bean	Oilseed rape	Wheat
N(Replicates)	10	10	10	10	10
Mean µg	4.1	3.0	2.6	1.6	4.6
(±SD)	(±0.1)	(±0.1)	(±0.2)	(±0.)	(±0.2)
Median	4.1	3.0	2.6	1.6	4.7
*RSD%	4%	2.0%	7%	10%	5%
DFR Recovery%	82.0%	60.0%	52.0%	31.0%	89.0%
Mean (±SD)	(±2.9)	(±1.2)	(±3.6)	(±3.4)	(±4.0)
Anova significance	P≤ 0.005				
Shapiro-Wilk Normality test (P value) (Significance P=0.05)	0.3	0.4	0.9	0.4	0.8

Table 6.1 elucidates *the descriptive statistics of difenoconazole EC 10% (w/v) DFR on 5 selected plants. Each treatment group had 10 replicates, and each replicate consisted of two treated leaves. A significant difference between all tested groups was observed when tested with ANOVA. The mean difference is significant at the 0.05 level (P ≤ 0.05). Data represent the mean (±SD) of n = 10 determinations. SD± mean is the standard deviation. RSD % is the percentage of relative standard deviation among samples of each tested group. All RSD% values are below 11%.*
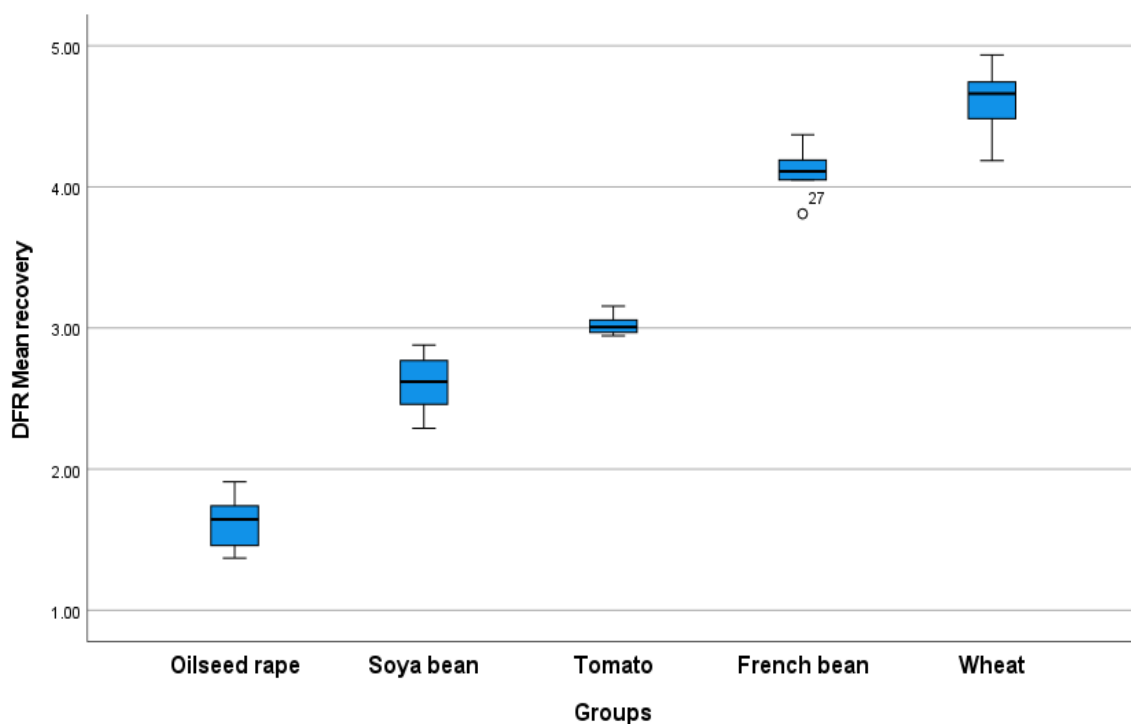
Figure 6.2, a box plot chart, compares the DFR means of different leaf types from five crops (soya bean, tomato, French bean, wheat, and oilseed rape). The box plot elucidates the DFR mean difference of difenoconazole EC 10% (w/v). 10 replicates were chosen for each treatment group, and every two treated leaves represented one replicate. A significant difference between all tested groups was observed when tested by ANOVA. The mean difference is significant at the 0.05 level ($P \leq 0.05$).

Figure 6.2: Box plot chart showing the degree of DFR means from different leaves treated with difenoconazole EC 10% (w/v) using the micropipette method.

6.4.2 The degree of DFR from different leaves treated with difenoconazole EC 10% (w/v) using the Track sprayer method.

The surface area of the Petri dishes was approximately 100 cm^2, and the average spray weight in an empty Petri dish was 0.22 grams. The average surface area of the Petri dishes and the average spray weight were used to calculate the application rate per Hectare by conversion from cm^2 to a hectare. The application rate per hectare using the track sprayer at the speed of 50 cm second^{-1} was 137.5 g DFZ 220 L^{-1} hectare $^{-1}$.

The average concentration of the DFZ calculated in the 100 cm^2 Petri dishes was (0.41 g dish^{-1}), which was calculated from the DFZ spray concentration (0.625 mg mL^{-1}) and the average weight of the spray in the sprayed Petri dish (0.66 g). Therefore, the average DFZ per cm^2 of the Petri dish was estimated to be 0.001 mg cm^2 (dish surface area is 100 cm^2). This DFZ amount per cm^2 was then used to calculate the recovery % of DFZ from the track sprayer on the targeted leaves for each replicate after calculating the surface area of each washed replicate (double-sided).

Table 6.2: Track sprayer calibration using the weight difference of Petri dishes before and after four rounds of a spray.

Petri dishes	Petri dishes weight (g)		Weight difference
	Before the spray	After the spray	(g)
Petri 1	18.86	19.52	0.66
Petri 2	16.27	17.01	0.74
Petri 3	16.37	17.01	0.64
Petri 4	16.37	16.97	0.60
Total spray weight for all dishes (g)			2.64
Average spray weight per dish (g)			0.66
Average spray weight per round from 3 spray rounds (g)			0.88
Spray weight per dish from 4 Petri dishes (g)			0.22

Table 6.2 shows the weight difference between empty Petri dishes before and after water spray at a speed of 50 cm sec $^{-1}$ with a Teejet 8001EVS nozzle at the height of 65 cm from the treatment shelf (approximately 35 cm from the targeted leaves).

Table 6.3: The effect of different leaf textures/crops on the DFZ EC 10% (w/v) DFR using the track sprayer method.

Descriptive statistics	French bean	Tomato	Oilseed rape
(T0 hour) Mean (mg/cm^2) (N=2)	0.0001	0.00006	0.00005
(T3 Hours) Mean (mg/cm^2) (±SD) (N=5)	0.0001 (±0.00003)	0.00008 (±0.00001)	0.00002 (±0.000005)
*RSD%	30.0%	13.0%	25.0%
Mean DFR Recovery % from DFZ concentration in 1 cm^2 leaf (0.001 mg cm^{-2}) (±SD)	8.0% (±1.9)	6.0% (±0.7)	1.0% (±0.3)
ANOVA significance	P≤ 0.00005		
Shapiro-Wilk Normality test (P value) (Significance P=0.05)	0.6	0.1	0.5

Table 6.3 elucidates the descriptive statistics of difenoconazole 10% EC DFR on 3 selected plants. Each treatment group had 7 replicates, each consisting of two treated leaves. The first two replicates were used to estimate the recovery just after the application (T0), while the remaining 5 replicates were left 3 hours for the residue to dry (T5). A significant difference between all tested groups was observed when tested with ANOVA. The mean difference is significant at the 0.05 level (p ≤ 0.05). Data represent the mean (±SD) of n = 5 determinations. SD± mean is the standard deviation RSD % is the percentage of relative standard deviation among samples of each tested group. All RSD% values are below 30%.

DFR recovery % (±SD) of the DFZ EC 10 % (w/v) on the three tested crops/leaves are shown in Table 6.3 and Figure 6.2. The residue retention on French bean leaves proved to be the highest, with a DFR recovery percentage of (8.0 ± 1.9%) followed by tomato (6 ± 0.7%) and oilseed rape (1 ± 0.3%). The recovery % was calculated based on the average concentration of the DFZ spray in 1 cm^2 of the sprayed Petri dishes during the calibration step. The average DFZ per cm^2 of the Petri dish was estimated to be 0.001 mg/ cm^2. In addition, the control groups of each leaf/crop tested had no detectable residue, all below the LOQ level.

The track sprayer method's relative standard deviation (RSD%) in all sample/leaf washes was below 30%. The DFR data of DFZ EC 10% (w/v) using the track sprayer method in all plants proved to be normally distributed when tested by the Shapiro-Wilk test using SPSS with no P-value below 0.05, as shown in Table 6.3. This result was also confirmed by looking at the plotted normal quantile-quantile(Q-Q) plots, where all data points lay around the mean of each tested group. Data points falling along a straight line in the Q-Q plot provided evidence that the data came from a uniform distribution with no skewness or kurtosis out of the normal range detected. In addition, skewness and Kurtosis values were within the acceptable range of the normal distribution (-1,1) and (-3,3), respectively. The comparisons between all tested groups have been performed using Tukey's HSD.

In the track sprayer experiment, the DFR recovery from DFZ EC 10% (w/v) showed a significant difference among all treated leaves/crops compared at $P \leq 0.05$. In other words, the one-way ANOVA, along with Tukey's HSD test, showed that there was a statistically significant difference in DFR mean values between all treated groups ($F_{(2, 12)} = 45.4$, $P \leq 0.00005$).

Figure 6.4 shows the plants ready for the track sprayer treatment on the treatment shelf. The nozzle height was adjusted to be 35 cm from the targeted leaf in all the sprayed plants. Seven pots of each plant were placed on the treatment shelf in one raw and sprayed simultaneously.

Figure 6.3: picture showing the plants prior to the track sprayer treatment.

6.4.3 Contact angle measurements and comparison from different leaves treated with different DFZ 10% formulations.

As presented in Table 6.4 below, the difference in the mean contact angles in DFZ WP 10% (w/v) droplets was greater than DFZ EC 10% (w/v) on all tested plants/ leaves. In the case of French beans tested with EC 10%, the difference in the average contact angle was the lowest (13.8˚) compared to all other formulations/leaf combinations.

On the other hand, the difference in oilseed rape mean contact angles were the highest when tested with both DFZ formualtions. Although the mean oilseed rape contact angle at the initial time of the measurement was the highest with both DFZ 10% formulations (i.e., EC and WP) (71.7 ± 3.7) and (67.6 ± 13.1), their decline rate was also the highest among all test formulation/crop combinations, demonstrating rapid droplet spreading. The mean contact angles of French beans were relatively higher in both EC and WP formulations (56.7 ± 5.9), (53.2 ± 6.3) compared to tomato mean contact angles (54.6 ± 5.0), (53.1 ± 5.3), respectively. The decline in the mean contact angle in both tomato and French bean was relatively comparable with both DFZ formulations tested.

Table 6.4: The mean contact angle measurements for difenoconazole EC 10% (w/v) and WP 10% (w/v) droplets on different leaves.

Contact angles time (seconds)	Formulation/crop combinations Average (± SD) contact angle measurements (degree)					
	French bean		Tomato		Oilseed rape	
	EC 10%	WP 10%	EC 10%	WP 10%	EC 10%	WP 10%
Impaction time (0 sec)	56.7 (± 5.9)	53.2 (± 6.3)	54.6 (± 5.0)	53.1 (± 5.3)	71.7 (± 3.7)	67.4 (± 13.1)
120 sec.	43.0 (± 4.3)	33.0 (± 11.3)	39.2 (± 13.9)	32.5 (± 12.9)	50.7 (± 3.5)	39.0 (± 7.0)
Contact angle difference	13.8	20.2	15.4	20.6	20.9	28.5

Table 6.4 shows the mean contact angle measurements for 2 µL droplets of DFZ EC 10% and WP formulations on different crops/leaves (i.e., French bean, tomato and oilseed rape). The contact angle measurement was measured using DataPhysics instrument – Model OCA 25-Contact Angle Measuring and Contour Analysis System purchased from DataPhysics Instruments GMBH, Germany and available at Syngenta International Research Station, UK.

Figure 6.5 below demonstrates a dynamic decline in the mean contact angle (right and left angles) of the DFZ EC 10% (w/v) and WP 10% (w/v) with a droplet size of 2 µl on different leaves (i.e., French bean, tomato and oilseed rape). The contact angle filming was for 150 seconds using Physics instrument – Model OCA 25-Contact Angle Measuring and Contour Analysis System purchased from DataPhysics Instruments GMBH, Germany and available at Syngenta International Research Station, UK.

The French bean's average contact angle decline rate was faster in the DFZ WP 10% (w/v) droplets compared to the EC 10% droplets. However, the contact angle decline rate for DFZ EC and WP droplets was similar in tomato and oilseed rape-tested leaves, as shown in Figure 6.5 below.

Figures 6.6, 6.7 and 6.8 below show the images of 2 µL droplets of DFZ EC 10% (w/v) and WP 10% (w/v) on French bean, tomato and oilseed rape leaves, respectively. In all Figures, image (A) and (B) shows the DFZ EC 10% droplet formation at the impaction time (zero time from the droplet dispensed) and after 120 s, while images (C) and (D) shows the DFZ WP 10% droplet formation at the impaction time and after 120 s.

In Figure 6.6, in the DFZ EC 10% (w/v) dispensed droplet on French bean, the estimated average contact angles (right and left) declined from (56.7 ˚to 43.0°)at 120 s. On the other hand, the DFZ WP 10% droplet mean angles dropped faster from (53.2˚ to 33.0°) after 120 s compared to EC contact angles decline. Images (C) and (D) in Figure 6.6 highlight the faster decline in the mean contact angle in the DFZ WP 10% formed droplet on French bean leaves.

In Figure 6.7, Images (A) and (B) show the EC droplet formation on the tomato leaves at zero and 120 s after dispensing the droplet on the leaf surface. From the estimated mean contact angles (right and left), a faster decline in the contact angle mean was observed in the WP-formed droplet from (53.1° to 32.5°) compared to the EC droplet decline from (54.6° to 39.2°) in images (A) and (B). Images (C) and (D) in Figure 6.7 highlight the faster decline in the mean contact angle in the DFZ WP 10% (w/v) formed droplet on tomato leaves.

In Figure 6.8 below, Images (A) and (B) show the EC droplet formation on oilseed rape leaves at zero and 120 s after dispensing the droplet on the leaf surface. From the estimated mean contact angles (right and left), a significantly faster decline in the contact angle mean was observed in the WP-formed droplet (from 67.4° to 39.0°) as shown in images (C) and (D) compared to the EC droplet (71.7° to 50.7°) in images (A) and (B). Nevertheless, regardless of the formulation, the mean contact angle of the DFZ formed droplets at the impaction time and, after 120 s in both French bean and

tomato, showed relatively close values compared to the higher mean contact angle on oilseed rape.
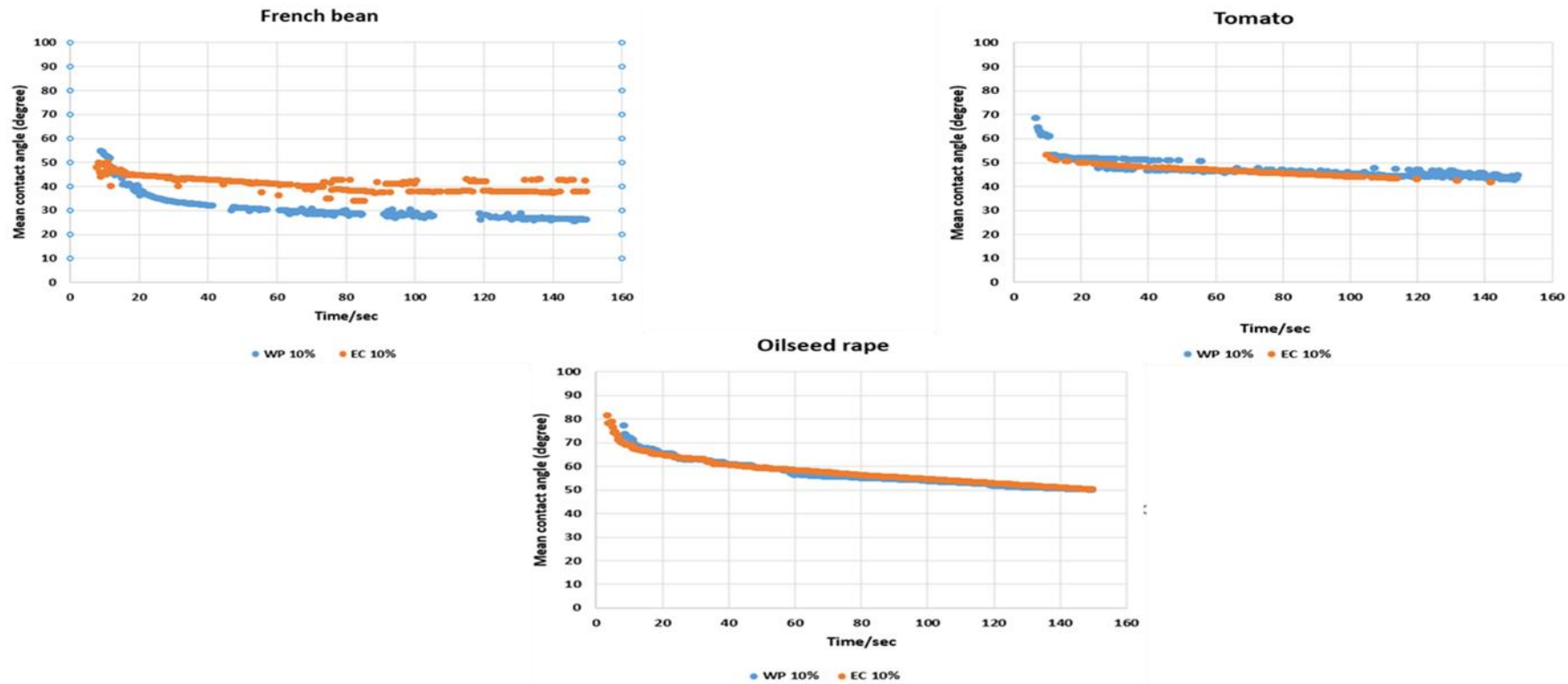
Figure 6.5 below a dynamic decline in the mean contact angle (right and left angles) of the DFZ EC 10% and WP 10% 2 µl droplet on different leaves (French bean, tomato and oilseed rape) for 150 s from a video analysis using Physics instrument – Model OCA 25-Contact Angle Measuring and Contour Analysis System purchased from DataPhysics Instruments GMBH, Germany and available at Syngenta International Research Station, UK.

Figure 6.4: Dynamic average contact angles decline rate for DFZ EC 10% and WP 10% formulation on different leaves/crops.
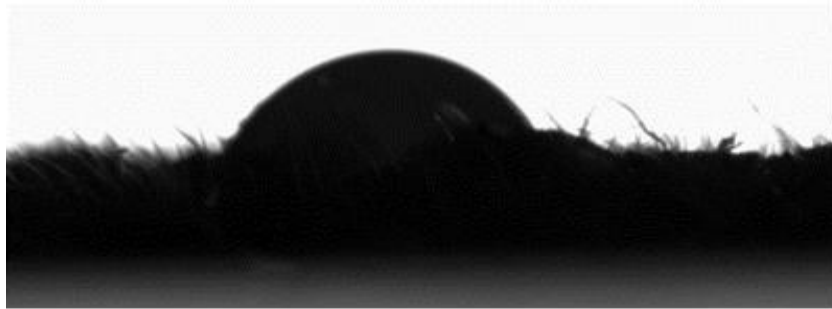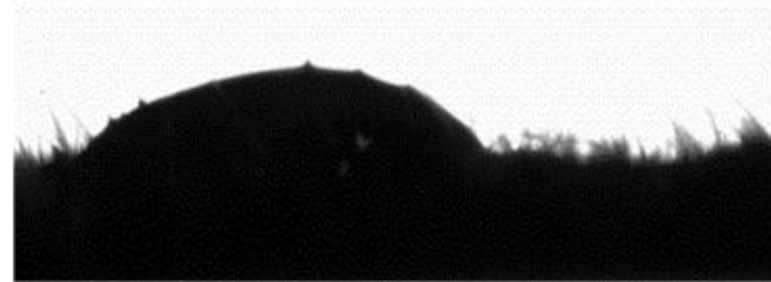
Figure 6.6 shows the images of 2 μL droplets of DFZ EC 10% (w/v) and WP 10% (w/v) on French bean leaves at impaction time (zero time) and after 120 s. (A) is a DFZ EC 10% droplet on French bean leaves at 0 time with a contact angle of 56.7˚. (B) is a DFZ EC 10% droplet on French bean leaves after 120 s with a contact angle of 43.0˚. (C) is a DFZ WP 10% droplet on French bean leaves at 0 time with a cntact angle of 53.2˚. (D) is a DFZ EC 10% droplet on French bean leaves after 120 s with a contact angle of 33.0°.

Figure 6.5: Images of 2 μL droplets of DFZ EC 10% and WP 10% on French bean leaves at impaction time (zero time) and after 120 seconds.
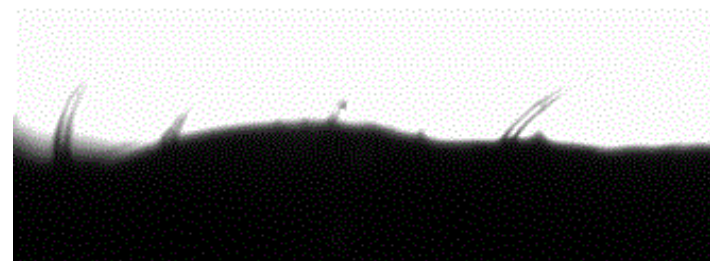
*Figure 6.7 shows the images of 2 µL droplets of DFZ EC 10% and WP 10% on tomato leaves at the impaction time (zero time) and after 120 seconds. (**A**) is a DFZ EC 10% droplet on tomato leaves at 0 time with a contact angle of 54.6°. (**B**) is a DFZ EC 10% droplet on tomato leaves after 120 s with a conatct angle of 39.2˚. (**C**) is a DFZ WP 10% droplet on tomato leaves at 0 time with a contact angle of 53.1°. (**D**) is a DFZ EC 10% droplet on tomato leaves after 120 s with a contact angle of 32.5°.*

Figure 6.6: Images of 2 µL droplet of DFZ EC 10% and WP 10% on tomato leaves at impaction time (zero time) and after 120 seconds.
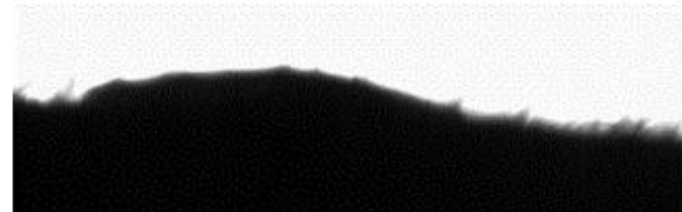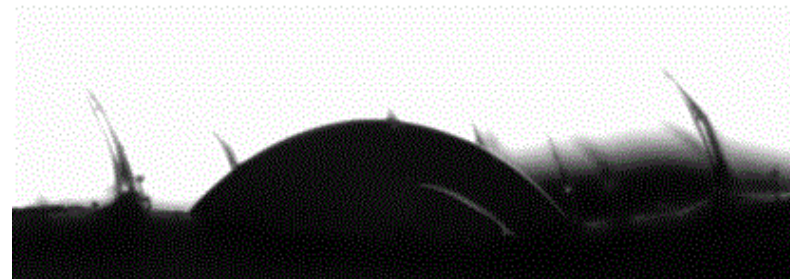
Figure 6.8 shows the images of 2 µL droplets of DFZ EC 10% and WP 10% on oilseed rape leaves at impaction time (zero time) and after 120 s. (*A*) is a DFZ EC 10% droplet on oilseed rape leaves at 0 time with a contact angle of 71.7°. (*B*) is a DFZ EC 10% droplet on oilseed rape leaves after 120 s with a contact angle of 50.7°. (*C*) is a DFZ WP 10% droplet on oilseed rape leaves at 0 time with a contact angle of 67.4° (*D*) is a DFZ EC 10% droplet on oilseed rape leaves after 120 s with a contact angle of 39.0°.

Figure 6.7: Images of 2 µL droplet of DFZ EC 10% and WP 10% on oilseed rape leaves at impaction time (zero time) and after 120 seconds.

6.5   Discussion

Previous research has shown that the leaves of tomatoes are of a Wenzel type. In contrast, French bean leaves are proposed to be of a Cassie-Baxter type due to the air sacs reported between the spray and the leaf surface, similar to the Cassie – Baxter interaction on hairy surfaces (Nairn et al., 2013; Ron M. Dahlin, 1992). Moreover, it has been shown that the trichome types found on both tomatoes and soybean are of a similar type (Franceschi & Giaquinta, 1983; Li et al., 2018). In Wenzel-type trichomes of hairy leaves, the deposited droplets can penetrate through these trichomes and reach the leaf epidermis (Wang et al., 2014, 2015). Conversely, the Cassie-Baxter types, where the deposited droplet is held above the leaf surface, preventing it from reaching the leaf epidermis, could lead to the more DFR, as illustrated in Figure 6.9 below, adapted from (Nairn et al., 2013). The droplet attachment model that prevailed also seemed dependent on the density of the hair mat (Nairn et al., 2013).
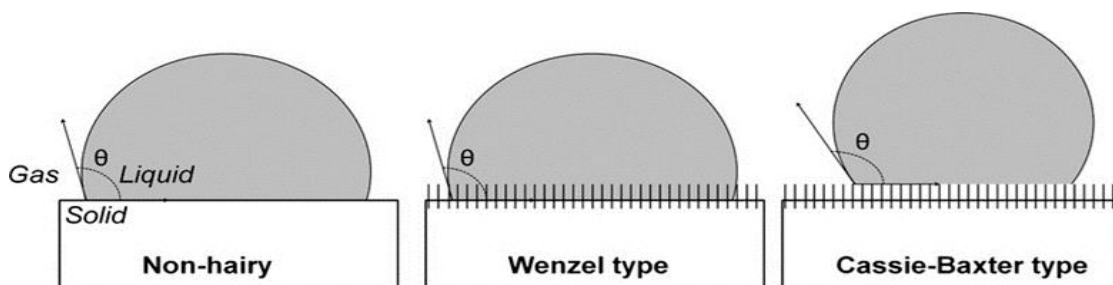
Figure 6.9 shows different types of droplet interactions on hairy leaf surfaces, namely Wenzel- and Cassie–Baxter-type adhesion. In the latter, the droplet is held on the tip of the trichomes preventing it from reaching the leaf epidermis and leaving an air pocket between the droplet and the leaf surface. In the Wenzel type, the droplet bursts and reaches the epidermis with a lower contact angle compared to the Cassie-Baxter type enhancing the absorption (Nairn et al., 2013)
Figure 6.8: Different types of droplet attachment for smooth and hairy surfaces.

The Cassie–Baxter model is often only meta-stable, with the droplet spontaneously (or with vibration to get over the energy barrier) switching to the Wenzel model (Nairn et al., 2013). The time needed for this transition could vary from plant to plant. Nevertheless, the time required for this transition would delay the droplets from reaching the epidermis and, in return, would also increase the DFR (Nairn et al., 2013).

From the DFR recovery % results in the first experiment using the controlled droplet method, the recovery % on tomato and soya bean from DFZ EC 10% (w/v) showed a significant difference (P=0.008) and a relatively close mean recovery % (60 ± 1.2%) and (52 ± 3.6%) respectively. The close DFR recovery % of tomato and soya bean could be due to both leaves exhibiting similar trichome types on their surface (Wenzel type). However, the statistical difference between them (P=0.008) could be due to the different percentages and coverage of these trichomes on their surfaces (Franceschi & Giaquinta, 1983). Notwithstanding that French bean leaves can also be considered hairy (similar to the tomato and soya bean leaves), the DFR recovery % on French bean leaf surfaces proved to be significantly higher (82 ± 2.9%) in comparison.

The literature hypothesises that in tomatoes and soybean, the trichomes on their surfaces puncture the surface of the impacting droplet, which promotes droplet shatter over adhesion or bounce, hence reaching the epidermis where absorption starts (Franceschi & Giaquinta, 1983; Li et al., 2018). On the other hand, and unlike what is happening with other leaves, spray droplets on French beans were proposed to adhere to the hairs' tips following the adhesion of Cassie–Baxter-type. Consequently, the droplets are leftover as DFR where absorption ceased or was delayed compared to other tested leaves. The same has also been evident in the research conducted by Dahlin (1992) on French bean trichomes, showing that the trichomes conferred resistance to leaf rust. This resistance was achieved by preventing

dew and other free water from coming into contact with the leaf surface epidermis, often leaving a layer of air between the water and the leaf surface (Ron M. Dahlin, 1992). That is like the Cassie–Baxter type illustrated in Figure 6.9 (Nairn et al., 2013). This explains the higher DFR recovery percentage (82.0 ± 2.9%) on the French bean leaf and its significance ($P \leq 0.05$) when compared to all other types of leaves/crops when treated with DFZ EC 10% (w/v). In the first experiment, the volume of the droplets used was 0.2 µL, the smallest reproducible droplet that could be generated using an electronic micropipette. Despite that, this droplet volume could be relatively bigger than those droplets delivered in the field using modern agriculture spray machinery where more sophisticated nozzles and spray pressures are employed. Consequently, the behaviour of the droplet and its capturing over the leaves in the field could deviate from the explanation above.

In addition, despite the dense trichome presence on French bean leaves imaged, DFZ spray droplet (EC or WP) using a bigger droplet of 2 µL showed no trichomes holding the droplet on its tip (Figure 6.6). This could be due to the bigger diameter of the 2 µL droplet compared to the one applied during the lab experiment (0.2 µL), which is even bigger than the in-field or track sprayer-generated droplets. This is in line with the findings that Cassie–Baxter-type of adhesion is limited under the assumption that the surface texture (trichome) is much smaller than the size of the liquid droplet or vice versa (W. Choi et al., 2009).

Nevertheless, it was evident that the leaves with trichomes also are more water repellent than leaves without trichomes or less dense in their trichome. This was observed from the difference in mean contact angle in both DFZ formulations, where both contained a continuous aqueous phase. The repellency of water or water-soluble solutions is associated with trichome density and the fact that trichomes prevent water
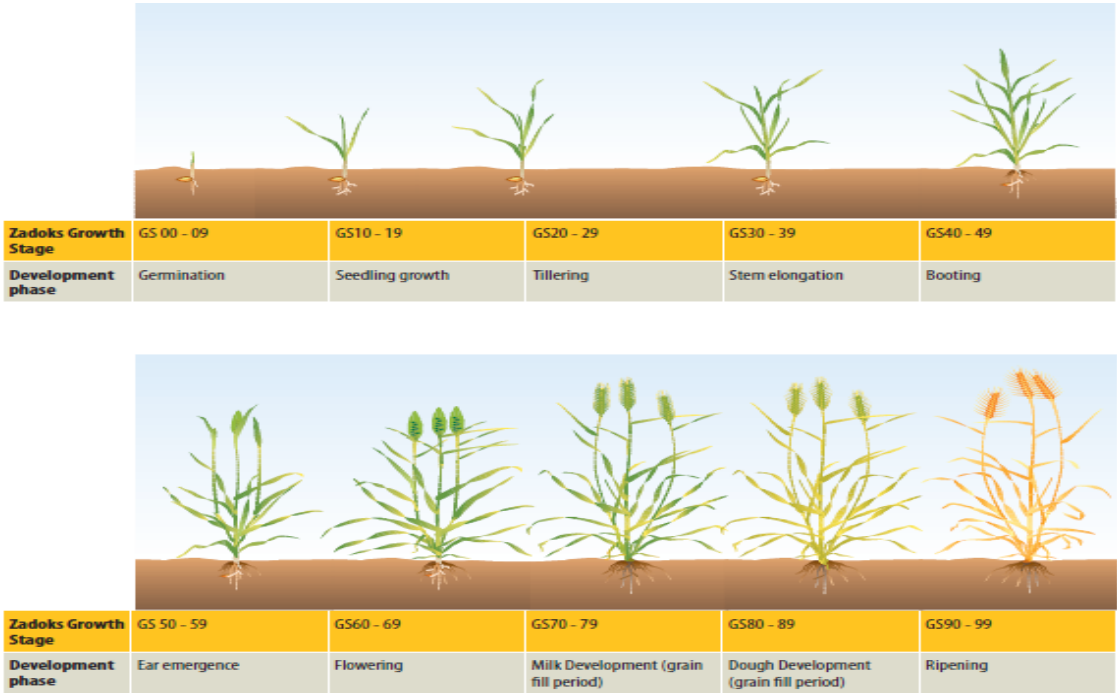
droplets from reaching the epidermis, resulting in relatively low droplet retention on leaves (L. Xu et al., 2011). As clearly shown in the images of Figure 6.6, there were trichome dense on the French bean leaf surface, and this could be the reason for absorption impairment and higher DFR%, especially with a bigger volume of droplets (0.2 μL or 2μL), unlike the DFR recovery % in the case of track sprayer experiment which was associated with smaller droplets from the spray jet. This is not to withstand that in each experiment (controlled DFR lab study or track sprayer), the residue pattern across different leaves was similar, with the French bean having more residue than other leaves tested, which could be due to the reasons explored above.

Other research also well-established that tomato and soya bean leaves exhibit similar morphological characteristics and trichome types (Franceschi & Giaquinta, 1983; Li et al., 2018). Both leaves bear glandular and non-glandular trichomes on their surfaces with different percentages. The overall trichome density and type on both leaves are known to differ. Trichome density on the soybean adaxial leaf surface is about 1300 cm^2, of which ~65% bare non-glandular (Type V) trichomes; while in the case of the tomato, the trichome density is 1800 cm^2, with ~48% of the trichomes being non-glandular of differing types (Franceschi & Giaquinta, 1983; Li et al., 2018). The different percentages of trichomes coverage on tomato and soya bean could contribute to the statistically significant difference in DFR between both leaves when tested using DFZ 10% EC. The latter suggestion is also in line with Xu et al. (2011), who concluded that "*Hairs on the surface give added complexity with differences in density (trichomes per mm²)*" (L. Xu et al., 2011). Therefore, it could be misleading to group all hairy leaves under the same categorial response for the DFR but classify them according to the type, and trichomes density on their surface which could provide a more realistic framework for predicting DFR outcomes.

The DFR % for difenoconazole EC 10% (w/v) from the treated wheat plants (*Triticum aestivum) was* unexpectedly the highest DFR recovery % (89.0 ± 4.0%), especially when compared to oilseed rape (31.0 ± 3.4%). Despite both plants being considered to possess waxy leaves, both leaf types could have different levels of epicuticular wax on their leaf surfaces. The level of epicuticular wax on the plant surface is a determining factor when testing the level of residue that could be left over after the pesticide drop dries on the surface (L. Xu et al., 2011). The reduced DFZ EC 10% (w/v) DFR% on oilseed rape could be due to the possibility of rolling the droplets from the leaves. This is in line with other research findings where spray droplets on water-repellent leaf surfaces result in unavoidable bounce, splash and run-off of the spray (Liu et al., 2021). This unexpected, elevated residue level on wheat leaves could be due to those treated leaves being at an early growth stage GS13-3 (unfolded leaves with the first tiller emerging from the first leaf axial).

Nevertheless, it is crucial to note that the current study's result would not be comparable with the data from an actual DFR experiment in the field. That is due to later growth stages are usually targeted for spraying between GS30-39 (Stem elongation stage) to GS90-99 (ripening stage), according to the Grains Research and Development Corporation (GRDC, 2005). The GRDC staging identifies wheat growth stages, from germination (GS 00-09) to ripening (GS 90-99), as shown in Figure 6.10. The explanation that the DFR may differ between the growth stage employed in the current laboratory study is in line with some field tests that Butler et al. (2004) concluded. These trials demonstrated experimentally that a single plant (i.e., wheat, oat, and barley) would have leaves with a range of ages and, therefore, a degree of wettability and waxy cuticles when tested (Butler et al., 2004). In their study, the wettability on the plants increased from 15% to 80% as the growth stage increased (Butler et al., 2004). Comparably, when applying the 15-80 % factor of residue

reduction, the DFR residue of the current experiment could indicate a residue level at higher growth stages that reach approximately 8-11% if the same hypothesis is applied. In the present laboratory experiment, higher wheat growth stages were not selected due to the limited growing space available for tall plants within the controlled growth chambers. The potential benefits of highly controlled growth and droplet application that can be achieved within a laboratory compared to in a field test are therefore counterbalanced by the potential impact of leaf age on DFR. Testing the effect of waxy leaves at different growth stages on the degree of DFR is recommended for further research.

Zadoks Growth Stage	GS 00 – 09	GS10 – 19	GS20 – 29	GS30 – 39	GS40 – 49
Development phase	Germination	Seedling growth	Tillering	Stem elongation	Booting

Zadoks Growth Stage	GS 50 – 59	GS60 – 69	GS70 – 79	GS80 – 89	GS90 – 99
Development phase	Ear emergence	Flowering	Milk Development (grain fill period)	Dough Development (grain fill period)	Ripening

According to the Grains Research and Development Corporation, figure 6.5 shows wheat's general growth stages. The figure above shows the different growing stages of the plant, including the stages from germination (GS 00-09) to ripening (GS 90-99) (GRDC, 2005).

Figure 6.9: Wheat General Growth stages according to the Grains Research and Development Corporation (GRDC, 2005).

When different plants were treated at the same application rate, the DFR from different leaf types demonstrated that hairy leaves, in general, possessed more residue than waxy leaves (when the result obtained from the wheat leaf was disregarded due to the reasons described above). The latter finding agrees with the classification that the USA Agriculture Taskforce has set (ARTF), which grouped the leaf types statistically based on their texture effect on the DFR into three categories (Bruce & Korpalski, 2008). The categories are smooth, waxy, and hairy. The degree of DFR was reported to follow a statistical trend of **hairy > smooth > waxy** leaves as the hairy leaves had more residue than waxy leaves (Bruce & Korpalski, 2008). However, it is critical to note that this classification was carried out by analysing different data from the literature. Furthermore, it did not identify the crops, pesticides, growing conditions and growth stages involved. Nevertheless, the current findings confirm this result by proving that different hairy leaves (i.e., French bean, soya bean, tomato) result in more DFR than the waxy leaves tested (oilseed rape). In addition, the results showed further evidence that hairy leaves could act differently in response to the residue based on the type and density of the trichomes found on their surface.

The above residue trend was also confirmed in the second experiment when the plants were sprayed with a similar application rate but using a different application method (track sprayer) that resembled the in-field conditions. The mean DFR recovery % was the highest in the case of French bean (8.0 ± 1.9%), followed by tomato (6.0 ± 0.7%) and oilseed rape (1.0 ± 0.3%). Despite the variations that may be encountered in the track sprayer experiment and expressed in an RSD of s maximum of 30%, which could be due to the spray angle, speed and pressure of the spray, the residue trend showed

statistically that hairy leaves (i.e., French bean, tomato) had more residue than waxy leaves (oilseed rape). That is, even though the droplets generated from the track sprayer were much smaller than those generated by the micropipette. The recovery % was estimated based on a calibration estimate of the maximum quantity that could reach the Petri dishes lying on a flat surface. However, this was the best way to estimate the maximum residue; it does not reflect the actual scenarios where different leaves have different orientations and angles during the spray, which could be more or less exposed to the spray jet. Furthermore, the speed and the coarse spray droplets are known to wet the surfaces more quickly than bigger droplets. This could be another reason for the less residue magnitude observed in the track sprayer experiment compared to the DFR lab-controlled method (X. Dong et al., 2015).

Droplet impaction and deposition on the leaf surfaces generally comprise a complex and dynamic two-phase flow process. This includes droplet impact, rebound or retention and spreading (X. Dong et al., 2015). The probability of spray rebound and whether losing its target or forming smaller droplets on the surface is higher with waxy leaves than with hairy leaves. In such case, If retained, smaller droplets will be subjected to easier absorption and/ or faster evaporation from waxy surfaces (X. Dong et al., 2015). This, in return, cause less residue in comparison. These findings are supported by the low residue recovered directly after the spray (To) from each cm^2 of oilseed rape leaves (waxy leaf) (0.00005 mg cm^{-2}) compared to tomato (0.00006 mg cm^{-2}) and French bean (0.0001 mg cm^{-2}). This is also in line with Dong's (2015) findings and conclusion on waxy leaves, where These microscopic processes of droplet impact and deposit formation demonstrated that droplet diameter, droplet impact speed, and waxy leaf surface were the essential factors affecting the fate of droplets on leaves (X. Dong et al., 2015).

The droplets on the leaf surfaces were imaged by applying a droplet volume (2 µL) associated with a higher diameter than the droplets generated by the micropipette in the DFR lab experiment and those generated by the track sprayer jet. This was, however, the smallest droplet that could be imaged using the instrument. These images provided insight into the contact angles but could, on the other hand, differ from the actual estimates that could exist in the field or the DFR lab experiment.

The dynamic decline in the average contact angle in both DFZ formulation droplets (EC and WP) showed that both formulations declined relatively at a similar rate on tomato and oilseed rape. At the same time, there was a relatively faster decline in the contact angle of the WP droplets on the French bean compared to the EC suspended droplet, as shown in Figure 6.5 and Table 6.4 above. The contact angle decline could indicate the spray's ability to wet the leaf surface. These findings support the non-significant difference detected between DFZ EC 10% and WP 10% on French beans in the Chapter 5 experiment (see Table 5.3). The mean DFR recovery % of EC 10% on French bean was (82 ± 3%) compared to the lower value for WP DFZ recovery % (74 ± 13.1%). In addition to the explanation of the DFZ EC and WP formulation difference elaborated in Chapter 5, and despite such insignificance, it was clear from the images in Figure 6.6 of both formulation droplets at the impaction time and after 120 s, that the wetting of the spray, and consequently the absorption into the leaf, could be enhanced in case of the WP formulation on French bean leaves. This DFR recovery % insignificant difference could be due to the enhancement of the WP formulation discussed in Chapter 5. However, it must be stated that the absence of the emulsified internal phase in the WP formulation may also be associated with more rapid evaporation of the active substance from the leaf film. To investigate the relative contribution of evaporation, it would be necessary to image the droplet spreading dynamics from multiple observation angles and for a more extended period of time.

From the images of DFZ WP 10% on tomato leaf in Figure 6.7, it was clear that the spreading and possibly the absorption of the droplet enhanced on the surface (image D) compared to the EC 10 % droplet (image B) after 120 s. This was also the case when both mean contact angles were compared after 120 s, as shown in Table 6.4, where the contact angles were 39.2° and 32.5° on EC and WP droplets, respectively. Furthermore, the DFR recovery % reflected a significant difference between both formulations on tomato leaves when tested in Chapter 5 (see Table 5.3), with less mean DFR recovery% (39.0 ± 5.0%) in the case of WP 10% than EC 10% mean DFR recovery % (60.0 ± 1.2%). The difference in the contact angles of both DFZ formulations discussed above is comparable to the DFR % mentioned. Ultimately, these results support the explanation of the formulation difference effect of DFZ on tomato leaves, as stated in Chapter 5, and the above explanation of tomato hydrophilic trichomes that exist on the surface, which are hypothesised to have facilitated the absorption and spreading of the spray. In addition, the density of the trichomes on the surface of tomato leaf is not comparable to that on French bean leaves, and hence better spreading was observed.

In the case of waxy leaves such as oilseed rape, the images of the droplets and the mean contact angle measurement for both DFZ formulations (EC and WP) showed a bigger contact angle (71.7 °and 67.4°) at the impaction time of the droplets which were then reduced significantly compared to other leaf surfaces to (50.7° and 39.0°) respectively as shown in Table 6.4. The absence of these fine structures (trichomes) on the waxy surface could increase the exposure of these droplets to the ambient conditions and hence, increase the evaporation and degradation of the DFZ from the droplets than those existed and encountered within the complex architecture of the leaves.

When foliar uptake of lipophilic solutes such as those in the DFZ is considered, one has to be aware that solute mobilities determine the rate of the cuticular penetration in the cuticle with some driving forces caused by the thermodynamic activity of the AI within the formulation film. The sorption process is affected by numerous factors that change during droplet drying and can rarely be analysed individually (Buchholz, 2006).

The relevant factors that affect the sorption process are the physicochemical properties of the active substance. For example, low water-soluble molecules such as DFZ will result in minor cuticular wax sorption, although partitioning across waxy layers could be high (Buchholz, 2006). But the spray solution comprises, besides the active substance, other formulants such as adjuvants, surfactants, etc., which also facilitate and enhance the solubility or prevent AI crystallisation and agglomeration during droplet evaporation (such as those surfactants collaborated in the DFZ formulations and the surfactants that are normally present in EC formulations as a major part of the formula). In addition, other driving forces, such as environmental factors, govern the velocity of this dynamic process. Hence in the case of an active substance that is less soluble in water, there could be a rapid distribution and partitioning of the active substance in the cuticular layers or apoplast (with potential access to the symplast after traversing the biomembrane (Buchholz, 2006). The apoplast is the intercellular space involved only in water and nutrient transport and separates the "dead" apoplast from the "living" symplast (Farvardin et al., 2020). In other words, The apoplast pathway is where the water goes from cell wall to cell wall, not entering the cytoplasm at any point. The symplast pathway is where water moves between cytoplasm/vacuoles of adjacent cells. In line with other experiments, volatile solvents (also water) evaporate from the droplet where solutes and surfactants penetrate the cuticle membrane during this evaporation to an unknown extent (Arand et al., 2018a).

This could explain the quick decline in the mean contact angle of the DFZ WP 10% formulation droplet (see Table 6.4) and also could add an explanation of why the mean DFZ DFR recovery % on oilseed rape is lower than all other tested leaves in both EC 10% (31.0 ± 3.3%) and WP 10% (37.0 ± 6.1%) formulation as discussed in Chapter 5.

6.6   Conclusion

The results of this study shed light on the importance of the leaf texture as a factor that could affect the intensity of DFR. Understanding and investigating this factor could be of utmost importance for the agrochemical industry and the pesticide regulatory bodies. The current work showed that different leaves could act differently in response to the foliar application of difenoconazole EC 10% (w/v) and WP 10% (w/v). Moreover, grouping different leaves/crops based on their roughness (i.e., hairy or waxy) could be relevant and applicable since hairy leaves had more difenoconazole DFR than waxy leaves when tested regardless of the formulation used. However, an accurate approach to the classification would better involve the coverage degree and types of trichomes in different hairy leaves since hairy leaves may act differently based on these characteristics.

Another factor that could play a role and should not be overseen in such classification is the level of epicuticular wax found on the surface at different plant growth stages. For example, plants such as wheat "Triticum aestivum" could have different levels of epicuticular wax on their surface across various growth stages. That requires the selection of the plant growth stage most appropriate to the time of application of PPPs when designing the DFR study in the lab or the field. Using the study findings with the future exploration of the leaf texture as described by imaging the spray droplets on the leaves along with estimating the contact angles of the droplets could facilitate exploring the driver of the DFR intensity.

Generating more data using the laboratory DFR controlled method introduced and relating it to the leaf texture as a determining factor that could affect DFR could lead to a deeper understanding of the factor and, consequently, more evidence on the possible extrapolation between DFR field studies. In return, this could save the regulatory bodies and the PPPs registrants time and resources.

Chapter 7 : General Discussion

Plant protection products (PPPs) are used worldwide in agricultural settings, commerce, and individual households, resulting in increased productivity and continued human exposure to pesticide residue (Bonner & Alavanja, 2017; Caberera, 2017). It was estimated that without pesticides, 70 % of crop yields could have been lost due to pest infestations (Oerke, 2006). Humans are exposed to these chemicals from various sources, with multiple and different exposure levels. Generally, high exposure occurs in occupational, agricultural, or residential settings when pesticides are applied, mixed, and loaded from one place to another (Caberera, 2017). Pesticide exposure has profound effects on human health, including an increased risk of cancer, diabetes, genetic disorders, and neurotoxicity (Caberera, 2017). Consequently, pesticide residues on or in food or feed crops have the potential to impact human health if exposure results in an unsafe dose (Rani et al., 2021).

Therefore, pesticide use is determined by regulatory agencies worldwide to ensure the proper, safe, and consistent use of pesticides. Accordingly, a pesticide risk assessment is considered an essential component of pesticide regulation in most of the developed world (Krieger & Ross, 1993). In the European Union (EU), as published in the most recent European Food Safety Authority (EFSA) guidance for Regulation (EC) No 1107/2009, the risk assessment for plant protection products (PPPs) must be carried out for all exposure scenarios for operators, workers, residents, and bystanders that can be expected to occur as a consequence of the proposed uses of PPPs (Charistou et al., 2022). Most of these scenarios will fall into a category for which standardised first-tier exposure assessment can be carried out according to the guidance, using previously set default input values. However, for scenarios that are not covered or do not satisfy the first-tier assessment, the applicant may also use an ad hoc, higher-tier

exposure assessment by generating experimental data based on actual exposure (Charistou et al., 2022). Residue decline studies that estimate the half-life (DT_{50}) of the applied PPPs are also an essential part of risk assessment processes. Despite the high associated costs of field studies, they offer numerous advantages over studies that only measure residues at harvest. They provide data on residue behaviour over time, allowing reliable estimation of residues at any point up to the harvest point (European Commission, 2019b).

Nevertheless, the latest EFSA guidance identified gaps and uncertainties in the available data for worker exposure; hence, the EFSA working group (WoG) strongly recommended further collection and production of data on specific parameters related to worker exposure (Charistou et al., 2022; EFSA, 2014a). Since the most significant route of exposure for workers is dermal, these parameters are the transfer coefficient (TC) and the dislodgeable foliar residue (DFR) (Charistou et al., 2022; EFSA, 2014a). In the absence of experimental DFR data, and data for the active substances in question are not included in the EFSA 2014 guidance appendices, default values for DFR and DT_{50} would be used in the first-tier assessment (EFSA, 2014a). These default values are 3 µg active substance cm^{-2} of foliage per kg active substance applied/ha for the DFR and 30 days for the DT_{50} (Charistou et al., 2022; EFSA, 2014a). Nevertheless, the DFR default value was regarded as highly conservative (Charistou et al., 2022; Lewis & Tzilivakis, 2017b), and the DT_{50} default value was limited and relied on "a now-outdated" statistical analysis. In addition, residue dissipation data tends to be highly variable depending on how the dissipation rate was measured (e.g., on the crop surface or inside the crop tissues) and on the part of the plant tested (foliage, stems, fruit, etc.)(EFSA, 2014d). In addition, 13% of pesticides in the United States Department of Agriculture (USDA) data set had DT_{50} values reported as 30 days or more, indicating such conservatism (EFSA, 2014d).

Despite the presence of the OPPTS Guidelines 875.2000 and 875.2100 for conducting DFR studies in the field, there is still no harmonised method for conducting DFR studies in the open literature, and most of the current studies follow the previously mentioned method with minor differences (BfR, 2020). Nevertheless, the test guidelines (OPPTS 875.2100) are referenced by the European Commission (EC) in a document on the authorization of plant protection products in Europe (European Commission, 2020), which mandated the study in Europe to follow the Good Laboratory Practice standards (GLP) as recommended also in the latest EFSA guidance (Charistou et al., 2022).

The variability that exists among DFR field studies is often attributed to the seasonal nature of such studies, which usually encompasses non-controllable effects, such as changes in the meteorological conditions. Besides the different techniques, units, and formulations used in the available DFR studies, a direct comparison between these studies was not possible (Badawy et al., 2022; Charistou et al., 2022). Furthermore, The DFR definition considers that all pesticides that exist on the leaf surface after the pesticide dries are dislodgeable and require quantification during worker re-entry activities, but the initial guidelines of the current method did not include any validation of the wash-off solution used nor its efficiency in dislodging all the residue from the plant surface (Badawy et al., 2022). Moreover, the method guidelines emphasized the need for further validation requirements on the efficiency of the dislodging procedure (EPA, 2009). That highlights the importance of validating the volume of wash-off solution needed for each crop or leaf type before conducting the DFR field studies required by the non-dietary risk assessment of the PPPs (EFSA, 2014c).

Data on the DFR of PPPs are scarce in the literature (Badawy et al., 2021; Charistou et al., 2022), and EFSA has acknowledged this in their guidance in assessing the PPPs exposure of operators, workers, bystanders and residents. Hence, a recommendation

for generating more DFR data to reflect a realistic default value was made (Charistou et al., 2022; EFSA, 2014a). However, the public consultation on the same guidance also emphasised the lack of data correlation between factors influencing DFR and its values. Such a gap in the literature could be due to the high cost associated with conducting DFR GLP studies for PPP registration purposes, along with the seasonal nature of the field studies, as well as the ownership and confidentiality problems of the data. For these reasons, extrapolations between DFR studies are not favoured nor supported by regulatory authorities like EFSA due to the lack of complete knowledge of the nature of DFR.

All the challenges mentioned above in the non-dietary risk assessment of PPPs were recognised while establishing the aims and objectives of the current PhD project, and they were all targeting studying factors that may affect DFR and understanding the nature of these residues as summarised in the three aims below,

- Investigating any possible correlations between the dietary and non-dietary (DFR) decline data (DT_{50}). A protective and slower decline in the dietary residue compared to the non-dietary could allow the abundant decline data of the dietary residue to be used as a surrogate to DFR decline data and eventually derive a more realistic DT_{50}, which would be used in the non-dietary risk assessment. This would also help determine the length of the DFR studies, or even shorten the experimint period and eventually saving time during PPPs registration and approval.

- Developing a simple, reproducible laboratory methodology that facilitates investigating factors that may affect DFR. The newly developed method should be fast, cost-effective and controllable, managed and operated in different

desirable conditions and seasons to overcome the seasonality and costs associated with the DFR GLP field studies. In addition, this method should allow the generation of more data and studying potential factors that affect DFR solely or in conjugation to allow further extrapolation and comparisons in the future with the available field data.

- Using the DFR developed laboratory method to investigate factors that affect DFR (i.e., different leaf textures, pesticide formulation types, co-formulants). Such investigation would provide a further understanding of these residues and provide more data, and scientific evidence should any extrapolation be sought. In return, this would allow for faster registration of PPPs and more certainty in proposing default values or extrapolations rules between available studies. Eventually, this could help the regulatory authorities and the agrochemical producers save time and resources.

To address the first aim, an analysis to investigate the correlation between the two types of available decline residue data (i.e., DFR and dietary residue) was performed in two stages. First, on high-quality field trial data (Syngenta's data) and second, based on data that was extracted from the peer-reviewed residue studies or research in the literature (Pesticide Properties Database (PPDP))(Lewis et al., 2016). The selected studies were paired on the basis of providing data for both residue types for the same specific crop/active substance.

The first stage included studying six pesticide-active substances lambda-cyhalothrin, ADEPIDYN™ (pydiflumetofen), cyantraniliprole, cyprodinil, emamectin-benzoate and difenoconazole (see Table 3.1 In Chapter 3, Section 3.3.1). These studies had detailed data available, and there was confidence that the paired studies were conducted under

very similar experimental conditions. All these paired studies followed the Good Laboratory Practice (GLP) standards. Both types of trials (DFR and total residue) included data on the dissipation of the active substance residue over time (at least 5 points per trial) to allow the calculation of each active substance's DT_{50} for each trial independently using the first-order kinetics model in the CAKE software. As illustrated in Chapter 3 (Table 3.1 and Figure 3.2), the calculated dietary mean residue values for the six tested pesticide active substance were higher compared to the DT_{50} of DFR mean values. Statistical significance was achieved in 5 of the 6 tested active substances (i.e., lambda-cyhalothrin, ADEPIDYN™ or Pydiflumetofen, cyantraniliprole, cyprodinil, emamectin benzoate) using the non-parametric statistical test (i.e., Mann–Whitney U-test). Dieatry DT_{50} value of ADEPIDYN™ or Pydiflumetofen dietary DT_{50} was not statistically different (P = 0.053) from the DFR DT_{50} using the parametric T-Test, despite the significant difference observed with the non-parametric test. This was attributed to the small sample size tested in this active substance due DFR data limitation. On the other hand, there was no significant difference between dietary residues and DFR using either statistical test for difenoconazole. The non-significance observed in the case of difenoconazole residue decline using both types of statistical tests for the DFR and dietary residue tested from Syngenta's trials was attributed to the small sample size tested (n = 7) on one crop (i.e., apple), unlike the other active substances, where at least two crops were tested.

Despite the positive medium correlation coefficient (r=0.51) between the two residue types using the field trial data, it was evident that for all the active substances tested, the dissipation rate for the dietary residues was higher than that for DFR. Hence, accepting the limitation caused by the small number of studies, this work does provide some initial evidence that the dietary DT_{50} values are a reasonable surrogate for absent foliage DFR DT_{50} values.

In the second stage, there was no statistical correlation between 31 residue studies extracted from the PPDP on the 20 matching crops available for comparison purposes, which could be due to the vast variabilities mentioned in the open literature studies gathered in the database. At the same time, most of the data (75%, n = 42) followed the same pattern of higher DT_{50} of total residue compared to the DFR DT_{50} mean values, as shown in Chapter 3 (see Figure 3.2 in Chapter 3, Section 3.4.2).

From this novel research, the project's first aim was addressed, and there is initial evidence that the DT_{50} of the dietary residue studies could potentially act as a conservative surrogate for DFR DT_{50} on a specific crop/active substance combination or even on different crop matrices. This could be particularly useful if there is a lack of DFR studies and could also be used as a reference to anticipate the appropriate length of the DFR studies in the field. In addition, this study would encourage sharing confidential residue studies to ascertain this correlation which will improve the knowledge of the crop protection industry, regulatory bodies, stakeholders, and environmental scientists, leading to more robust regulation of pesticide use in a way that does not compromise human or environmental safety.

The second aim of the project was addressed by developing a new laboratory DFR method which was proved to be accurate and precise following the latest guidance for conducting studies of occupational exposure to pesticides OCDE/GD(97)184 during agriculture application. The laboratory method reflected the available field DFR methodology. Still, it involved controlled application of droplets to leaves and validation of the wash-off process used to remove the residue from the leaf surface before the analytical quantification. As a result, a very high level of accuracy (99.7-102.1 %) and precision (±1.5%) was achieved (see Table 4.4 in Chapter 4, Section 4.5.1). Residue data generated from the illustrated application of the method showed a

robust normal distribution, unlike field studies. The validation of the technique included testing different difenoconazole 10% formulations (EC and WP) by validating the effect of the plant matrices on the analysis to ensure that it does not affect the DFR recovery % (see Chapter 4, Section 4.5.2) along with validating the accurate wash-off volume required for every tested crop/ formulation (see Chapter 4, Section 4.5.3). A summary of the wash-off solvent required for each DFZ formulation/crop combination, and the amount calculated for each cm^2 foliage treated was then tabulated (see Table 4.9 in Chapter 4, Section 4.5.2).

From the data generated in Chapter 4, different tested leaves/crops (i.e., French bean, tomato, soya bean, oilseed rape and Wheat) required different wash-off solution volumes to rinse all the pesticide residue from the leaf surface (see Table 4.10, 4.11, 4.12 in Chapter 4, Section 4.5.3). Therefore, dislodging all leaves in the same volume of the wash-off solution regardless of the existing differences between them could underestimate the dislodged fraction of the pesticide leading to misleading or at least inconsistent quantification of the DFR and consequently poor comparison between different DFR estimations. Furthermore, such a gap in the literature could lead to an inaccurate estimate of the non-dietary risk associated with using PPPs. Eventually, the newly developed method was deemed to be controllable, cost-efficient, and time-saving, taking hours rather than days. This enables the generation of more data to allow extrapolation between the generated data through the investigation of multiple factors that may influence DFR.

To this end, it was crucial to use the developed method to investigate factors that influence DFR, however, many factors could potentially affect DFR. Due to the project timeline, some of these factors were investigated in Chapters 5 and 6. Chapter 5 discuss the effect of different difenoconazole formulation types (EC 10%, WP 10%, and

another EC 10% formulation with different co-formulants (i.e., different solvent system) denoted as EC(X) 10% (w/v)) on various crops (i.e., French bean, tomato and oilseed rape). Despite the pressing argument that the most significant effect on leaf wettability is the surface structures, some physical tests were also considered crucial (i.e., dynamic surface tensions, contact angles etc.) to understand the spray behaviour (Taylor, 2011). Therefore, studying the dynamic surface tension of multiple spray solutions would give an idea of the expected retention and consequently could be compared to the DFR magnitude. Therfore, the dynamic surface tension (DST) measurements for all formulations were investigated using the bubble pressure tensiometer BP100 from Kruss GMBH (Hamburg, Germany) (see Figures 5.5 and 5.6 and Table 5.8 in Chapter 5, Section 5.4).

A statistically comparable DFR% was retained from DFZ WP10% (w/v) and EC 10% (w/v) on most of the crops tested except for tomato, where lower DFR % was retained in the case of WP compared to EC formulation. The cause of this lack of difference could be due to the presence of non-ionic surfactant in the WP-tested formulation. The existence of the non-ionic surfactant in a WP formulation is not an old practice as the major inert ingredients of (WP) formulations are used to be wetting agents, dispersants, and diluents; other inerts may include anti-foaming agents, binders, and disintegration agents (De Schampheleire et al., 2009). Therefore, Syngenta's WP formulation could have benefited in terms of improved DFR% from the inclusion of the non-ionic surfactant in its formulation, equating to the EC formulation's effect. This result also agrees with the findings of many researchers where the EC formulations of the different pesticides provided better retention leaving more residue than the WP-tested formulation (Buzzetti, 2017; Yao et al., 2014). In contrast, the data gathered by the USA ARTF proved the proximity of EC and WP DFR on different leaves (Bruce & Korpalski, 2008).

On the other hand, the reason for the lower DFR % in the case of tomatoes was attributed to the leaf architecture and the WP formulation enhancement. it is hypothesised that tomato leaves, unlike the other leaves, bear trichomes that puncture the spray droplets into smaller droplets. These smaller droplets could reach the epidermis more easily and enhance the absorption before the pesticide is dry (Franceschi & Giaquinta, 1983; Li et al., 2018). In addition, tomato trichomes are known to be hydrophilic and increase the affinity of the pesticide spray for adhesion to the epidermis (Kasiotis et al., 2017). Ultimately, the absorption of the DFZ WP 10% (w/v) formulation increased with the aid of the non-ionic surfactant initially incorporated in it, leaving less DFR on the surface.

Adjuvants play a significant role in droplet size distributions, deposit patterns, foliar residues (both dislodgeable and penetrated), and persistence characteristics of pesticides (A. Sundaram et al., 1985; K. Sundaram & Sundaram, 1987). The adjuvant choice depends on the physicochemical properties of the active substances and the types of formulation (EC, WP, solution, granules, etc.) (Mesnage & Antoniou, 2018). Some of the most common adjuvants are surfactants (Penner, 2000). The primary function of surfactants is to reduce the surface tension within the external surface layers of water. The lower the surface tension in a pesticide solution, the better the pesticide coverage, allowing more pesticides to reach their target, as well as improving droplet spreading on the leaf surface (Hall et al., 1999). Additionally smaller droplets can be achieved during spraying due to reduction of surface tension. Theoretically, this will eventually lead to more absorption and less DFR on the surface. The most common surfactants are non-ionic surfactants such as Tweens. A further commonly-used adjuvant class are as "*plasticisers*" or "*accelerators*", which accelerate the mobility of active substance absorbed into the leaf via the lipophilic pathway (Schreiber, 2005). An example of a plasticising molecule is the organophosphate tris(2-ethylhexyl)phosphate

(short: TEHP) (Arand et al., 2018b). Both DFZ EC 10% and WP 10% formulations were mixed with different adjuvants (i.e., Tween-20 or TEHP) at concentrations of 0.1% (w/v) to investigate the effect of adjuvant addition on the DFR.

The study used the active substance difenoconazole as both an emulsifiable concentrate (EC 10%) and a wettable powder (WP 10%) with and without adjuvants (Tween 20, and TEHP) on French bean leaves. There was no statistical difference in the DFR recovery % between the WP 10% and the WP10% with either mixed adjuvants (i.e., Tween 20% and TEHP) (see Table 5.4 and Figure 5.2, in Chapter 5, Section 5.4). The reason for the DFZ WP 10% showing no significant improvement in the DFR intensity when mixed with either adjuvants tested could be due to the nature of the WP formulations. WP formulations form suspensions after dispersion in water and already contain a non-ionic surfactant as a suspending agent. Therefore, further addition of other adjuvants in the presence of the non-ionic surfactant initially in the WP did not impact the DST of the formulations at the impaction time. This could be due to having the right concentration of the initially incorporated adjuvant in the WP formulation, which enabled the utmost mixing possible between the active substance and the intial surfactants present in the formualtion . From the literature, It is evident that the stronger the bond between the active substance (AS) and the adjuvant in the formulation, the better the adjuvant's biological effect (Faers & Pontzen, 2008). Furthermore, and from the above, the lower miscibility of the AI in the DFZ 10% WP formulation and the expected fast dryness of the AI on the leaf could be the leading cause of the poor functionality of both adjuvants tested (i.e., Tween-20 and TEHP). This non statistical difference in case of WP compared to WP with both adjuvants was also evident by The DST measurements at the impact time for the WP formulations (i.e., WP10%, WP10% +Tween-20 0.1% (w/v) and WP 10% +TEHP 0.1% (w/v)) which was proved to have very close DST, as shown in Table 5.8 in Chapter 5, Section 5.4. This was

attributed to the intial surfactant present already in the formulation and not the added adjuvnats.

On the other hand, the good and homogenous miscibility of the AI in the emulsion formed from the DFZ EC 10% (w/v) was found to enhance the absorption of the DFZ into the French bean leaves when the DFZ EC 10% was mixed with TEHP 0.1% (w/v) but not with WP 10%. From this result, it was evident that the size of suspended particles in (DFZ) could be another factor limiting the functionality of the adjuvants when mixed with the WP formulation. This is because of the smaller the particle size in the formulation, consequently the easier the penetration. If compared with the ECs, WPs dispersing particles are generally larger (De Ruiter et al., 2003; R. Singh & Arora, 2016).

EC formulations showed a slower rate of reaching equilibrium surface tension compared to the DFZ WP formulations. This relatively rapid decrease in the surface tension of the WP formulations indicated better incorporation of the inital adjuvant/surfactants in the WP formulations compared to the EC formulations, which enabled the WP formulation to perform relatively similar in terms of the DFR % recovered despite the better-known characteristics of the emulsion formed from the EC dilution. The presence of an emulsified phase within the spray formulation may have a number of effects, including the retardation of molecular diffusion by a high viscosity emulsion. Additionally, the surfactants must equilibrate between the internal droplet phase, the droplet-continuous phase interface as well as the droplet surface, affecting wetting of the leaf surface. This dynamic behaviour could impact on the rate at which equilibrium surface tension was achieved, and therefore spreading of droplets.

The findings of this research shed light on the importance of the formulation as a factor that may affect DFR. from the results. It was observed that the DFZ WP 10% (w/v)

formulation performed well compared to the DFZ EC 10% (w/v) formulation. That gives an indication that WP formulation was not the worst formulation, unlike previous descriptions in the historic literature. Even though there is a difference between DFZ EC and WP formulations, especially with WP formulations forming suspension on dilution, the innovation in the formulation science has succeeded in formulating DFZ WP formulation with effective adjuvants incorporated that enhanced its retention/absorption to the degree that equated the EC efficacy and beyond. That was also obvious from the DST values recorded at the initial and impaction time of the spray.

Experiments in Chapter 6 were then designed to investigate the leaf texture of different crops (i.e., French bean, tomato, oilseed rape, soya bean and wheat) as a factor that may affect DFR. This research used the newly DFR validated and developed laboratory method, but this was also extended to investigate the same with an application method of DFZ EC 10% resembling the field condition (i.e., track sprayer method). The track sprayer was calibrated to deliver a similar application rate to the one used in the micropipette controlled method. The reason for the latter experiment was to test the applicability of the lab experiment when variabilities similar to the field conditions exist (i.e., the angle of the spray, speed and pressure of the spray jet). The droplets on some leaf surfaces tested (i.e., French bean, tomato and oilseed rape) were imaged by applying a droplet volume (2 µL) associated with a higher diameter than the droplets generated by the micropipette (0.2 µL) in the DFR lab experiment and those generated by the track sprayer jet. The rate of decline of the contact angle during spreading on the leaf demonstrated the speed of droplets spreading over the leaf surfaces and was linked to the DFR recovery % (see Table 6.4 in Chapter 6, Section 6.4.3).

From the results, it was clear that despite the differnt application method and the variabilities in the track sprayer method, tested leaves responded differently in their DFR recovery % with less residue magnitude in the case of the track sprayer compared to the micropipette application method. Nevertheless, the current study's findings confirmed other research results (Bruce & Korpalski, 2008) by demonstrating that different hairy leaves (i.e., French bean, soya bean, tomato) in general retained more DFR than the waxy leaves tested (oilseed rape). Furthermore, the results showed another piece of evidence that hairy leaves acted differently in response to the residue based on the impact of type and density of the trichomes found on their surface. Therefore, grouping different leaves/crops based on their roughness (i.e., hairy or waxy) could be relevant and applicable when tested. However, an accurate classification approach would better involve the coverage degree and types of trichomes in different hairy leaves, as hairy leaves may act differently based on these characteristics (see Tables 6.1, 6.3 and Figure 6.2 in Chapter 6 Section 6.41 and 6.4.2).

7.1   General Conclusion

The work conducted in this thesis demonstrated that the dietary and non-dietary decline residue correlation exists in most cases in the currently approved pesticide-active substances studied. Furthermore, It appeared to have DFR DT$_{50}$ values much lower than the 30-day default currently in use. This finding will help to re-ignite debate in this area, aiming to refine the non-dietary risk assessment of the PPPs.

Also, the new laboratory DFR developed method proved to be precise, accurate and validated to help the regulatory authority, residue scientist and the agrochemical industry to generate more robust DFR data. Due to the scarcity of DFR field data, its seasonal nature, and the high costs associated with the field experiment, this study would allow fast and robust generation of DFR data in the laboratory to help explore

the residue behaviour under different controlled conditions. In addition, this is hoped to identify the correlation between different variables and would finally allow direct extrapolation or the use of extrapolation factors.

The results of this thesis also shed light on the importance of the leaf texture, formulations and formulation additives as factors influencing DFR. In general, the results showed that hairy leaves retain more DFR than waxy leaves. However, further analysis and investigation of significant DFR recovery on different hairy leaf types (i.e. tomato and soya bean) showed that hairy leaves could differ in their DFR based on their trichome density, structure and type. The research also showed that different formulation additives (i.e., adjuvants, solvents) could influence DFR intensity.

7.2   General Reccomendations

The research of this thesis proved the accuracy and suitability of the developed DFR lab method however, It should be noted that some in-field applications and spraying methods could be challenging to correlate with the future generated data from this method. These challenging applications such as those driven from the drone/aerial spray or the ultra-low volume sprays. Nevertheless, studying the factors that may affect DFR using ultra-low volume or aerial spray (i.e., leaves, formulations, etc.) with such a method in the future could help estimate a correlation factor to predict and extrapolate between the in-field generated data if applicable.

Further research is also recommended for testing various leaves with different roughness and wax deposition with various PPPs active substances. This will help generate more data to support the residue pattern proposed in this thesis and its robustness. In return, this would be used by the PPPs regulatory authority and the industry to conclude the extrapolation possibilities between DFR studies. Further

studies could also add evidence that allows for waiving the requirements of conducting a DFR study for each crop/formulation during the registration/approval process easing the process and saving time and resources.

Some other adjuvants and additives, such as those having rain-fastness characteristics, would be very interesting to investigate as their influence on the formulation could alter the DFR behaviour compared to the formulation lacking such ingredients. Moreover, it is noted that some PPPs may have stereoisomers where a significant change could occur in the stereoisomeric composition during and after the application on leaves. EFSA has acknowledged this to add more uncertainty to the DFR fraction, and their behaviour and consequently will add more complexity to the non-dietary risk assessments (Bura et al., 2019). Therefore, further investigation of the factors in conjugations with these PPPs known to have stereoisomers is recommended.

This could include monitoring the decline rate and breakdown of residue on different leaf surfaces under certain environmental conditions and exploring if any of these decline derivatives are stereoselective in changing the stereoisomer configuration ratio and to what degree. This will avoid using conservative uncertainty factors that will cause compounded conservatism in the current non-dietary risk assessment.

Publication list

1- Mohamed H. Badawy, Neil Morgan, Kathleen A. Lewis, Correlation between dietary and dislodgeable foliar (DFR) crop residues decline data; A proposed approach to refine non-dietary risk assessment, Regulatory Toxicology and Pharmacology, Volume 127, 2021, 105049, ISSN 0273-3300, https://doi.org/10.1016/j.yrtph.2021.105049. (https://www.sciencedirect.com/science/article/pii/S0273230021001902)

2- Mohamed H Badawy, Darragh Murnane, Kathleen A Lewis, Neil Morgan, Development of a New Dislodgeable Foliar Residue Analytical Laboratory Method for Pesticides, *Annals of Work Exposures and Health*, 2022;, wxac045, https://doi.org/10.1093/annweh/wxac045

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