Investigating water-soluble polymers as rainfastness adjuvants for agrochemicals

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In dedication to my parents

Thank you for your love and support

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Brett Symonds

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List of publications

1. Symonds, B. L., Thomson, N. R., Lindsay, C. I. & Khutoryanskiy, V. V. Rainfastness of Poly(vinyl alcohol) Deposits on Vicia faba Leaf Surfaces: From Lab-scale Washing to Simulated Rain. *ACS Appl. Mater. Interfaces* **8,** 14220–14230 (2016).

2. Symonds, B. L., Thomson, N. R., Lindsay, C. I. & Khutoryanskiy, V. V. Chitosan as a rainfastness adjuvant for agrochemicals. *RSC Adv.*, 2016, **6**, 102206-102213 (2016).

List of oral presentations

1. “Polymers as retention and rainfastness aids for agrochemicals,” 11th International Saint-Petersburg Young Scientists Conference, November, 2015, St. Petersburg, Russia.
2. “Quantifying the rainfastness of fluorescently labelled poly(vinyl alcohol) deposits on Vicia faba leaf surfaces via fluorescent microscopy,” 15th European Student Colloids Conference, June, 2015, Kraków, Poland.
3. “Relationships between physical properties of poly(vinyl alcohol) and its rainfastness,” – UK and Ireland Controlled Release Society, Cardiff, UK.

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Abbreviations

2,4-D	Dichlorophenoyxyacetic acid
5DTAF	5-(4,6-Dichlorotriazinyl)aminofluorescein
6AF	6-Aminofluorescein
AP	Amidated pectin
AZ	Azoxystrobin
CMC	Carboxymethyl cellulose
CS	Chitosan
DA	Degree of acetylation
DDT	Dichlorodiphenyltrichloroethane
DoH	Degree of hydrolysis
DSC	Dynamic scanning calorimetry
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
FITC	Fluorescein isothiocyanate
GPC	Gel permeation chromatography
GS	Growth stage
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LP	Leaf position
MP	Methylated pectin
MS	Mass spectrometry
NAA	1-Naphthylacetic acid
NMR	Nuclear magnetic resonance
NOAA	2-naphthoxyacetic acid
NTAA	2-naphthylthioacetic acid
PDI	Polydispersity index

PEO	Poly(ethylene oxide)
PPO	Poly(propylene oxide)
PVA	Poly(vinyl alcohol-*co*-vinyl acetate)
PVP	Poly(vinyl pyrrolidone)
SD	Swelling degree
SEM	Scanning electron microscopy
Sulfo-NHS	Hydroxysulfosuccinimide sodium salt
T_g	Glass transition temperature
T_m	Melting temperature
UPLC	Ulta high pressure chromatography
VMD	Volume mean diameter
WAXS	Wide angle X-ray scattering
XRD	X-ray diffraction

Abstract

Rainfastness is the ability of agrochemical deposits to resist wash-off by rain and other related environmental phenomena. This work reports studies of the rainfastness of selected water-soluble polymers, including poly(vinyl alcohol) (PVA) and chitosan, on *Vicia faba* leaf surfaces. This was achieved using a novel method involving fluorescent microscopy combined with image analysis. The retention of polymer deposits was analysed via lab-scale washing to simulate rain. PVA over a threshold molecular weight and chitosan were shown to be excellent rainfastness aids. The washing method was 'scaled up' with the use of a raintower and it was shown that the lab-scale washing method was representative of low-to-medium intensity rain (10 mm/h).

Physical characterisation indicated that rainfastness correlated with polymer film dissolution, swelling and crystalline properties. It was established that the rainfastness of PVA scaled with increasing molecular weight and crystallinity. Chitosan proved the most effective of the polymers analysed and even samples of moderate molecular weight were able to resist the highest intensity simulated rain. Those polymers which exhibited rainfastness were only soluble in water with a stimulus, such as heating for PVA or decreased pH for chitosan.

The microscopy analysis was expanded to assess the rainfastening effect of these polymers on a model agrochemical. This was achieved by following the retention of azoxystrobin – a fluorescently active fungicide. Those polymers which showed retention alone also improved the retention of azoxystrobin. A 'spot and wash' method using mass spectrometry to quantify rainfastness performed alongside fluorescent microscopy analysis further validated the findings.

Chapter 1 Introduction

The world faces the crucial challenge of securing a sustainable food supply for a growing population. The Food and Agricultural Organization of the United Nations expects that global agricultural production will have to increase by 60 percent from 2005-2007 levels in order to feed an estimated 9 billion people in 2050.[1] Additionally, the reduction in arable land per capita means that the production increase must be made by increasing efficiency of current farming methods.[2] Pesticides are used to improve farming yields and adjuvants are formulated with pesticides to improve their efficacy. Water-soluble polymers often offer multifaceted benefits when incorporated into agrochemical formulations.[3] Typical uses include thickeners, stabilisers and droplet retention aids.[4] Many are non-hazardous and biodegradable.[5] Their use as rainfastness adjuvants offers the route to safer and more efficient agrochemical formulations but there is a lack of literature as to why they are rainfast.

Rainfastness is the ability of agrochemical deposits to resist wash off by rain and other related environmental phenomena.[6] Rainfast agrochemical deposits are less likely to be washed into soil or water sources by rain and are less likely to harm farmers who come into physical contact with residues while working in the field.[7] The quantification of rainfastness is of great interest to the field of agrochemical formulation development. Also important is to understand why some water soluble polymers can be used as rainfastness aids. Understanding these factors enables the design of improved agrochemical formulations to meet the aforementioned demands facing the agricultural industry.

The aim of this work was to develop novel methods for the assessment of rainfastness. Further aims were to use these methods to determine the rainfastness of selected water-soluble polymers and to characterise these polymers. This characterisation was undertaken to understand why these polymers were rainfast and which properties govern rainfastness of water-soluble polymers.

Chapter 2 is a review of the literature concerning topics such as the use of pesticides, agrochemical formulation and state of the art – especially regarding the use of polymers in agrochemical delivery. In chapter 3 the characterisation and fluorescent labelling of the polymers is described in detail. Chapter 4 is about the development of novel fluorescent microscopy methods used to study rainfastness of the polymers characterised in chapter 3. The physical properties of poly(vinyl alcohol-*co*-vinyl acetate) (PVA) and chitosan are related to their rainfastness. In addition, the suitability of these methods for measuring rainfastness is evaluated. In chapter 5, the fluorescent microscopy methods were adapted to determine if PVA and chitosan were able to rainfasten a model agrochemical compound. An additional quantitative mass spectrometry method to determine retention of these agrochemical formulations was used to verify the microscopy results. Chapter 6 summarises the work of this thesis and proposes avenues to continue the work in the future.

1.1 References

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Chapter 2 Literature review

2.1 Background on agrochemicals and plant protection

Agrochemicals are utilized to improve crop production and yield – they are pesticides, fertilizers, growth agents and adjuvants.[1]–[6] These treatments may take the form of seed or soil treatments or sprayed mixtures and are almost always a formulation of more than one component.[7], [8] Such formulations can be subject to losses from a number of sources no matter the method of application, and are prepared and applied so as to reduce these losses as much as possible, as well as with safety in mind.[5], [6], [9] There are few 'one size fits all' solutions for the many challenges of crop protection. Therefore, considerable effort is expended in research and development around the world in order to design and market improved formulations and crop protection technology.

While there are alternative methods towards the goals of achieving food security – such as via genetically modified plants and novel agro-ecological management – this work focuses on agrochemical formulations which are still the most important method for improving farming efficiency. This review will examine the background to agrochemical delivery and summarise the state of the art, with a particular focus on polymeric systems.

2.1.1 History of pesticide use

A brief history of pesticide use is discussed in order to establish the background to the field of agrochemical formulation. Pesticide use is almost as old as the domestication of crops – evidence suggests sulphur based pesticides were in use 4,500 years ago.[10] Ancient populations used basic pesticides derived from plants, animals or minerals.[11] These include mercury and arsenic compounds, tar, salt,

ash, various smokes as fumigants, copper sulphate and lime.[12] With the industrial revolution in Europe and the emergence of a chemical industry in the 18th century, by-products of industrial processes such as coal gas production could be employed as pesticides. These organic compounds included phenols and petroleum oils as insecticides and fungicides while inorganics such as ammonium sulphate were used as herbicides.[13] The drawbacks to these products were their phytotoxicity and lack of selectivity.

The modern era for pesticides began after World War Two. Particularly important was dichlorodiphenyltrichloroethane (DDT) – its insecticidal properties were discovered in 1939.[14] DDT was used to control insects such as lice and mosquitoes. It helped prevent diseases like malaria and typhus and won Paul Hermann Mueller the Nobel Prize in Medicine in 1948. Initially one of the perceived benefits of DDT was that it had low toxicity, especially compared with pesticides in use during the 20s and 30s such as arsenic compounds. However, by the 50s and 60s evidence was mounting that DDT had a negative impact on wildlife and could be toxic toward humans. It was banned by most developed countries in the 70s.[14]

Particularly important herbicides developed in the 20th century were 2,4-dichlorophenoyxyacetic acid (2,4-D) in the 40s and (N-(phosphonomethyl)glycine) (Glyphosate) in the 70s.[15] Unlike DDT, 2,4-D was specifically developed as an herbicide and is still widely used to this day. It is a synthetic plant hormone that causes uncontrollable growth in the selected plants which eventually leads to death.[16] Glyphosate is a simple natural amino acid analogue with herbicidal properties. It affects an enzyme responsible for production of aromatic amino acids in growing plants. Therefore, it has no use as

a pre-growth herbicide and has no effect on animals which gain these amino acids through their diet.[17] It has been described as an ideal pesticide due to its low toxicity and broad spectrum of use and it the most widely used pesticide in the world.[17]

In modern times much research has been dedicated to improving safety of pesticides.[18] Instead of a pesticide with a broad spectrum of effect, numerous specialised but safe compounds have been developed. Table 2.1 illustrates this trend in safety with common herbicides from the 20th century provided with respective concentrations of median oral lethal dose. This development has presented new challenges such as pest resistance towards these compounds. It is now common to explore natural sources for new pesticides. For example, the strobulin fungicides was discovered by investigating a species of mushroom that secreted compounds to kill competitive fungi.[19]

Table 2. 1 Herbicides, the decade they were developed and their oral median lethal dose.

Herbicide	Oral LD50 (mg AI/kg)
Arsenic acid (1900-1920)	48-100
2,4-D (amine) (1940)	1500
Altrazine (1950)	1600
Glyophosphate (Roundup) (1960)	>5000
Fenoxaprop-ethyl (Excel) (1970)	2565
Imazethepyr (Pursuit) (1980)	>5000
Nicosulfuron (Accent) (1990)	>5000

2.1.2 Commonly used pesticides

Pesticide is a term for any compound that is designed to destroy a pest organism – be it plant, fungal, insect or mammal. Industrially important are herbicides, insecticides and fungicides. According to the US Environmental Protection Agency these three pesticide types respectively made up 40%, 29% and 22% of

worldwide pesticide sales worth $40 billion in 2007.[20] Fumigants are another type of pesticide that see widespread use, but are worth little of market sales. Herbicides are by far the most used pesticide and a list of the most common pesticides and their types is provided in Table 2.2.

Table 2.2 The 14 most used pesticides in the US in 2007 along with the type and amount used in kilograms. Adapted from US Environmental Protection Agency data.[20]

Active ingredient	Type	Amount used (millions kg)
Glyphosate	Herbicide	81-84
Atrazine	Herbicide	33-35
Metam Sodium	Fumigant	22-25
Metolachlor-S	Herbicide	13-16
Acetochlor	Herbicide	13-15
Dichloropropene	Fumigant	12-14
2,4-D	Herbicide	11-13
Methyl Bromine	Fumigant	5-6
Chloropicrin	Fumigant	4-5
Pendimenthalin	Herbicide	3-4
Ethephon	Plant growth regulator	3-4
Chlorothalonil	Fungicide	3-4
Metam Potassium	Fumigant	3-4
Chlorpyrifos	Insecticide	3-4

Pesticides can be further classified by their mode of action – such as systemic or contact. Systemic pesticides have an effect by being taken into the plant, via the roots or through the plant cuticle for example, and the active ingredient is translocated throughout the plant for an effect. These may be herbicides to kill a weed or insecticide to kill pests that eat the plant. Contact pesticides work by contact – they have an effect when an insect or fungal spore touches a treated leaf. Herbicides can broadly be defined as post- or pre-emergent. Pre-emergent

herbicides prevent the weed shoots from growing from soil while post-emergent herbicides are applied to kill already growing weed plants.[21]

Agrochemicals are not always applied directly to plants. They may be added to soil to destroy pests or encourage growth. Seed treatments are a common form of agrochemical application. Seeds may be coated in a formulation that contains fertilisers and growth promotors. Alternatively, they can be coated in a systemic pesticide which protects the plant during its initial growth stages. Much less pesticide is needed to treat a seed than would later be needed to treat a growing plant.[22] In this way pesticide use can be reduced, and unwanted environmental contamination can be prevented.

The agricultural industry is facing several challenges. As already discussed, the world must secure its growing population a sustainable food supply using ever decreasing resources. The public is also concerned about the safety of agrochemical use and their impact on the general environment.[23] It is important for the industry to prove that its methods are safe for workers, consumers and the environment. While research and development is expensive, new technology can give an advantage to those that developed it. Development of formulations with improved efficacy and safety is increasingly a focus for the agrichemical industry.[24]

2.2.4 The leaf surface

The leaf surface is the target site for application of many agrochemical formulations. As part of the background to agrochemical delivery it is important to understand the surface characteristics of leaves. The exterior of the leaf is known as the cuticle and is somewhat analogous to skin. The cuticle (Figure

2.1A) covers all aerial plant organs without periderm; it is a permeability barrier preventing water and other vital molecules escaping the plant. Other benefits of the leaf cuticle are protection of the DNA from ultra-violet radiation, prevention of mechanical damage and as a microenvironment for organisms.[25] The cuticle is a composite material; a polymer framework known as cutin but sometimes made of cutan or lignin or a combination. Waxes on the surface of the cuticle are termed epicuticular and those set in the polymer framework are termed intracuticular. Further toward the epidermis wall is the layer consisting of polysaccharides such as cellulose and hemicellulose (Figure 2.1B).[26], [27]

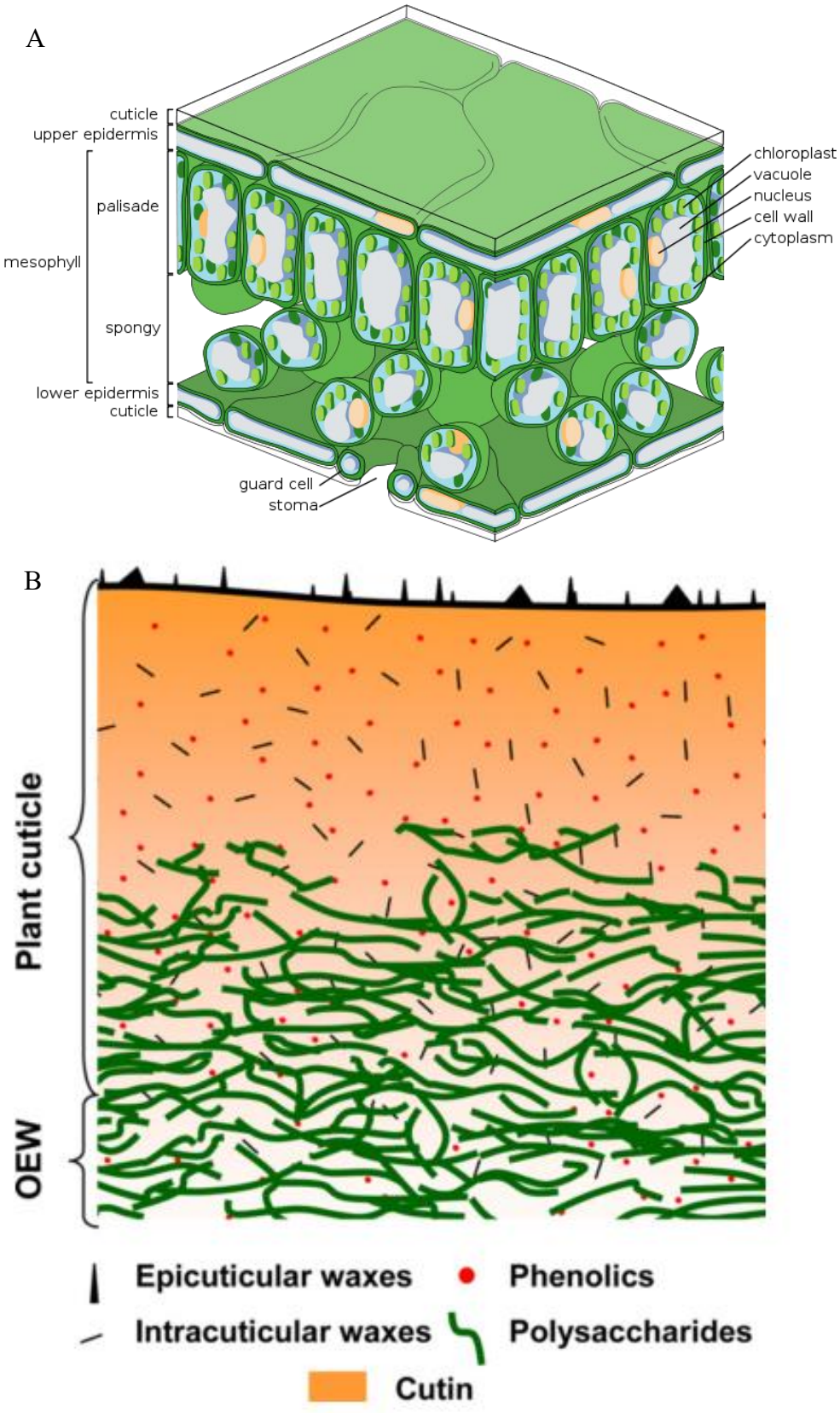

Figure 2.1 Generic cross-section illustrations of the entire leaf (above) and the leaf cuticle (below). Images taken by Wheeler and Dominguez *et al* respectively.[25], [28]

Leaves can exhibit hydrophilic or hydrophobic surfaces.[29] It has been established that the hydrophobic properties of leaf surfaces are a combination of micro- and nanoscopic architecture along with the chemistry of epicuticular waxes.[30] Epicuticular wax on the cuticle surface can form into tubules, platelets, films, rodlets and more complex hybrid structures. The cuticle surface on which the wax resides also has a variety of structures which affect surface properties. It is a combination of hydrophobic wax and the irregular surface structures that prevent water from wetting the most hydrophobic plant surfaces, such as the lotus leaf. Leaves which are hydrophilic tend to have smoother surfaces with less wax. The complicated architecture and wax structures are visible using scanning electron microscopy (Figure 2.2). Barthlott *et al* extensively reviewed the structures and chemistry of waxes for thousands of plant species.[31] They determined that epicuticular waxes comprise long-chain (C12-C60) aliphatic esters, alcohols, fatty acids and aldehydes. Less common but still dominant in wax composition were ketones, beta-ketones, secondary alcohols and cyclic compounds such as triterpenoids. Crystalloids of these varying components are responsible for the micro-morphology of plant waxes.

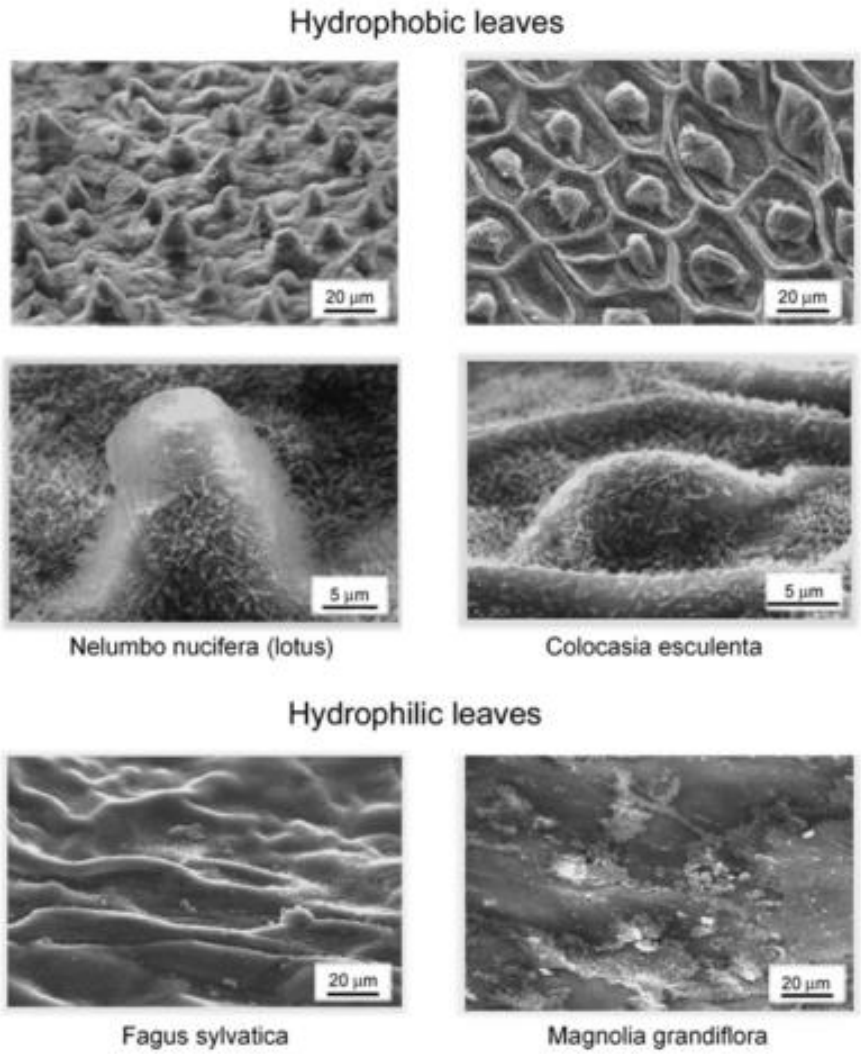

Figure 2.2 Scanning electron micrographs illustrating the differences between the hydrophobic and hydrophilic leaves. Image taken from Bhushan *et al*.[29]

Leaf wax composition was shown to change with age.[32] Wax from wheat leaves 24 days after germination contained 11% esters and 49% alcohols while wax from 100 days after germination contained 39% esters and 4% alcohols. It is likely that these changes are responsible for the varying conditions of leaf surfaces. Leaf hydrophobicity was shown to vary with age and the position on the leaf that was measured.[33] This is important information to take into account while evaluating results from studies that used real leaf surfaces. The cuticular pattern (or

microscopic structure) also contributes towards the hydrophobic nature of the leaf surface. This meta-structure can be a result of the cell wall shape, which lies beneath the cuticle, or of the carbohydrate polymer matrix which makes up the inner cuticle. Both result in epicuticular folding which gives the leaf a distinctive shape.[34]

2.2 Agrochemical formulation and delivery

2.2.1 Agrochemical delivery process

The basic agrochemical delivery process is visualised in Figure 2.3. There is potential at each step for losses to occur. These losses may begin during the initial spraying process – if weather conditions are not ideal then spray drift can occur.[4] This spray drift results in a waste of formulation and unnecessary pollution of the surrounding environment, not to mention the detrimental effects of unprotected crops.[5] Even when sprayed droplets hit their intended target they may have poor retention on plant surfaces. This is related to the physicochemical properties of the spray droplet during its formation from the spray nozzle and its initial impact with plant surfaces.[35]–[38] Once the droplet has retained, further losses can occur due to poor retention of this droplet or its dry deposit. This is often caused by microbial, photolytic or hydrolytic degradation or removal by adverse environmental conditions such as rain or even strong agitation such as by wind.[2], [4], [39] Considering the multitude of factors above, agrochemical formulations are subject to much research and development aimed at reducing and overcoming these losses.[40]–[53]

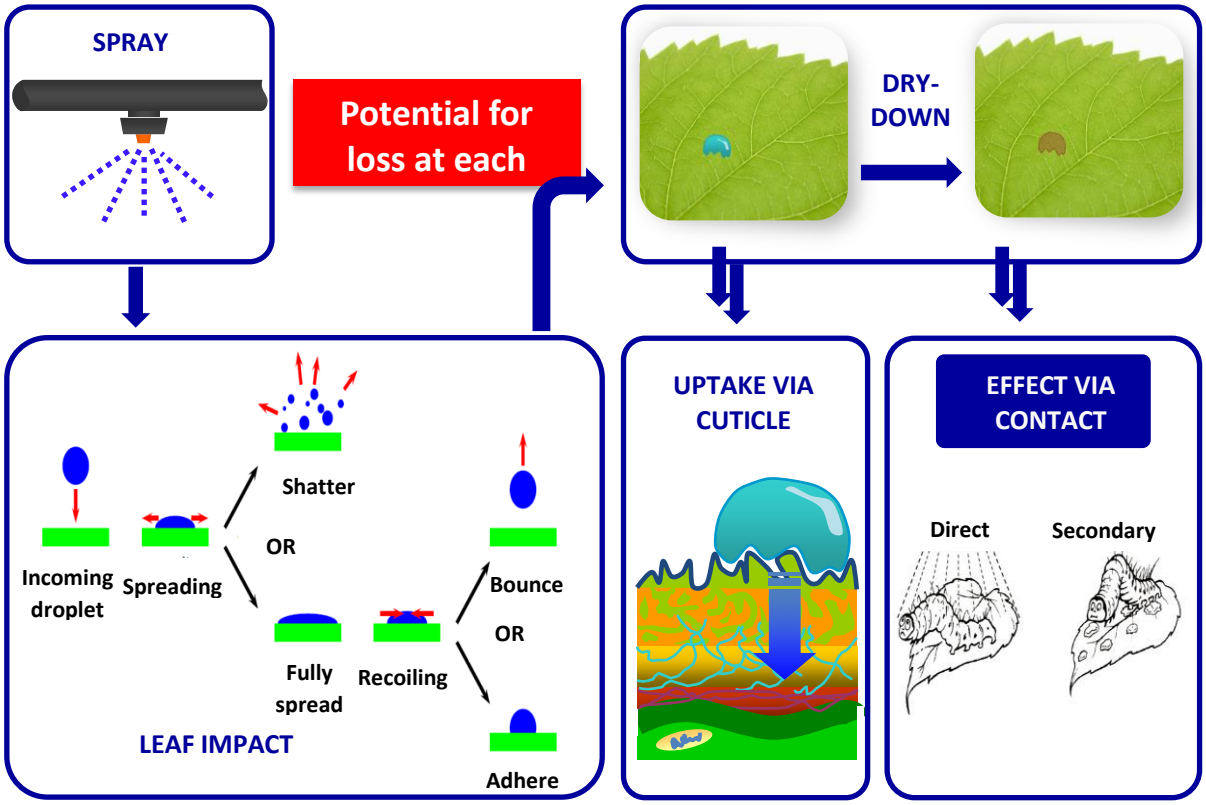

Figure 2.3 The basic agrochemical delivery process for a liquid formulation which highlights the potential losses of spray leaf impact as well as routes for an active ingredient to have efficacy.

Pesticides are therefore formulated with a number of adjuvants to overcome these losses. Adjuvants may take the form of spray modifiers, spreaders, UV protectants, retention aids, stabilisers, penetration enhancers and antifreeze. Adjuvants are most often oils, surfactants, polymers, solvents and emulsifiers.[5] Another aspect to the formulation of pesticides with adjuvants is the renewal of patents. As older patents expire and government regulations become stricter regarding new pesticides, patents are more commonly being filed for new formulations of existing products. Some formulation techniques and the adjuvants used are discussed here.

Controlling pH of a formulation can change its effects. For example, 2,4-D, when formulated at low pH takes its neutral form and is more lipophilic. At high pH it is ionised and exhibits better water solubility. The more lipophilic form is ideal for incorporating into a micellar formulation.[16] Spray drift and difficulties depositing droplets onto plant surfaces are another interesting aspect of adjuvant technology. Both spray drift and deposition are a result of droplet size, viscosity and surface tension of the sprayed solution. While fine droplets are more susceptible to spray drift, larger droplets are more difficult to retain on leaf surfaces after they impact due to their higher kinetic energy.[54] The solution is usually to increase droplet sizes while altering the dynamic surface tension of the solution with surfactants to aid the deposition of the droplets. Run-off can be another cause for concern – even if droplets initially adhere to a surface. If the droplets spread too well, they may run-off of the surface completely.[2] Water-insoluble pesticides can be formulated as emulsions. A variety of adjuvants are added to both the oil and water phases to stabilise the emulsion and to further enhance the formulation properties. Encapsulation offers sophisticated controlled release of pesticides. Microcapsules can be achieved by polymerisation of emulsions to form shells around pesticide containing oils.[55] Although encapsulation techniques can improve the stability and reduce phytotoxicity of pesticides they are very expensive and not realistically suitable for widespread use.

2.2.2 Rainfastness

Rainfastness is the ability of an agrochemical deposit to resist wash-off by rain and other environmental factors. The topic of rainfastness appears less frequently in the literature than other topics regarding adjuvancy of agrochemicals.

Consequences of poor rainfastness are the unprotected crops but also the unwanted pollution of soil and water sources with agrochemicals. Aquatic ecosystems are at particular risk from such eventualities.[56] Figure 2.4 illustrates the pathways for the environmental contamination by pesticides – most of which are a result of lack of formulation efficacy.

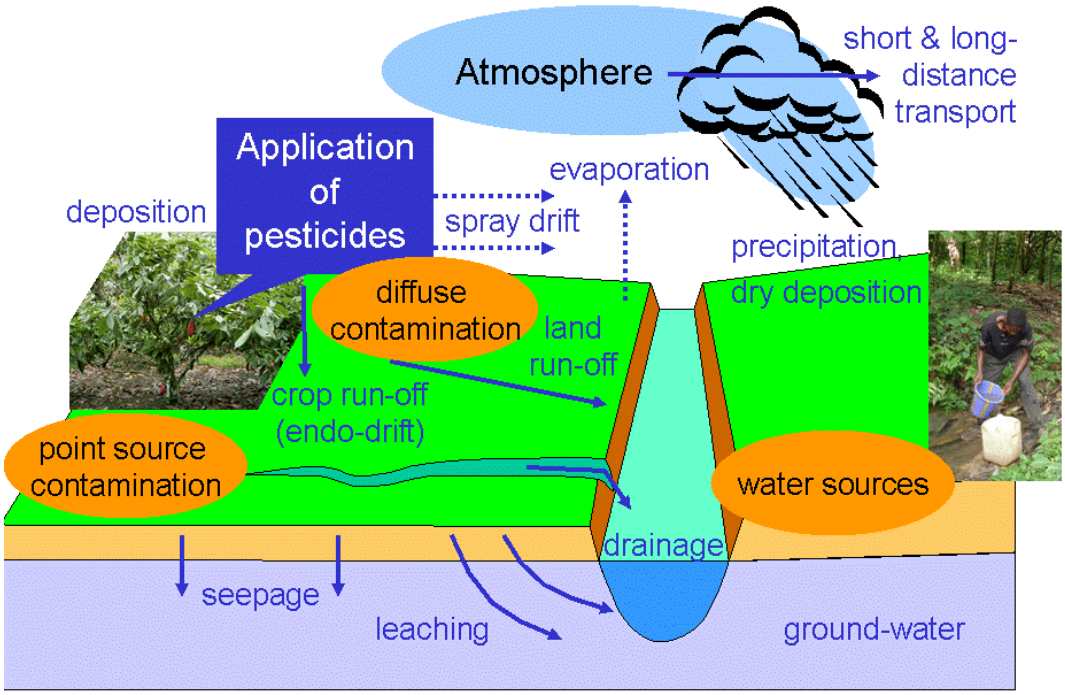

Figure 2.4 Pesticide pathways into soil and water sources. Image by Bateman.[57]

Pesticide formulations often come with rainfastness advice for the user. This might advise the operator to not apply the formulation directly before or after rain and the required rain-free period may be included in this advice. Researchers have investigated the rain-free period required for some herbicides to remain effective.[58] Rainfastness studies like this usually examine the effectiveness of treatments by observing the damage done to treated weeds that were exposed to rain. These studies can cost a considerable amount of time and resources as many

plants have to be used to test various rain conditions. The effectiveness of rainfastness adjuvants can be measured in the same way. The effectiveness after rain of glyphosate to control a weed species was shown to be improved with the use of a certain organosilicone adjuvant.[59] It was hypothesised that these silicone adjuvants were only effective rainfast aids for herbicides. This hypothesis suggested that the organosilicones were reducing interfacial tension and enabling droplets to spread and infiltrate leaf stomata.[60] Thus rainfastness is achieved by the rapid absorption of pesticide into the leaf. Specificity in efficacy towards weed species was also observed.[59] Later, non-spreading silicone adjuvants were shown to improve rainfastness of herbicides but the mechanism is unclear.[61]

An absorption study using a radiolabelled adjuvant was used to determine its retention.[60] Retention tests by capturing the wash-off eluent by using simulated rain is a commonly reported method for measuring rainfastness of adjuvants. The eluent is usually examined using the appropriate form of analysis (e.g. gas chromatography or spectrophotometry) to quantify the remaining agrochemical.[62], [63]

A patent claims modified copolymers of poly(ethyloxide) and poly(propyleneoxide) to be rainfast but does not describe the mode of action.[64] Ultimately there seems to be a lack of fundamental studies of the deposits and adjuvants themselves. Some common commercially available adjuvants include ‘Bond’, ‘Newman Cropspray 11-E’ (both de Sangosse) and ‘Nu Film’ (Miller). They are marketed as multi-functional adjuvants that can increase wetting, sticking, deposition and retention as well as rainfastness. The chemistry of Nu Film is based on polymeric terpenes, which cross-link and form films after application.[65], [66] Bond is comprised of ‘45% styrene-butadiene copolymer’

and ‘15% alcohol alkoxylate’ as surfactant. It is likely that the copolymer is stabilised in water by the surfactant and as it dries it is able to form a water-resistant film to provide rainfastness. In summary, most rainfastening adjuvants can be simplified as to having one of two modes of action: They either wet and spread the leaf so as to rapidly improve uptake and absorption of active ingredients so that they are inside leaves before a rain event or they form a hard to remove water resistant deposit.

2.2.3 Agrochemical delivery – current state of the art

This section has so far highlighted the importance of agrochemical formulation to increase efficacy of pesticides. In particular, discussion has focused on rainfastness, as it is the topic of this thesis. Further analysis of available formulation types and the techniques used to deliver agrochemicals is presented in this section. Knowles notes the relatively recent occurrence of multiple new formulation types based around improvement of efficacy and safety.[24] He summarised the main types of formulation currently in use (Table 2.3).

Table 2.3 Some commonly used formulation types and their international codes. Table adpated from Knowles.[24]

Formulation	International Code
Granules	GR
Solution concentrates	SL
Emulsifiable concentrates	EC
Dispersible concentrates	DC
Wettable powders	WP
Suspension concentrates	SC
Oil-based suspension concentrates	OD
O/W emulsions	EW
Suspoemulsions	SE
Microemulsions	ME
Water dispersible granules	WG
Microcapsules	CS
Seed treatments	DS, WS, LS, FS

Solid granules (GR) are applied to soil and are used to apply pre-emergent herbicides and to kill soil based pests. The granules are typically composed of a highly absorptive material so to be loaded with an oil based pesticide. Suitable materials include silica, attapulgite, montmorillonite, kaolin or recycled materials such as corn cob grits and walnut shells.[24] Wettable powders (WP) are solid pesticide processed into smaller particles (5-40 μm). Inert filler is required to prevent powder from fusing during processing/storage and dry surfactants and dispersants are added to improve their mixing with water. These inerts may be the same materials as used for granules. Water-dispersible granules (WG) offer a safer alternative to wettable powers. Powders are sometimes harmful to the user as they are easily atomised and pose a fire risk. Dispersible granules offer convenience and safety for the user, as well as reduced packaging. Granules must

disperse quickly and completely in water – so are limited to water-soluble active ingredients. Granulation can be an expensive process but the benefits to the user are designed to outweigh their extra expense.

Solution concentrates (SL) may be the simplest liquid based agrochemical formulation. Active ingredients are dissolved in water, additional adjuvants are required and include wetting agents and antifreeze. The technique is limited by the water solubility of the active ingredients. Emulsion concentrates (EC) are the solution to formulation of those active ingredients which have poor water solubility. They represent the largest volume of pesticide formulations used worldwide.[24] Oily and waxy active ingredients are dissolved in non-polar hydrocarbon solvents such as kerosene or, historically, xylene. Efforts are being made to reduce the amount of volatile organic solvents so these types of formulations may gradually become less prevalent.[67] Surfactants are carefully chosen to stabilise the emulsion with water so that they are thermodynamically stable. Surfactant chemistry is varied – ranging from non-ionic and polymeric to ionic surfactants with relatively small molecule weights. Formulations are often formulated with a 'balanced pair' of surfactants to ensure emulsion stability under a range of conditions – one part of the pair being a non-ionic surfactant and the other being an anionic surfactant. It is important to select a surfactant with the optimum hydrophile-lyophile balance (HLB) for the desired formulation where more hydrophilic surfactants have a higher HLB.[24]

Suspension concentrates (SC) are another conventional formulation – they are used to formulate solid pesticides. Solid particles are suspended in a solvent (usually water). The pesticides are milled into small particles of 1-10 μm to be dispersed into water using surfactants. The particles are prevented from

aggregation by surfactants that strongly adsorb onto the particle surface. These types of formulation also require anti-settling agents to prevent the sedimentation of the particles during storage. Water-soluble polymers are used to adjust the rheological properties of the solution to further enhance the stability of formulations.[67]

Recently there has been a push to improve the safety of agrochemical formulations and efforts are being made to eliminate the use of volatile solvents and dusty powders and to reduce pesticide dosage. The trends and incentives are to reduce hazards to the operator and to improve biological efficacy. There are several types of emulsion that do not use organic solvents, including oil-in-water emulsions (O/W), microemulsions (ME), suspoemulsions (SE) and multiple emulsions (O/W/O or W/O/W). These emulsions offer slight differences over concentrated emulsions and each other. They typically contain much less active ingredient than concentrated emulsions. Microemulsions are emulsions of very small droplet sizes, typically 50 nm, and require more surfactant than typical emulsions. It has been suggested they offer improved biological activity due to the well dispersed active ingredients and high surfactant content.[68] Multiple emulsions offer sophisticated formulation of active ingredients. It has been suggested that these formulations are less orally toxic as the active ingredient is restricted to an internalised droplet phase.[24] Suspoemulsions are a combination of suspension concentrates and oil-in-water emulsions. They are convenient to combine multiple pesticides in one formulation and enable the operator to reduce the amount of spraying required. An illustrative summary shows the variety of formulations available on the market and their intended usage (Figure 2.5).

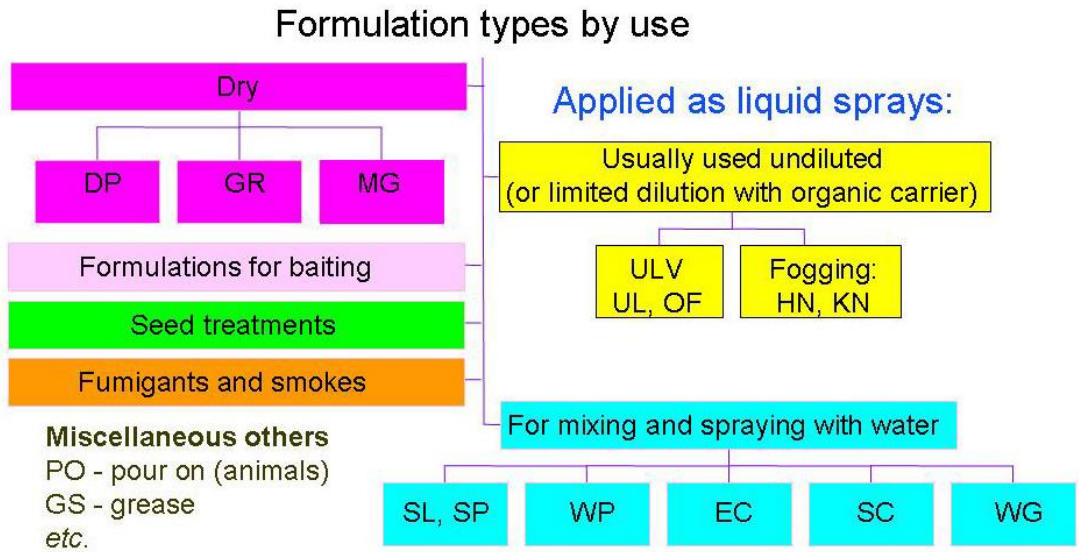

Figure 2.5 A list of used pesticides formulations illustrating the 'universe' of pesticide formulations and their intended form of use. [69]

2.3 Uses of polymers in agrochemical formulation

Polymers can provide multifaceted benefits when included in agrochemical formulation. Many uses of polymers in agrochemical formulation and delivery have been highlighted in the review so far. This section aims to broadly cover all major uses of polymers in the agrochemical delivery and formulation processes. Much of the published literature in this area takes the form of patents, due to the commercial applications.[70]–[76]

2.3.1 Organosilicones and other polymer surfactants

Also termed silicone-polyether block copolymers or organosilicone polyethoxylates, these adjuvants are notable for having 'spectacular wetting and spreading' properties.[77] A typical branched structure is provided (Figure 2.6) and due to the different copolymer variations a number of patented inventions exist.

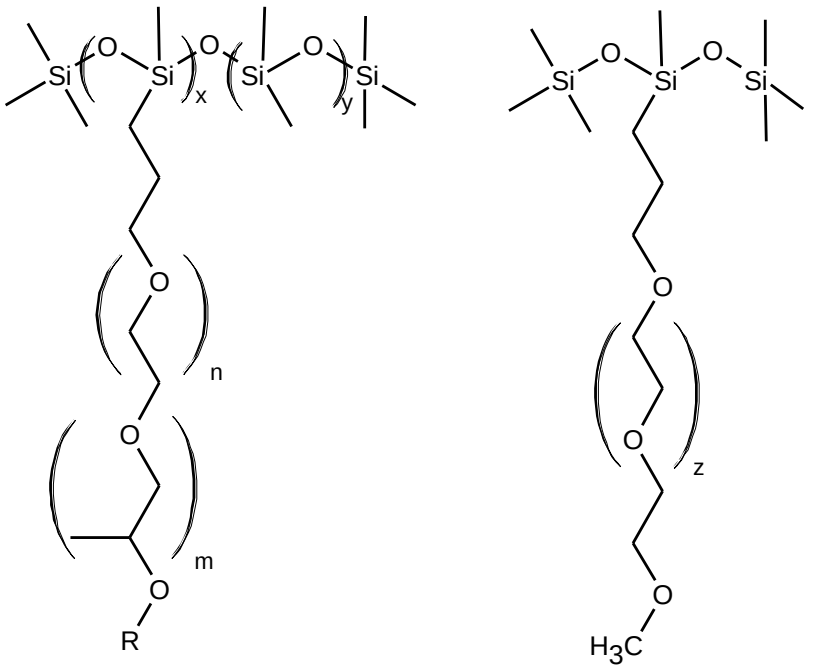

Figure 2.6 A generic structure for a branched siloxane copolymer where the properties depend on the composition of x, y, n and m, and a common trisiloxane, Silwet L 77, where z = 8.

By reducing surface tension of liquid formulations, these 'super-spreaders' are able to wet and spread extremely well over a leaf surface – herbicide activity was shown to be proportional to surface tension of the applied treatment.[77] Numerous commercial formulants are available under the trademark Silwet (Momentive) and the molecular weight of these products ranges from 600 – 29,000 daltons – although not all are suitable for agrochemical applications. Other brands of organosilicone polymers include Matrixx (Coastal), Herbex and Break-Thru (both Goldschmidt). Silwet L-77, a very commonly used trisiloxane are small molecules rather than polymers. Silwet DRS-60 is marketed as a spray drift-retardant while the Silwet L-27XX range is recommended for use when preventing foam is important.

This class of adjuvant offers a number of benefits when used as an agrochemical as they have the potential to improve the performance of a formulation in a

multifaceted way. Typically, the siloxane adjuvants are characterised by stomatal infiltration and poor hydrolytic stability.[77] However, the infiltration of stomata appears to be limited to the non-polymeric trisiloxanes only. As rate of evaporation of droplets is dependent on interfacial area, treatments formulated with these surfactants dry down to deposits quickly.

The hydrophobic silicone backbone is due to the presence of methyl groups. The flexibility of the silicone allows the methyl groups to interact at interfaces. The hydrophilicity of this surfactant comes from the polyethylene oxide (PEO) and polypropylene oxide (PPO) moieties either grafted or as part of a linear continuation of the silicone backbone. The extent of hydrophilicity can be further tuned by adjusting the ratio of the more hydrophilic PEO to less hydrophilic PPO. Most of the polymeric siloxanes do not spread on the leaf surface as well as Silwet L77 and other small trisiloxanes – despite having similar surface tension properties. The difference is thought to be due to the very small hydrophobe of Silwet L77 which is better able to adsorb to the advancing edge of a spreading solution – also called molecular ‘zippering’.[77]

The ability of these adjuvants to spread on the surface of leaves has led to their use with systemic adjuvants. Formulations with herbicides, growth regulators and foliar nutrients benefit from increased spreading on the leaf which allows for more of the active ingredient to penetrate into the leaf. One study showed that glyophosphate reached a maximum absorption into redroot pigweed leaves after just 1 hour when formulated with organosilicone adjuvants, compared to a maximum absorption at 24 hours for conventional adjuvants.[78] In this way, rainfastness is achieved via the very quick penetration of the active ingredient into the leaf.

Some drawbacks exist to this class of surfactant – excessive foaming is detrimental to the tank mixing and spray application process. Additionally, poor hydrolytic stability and cleavage of Si-O bonds at low pH mean that the formulation has limited utility outside of the pH range 6-8. When used with certain high flow; low-pressure spray nozzles the volume mean diameter (VMD) of droplets was reduced significantly. Lower VMD can result in losses via spray drift. Finally, the concentration and volume of surfactant application must be carefully managed to avoid causing run-off. By spraying a treatment of too high volume and concentration, spray droplets can coalesce and result in losses via run-off.[78] It is of benefit to maintain low surfactant concentration and overall formulation volume.

Recently there have been concerns about the safety and environmental impact of the organosilicone adjuvants. Concerns include learning impairment of honey bees after ingesting organosilicone adjuvants.[79] In a 2016 review, this toxicity to bees was reiterated and further concerns were raised about the lack of regulation regarding spray tank adjuvants, principally organosilicone adjuvants.[80] The review also suggests that pesticides thought to be non-toxic to humans may synergize with certain organosilicone adjuvants to become harmful.

In addition to the organosiloxanes, adjuvants with similar chemistries include alkylamine polyethoxylates and nonylphenol polyethoxylates. All share the polyethoxylate hydrophile, whereas the hydrophobe varies (Figure 2.7). Similar challenges and benefits exist regarding their use and safety.[81]

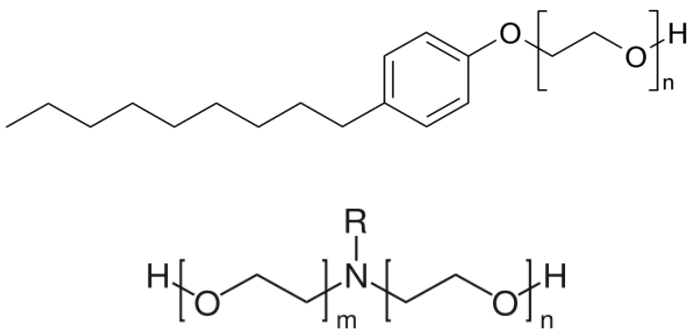

Figure 2.7 Structure for generic alkylphenol and alkylamine polyethoxylates.

2.3.2 Polymers in dispersions

Polymers for use in dispersions are an important adjuvant class in the agrochemical sector.[67] They are used in concentrated emulsions and suspoemulsions in tandem with surfactants such as those described in the previous section. A concentrated emulsion formulation would principally be made up of water and oil (either pesticide oil or an oil containing dissolved active ingredient) – with a further 10% being a polymer and surfactant to stabilise the emulsion of nano/micro-droplets. Alternatively, the oil could be absorbed by a hydrophobic polymer to form a latex dispersion to be stabilised by surfactants and polymers.[68] Solid particles can be stabilised by polymers as well (Figure 2.8).

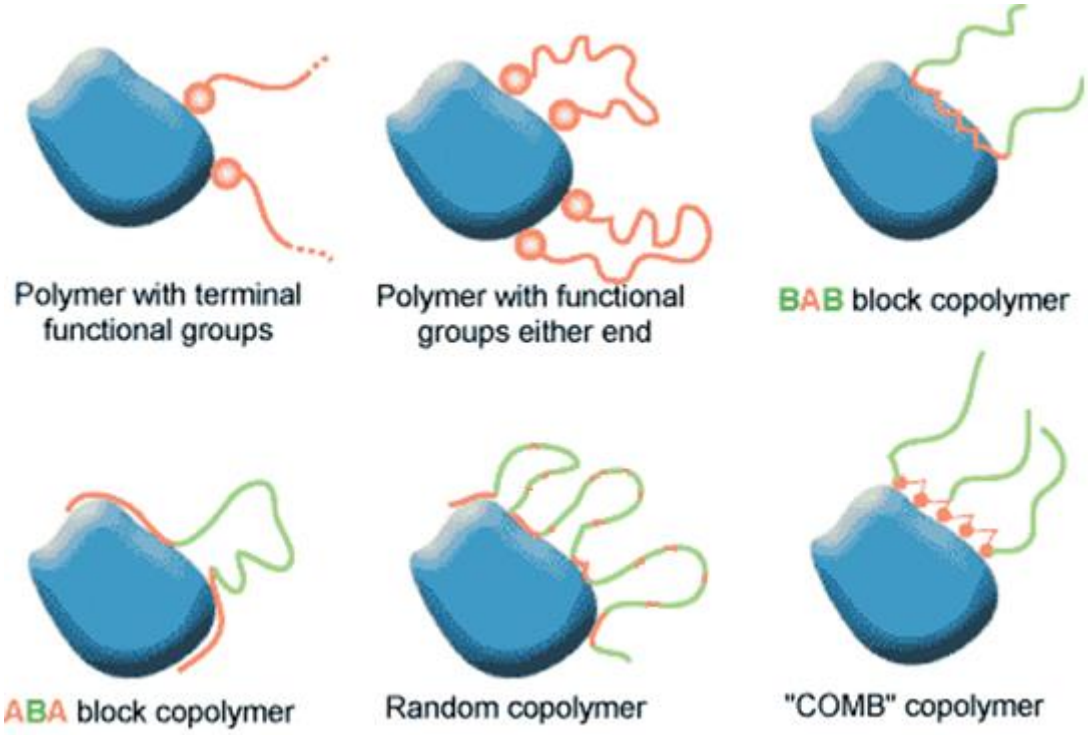

Figure 2.8 Basic illustration of a particle stabilised by various polymer types. Image adapted from the web.[82]

As the Figure 2.8 illustrates, a range of polymer configurations effectively stabilise particles in emulsions. The key is that a part of the polymer has affinity for the particle and the rest of the polymer has affinity for the solution. For example, particles may be stabilised by polymers that are terminated with functional groups on one or both ends. In other cases the one of the functional groups of a copolymer may stabilise the particle. These may be blocky or even random copolymers. Another type illustrated in Figure 2.8 are branched or 'comb' copolymers where polymeric chains are grafted to an anchor which has affinity for the particle. Many of these polymers are based on polyethylene oxide-*co*-propylene oxide (Figure 2.9) where the PPO acts as the hydrophobe and the PEO as the hydrophile. A straight chain PPO-PEO (A) copolymer as well as two

branched PPO-PEO (B and C) copolymers are shown. These could potentially be used as depicted in Figure 2.8 [68]

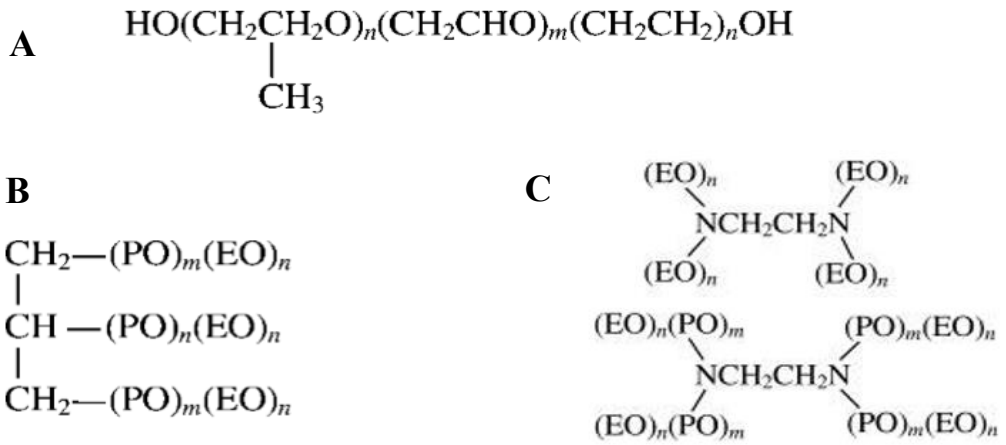

Figure 2.9 Ethylene oxide-propylene oxide based copolymer surfactants – straight chain and 2 branched variants are illustrated. Image adapted from Tadros.[68]

Adding water-soluble polymers as thickeners can stabilise an emulsion and prevent sedimentation in suspoemulsions. These adjuvants are also useful for altering the properties of the dried latex film. They may make the film more flexible or permeable. Many water-soluble polymers are used as thickeners in agrochemical formulations. There are numerous scenarios where modifying rheological properties of a formulation is advantageous. They can modify the properties of the spray from nozzle to leaf surface which can prevent spray drift and enhance the ability of droplets to adhere to leaf surfaces on impact.[83] The synthetic polymers include high molecular weight poly(ethylene oxide), poly(vinyl pyrrolidone), poly(acrylamide) and poly(vinyl alcohol).[24] Natural polymers include guar and xantham gums, soy lecithin compounds and cellulosic materials such as carboxymethyl cellulose. A recent study showed that viscoelastic fluids stabilise filaments formed during spray sheet breakup and supress the formation of fine droplets.[83]

2.3.3 Latexes

Copolymers of styrene, butadiene, isoprene, vinyl chloride and vinyl esters are examples of polymers used to form latexes. They act as sticking adjuvants and are designed to increase the amount of formulation that stays stuck onto the leaf surface. When the formulation dries, the latex forms a protective film. Initial approaches involved polymerisation of a monomer in the presence of a pesticide.[67] However, latexes may be used already prepared, and the oil/ active ingredient solution swells the particles. The aforementioned polymeric surfactants are usually dissolved in the oil phase prior to the dispersion. Ostwald ripening is a concern for conventional emulsions – where smaller droplets coalesce into larger ones – but is avoided by forming the latexes with relatively high molecular weight and hydrophobic polymers.[67]

2.3.4 Encapsulation using polymers

There are several methods to encapsulate pesticides for agrochemical application. One method is to produce a solid encapsulated formulation such as a granule or continuous tape. Methods for creating the products range from spray drying, pan agglomeration, extrusion and fluidized-bed granulation. Encapsulation can be a way to turn complex formulation into simple water dispersible solid component. Water-soluble and film-forming polymers are ideal as the outer encapsulation – such as poly(vinylpyrolidone) (PVP) and poly(vinyl alcohol-*co*-vinyl acetate) (PVA).[84] For PVP, an ideal molecular weight range of 40-60 kDa has been reported. For PVA, a molecular weight of approximately 8 kDa was reported.[85] As the molecular weight of PVA increases above 20-30 kDa the water solubility decreases significantly – therefore the water solubility of the granules can be tuned to be temperature-sensitive. The advantages of solid formulations are that

they are relatively simple to use and reduce exposure of the user towards the encapsulated pesticides. Biodegradable solid formulations could be applied to directly soils to control pests.[86] The fact that the polymer takes time to degrade means that the release time is delayed, ensuring that a continual dose is applied and that too much pesticide is not used.[67]

Microencapsulation of a formulation is a sophisticated route towards improving the efficacy of a pesticide and capsule technology is wide ranging.[72] Lignin microparticles can be developed and loaded with an agrochemical active. An organic phase containing lignin derivative and active is dispersed into water containing a surfactant after which the organic solvent is removed. The resulting active containing lignin microparticles are dispersed in water and can be used for controlled release.[70] By controlling the release of the active the phytotoxicity of the pesticide towards the treated plant or seed can be reduced. This allows a greater amount of active to be applied before an adverse effect is observed. Such microcapsules can also protect actives from environmental factors such as UV degradation in the case of the lignin particles.

Another method for producing microparticles is via Pickering emulsions. Pickering emulsions are stabilised by solid particles adsorbed to an interface.[87] An emulsion of oil in water stabilised by polymer particles can be formed into solid microparticles by carefully heating. Measures should be taken to avoid boiling the oil or water phase, but the incubating temperature should be sufficient to form a solid capsule. Alternatively the solid microparticle can be formed via interfacial polymerisation. Reportedly, a number of polymers are suitable, but in particular the biodegradable polyesters including poly(butylene adipate-co-terephthalate).[74]

2.3.5 Controlled release of agrochemicals using polymers

An area of academic interest is the conjugation of polymers with low molecular weight active ingredient molecules. The actives can be released from the polymer via cleavage of a linker such as by hydrolysis or photolysis on the leaf surface. Water-soluble polymers such as poly(acrylic amide), poly(acrylic acid), poly(vinyl alcohol), poly(ethylene glycol) and dextran have all proven to be suitable. Commonly used actives include 2,4-dichlorophenoxy acetic acid (2,4-D), 1-naphthylacetic acid (NAA), 2-naphthoxyacetic acid (NOAA) and 2-naphthylthioacetic acid (NTAA) – all growth regulators.[88], [89] However, this class of agrochemical suffers from notable disadvantages as the conjugation of actives is costly and not very adaptable.

More applicable are physical interactions between active ingredient and polymer, as this offers flexibility in the formulation. In the literature, a patent for poly(vinyl alcohol) and sulfonylurea mixtures indicates that hydrogen bonds form between the polymer and active.[73] The patent claims that formulations of various sulfonylurea herbicides with PVA reduced phytotoxicity towards wheat and rice and improved selectivity towards pests.

2.3.6 Other uses for polymers

Water-soluble polymer packaging is an ecologically friendly way of producing a pesticide formulation by reducing waste packaging. PVA is useful as a water-soluble, film-forming polymer that can be used to package a gel based formulation.[90] The advantage, as with granules, is that the finished product is simple for the consumer to apply by simply adding to the correct volume of water. Depending on the molecular weight of the PVA used to create the bag, the

temperature at which the bag is soluble could be tuned using higher molecular weight grades of PVA.[5]

Polymer hydrogels can be used for the controlled release of potassium nitrate into soil. A carboxy methylcellulose-g-polyacrylamide hydrogel was able to release potassium over a period of 7 days.[91] The benefits of this controlled release are the prevention of over application of fertilizer which can lead to pollution of ground water and waterways.

2.4 Background to polymers used in this work

This section will finish with a brief review of the two most significant polymers used in this work. Their uses in the literature are evaluated. Poly(vinyl alcohol-*co*-vinyl acetate) and chitosan were the two most important polymers examined in this work. Their general characteristics and properties and uses are outlined here as well.

2.4.1 Polymer preparation and uses

PVA is prepared from the deacetylation of poly(vinyl acetate) (PVAc) and most commercial grades of PVA are 80-99% deacetylated. It is a semi-crystalline water-soluble polymer as well as non-toxic and is one of the few polymers containing a carbon backbone considered to be biodegradable. The mechanism of chain cleavage occurs via enzymatic oxidation of the alcohol groups followed by hydrolysis of the resulting ketone.[92] It is the most important commercially available water-soluble polymer and has been described as a ‘green’ polymer due to the aforementioned biodegradability.[93] It is readily blended with other

polymers and natural materials – but its uses must be offset against its lack of long term life cycle.

The main use for PVA is as a precursor for poly(vinyl butyral) or emulsifying agent for the emulsion polymerisation of poly(vinyl chloride). The spun fibre (spun from a solution rather than melt) is used in paper making, cement reinforcement, canvas and fishing nets. As PVA is biodegradeable and water soluble it has attracted attention from plastic packaging manufacturers as a greener packaging material.[93] Its use as a soluble bag for agrochemical formulations and its use as a stabiliser agrochemical emulsion formulations has been highlighted as well.[5], [24]

Increasing the hydrolysis of PVA reduces water solubility while increasing solvent resistance, tensile strength, crystallinity and adhesion to hydrophobic surfaces. Tuning properties such as the degree of hydrolysis and molecular weight enables the tuning of the final product properties. PVA used in capsules for drugs must be much more soluble than PVA used in food packaging.[93]

Chitosan is prepared from the deacetylation of chitin, a naturally derived polysaccharide principally from sources such as exoskeletons of crustaceans and cell walls of fungi. Thus chitosan is somewhat analogous to PVA – it is also semi-crystalline, biocompatible, biodegradeable and water soluble.[94] Unlike PVA, deacetylation decreases chitosan crystallinity. Chitosan is antimicrobial and has been explored as a component in wound dressings and medical sutures.[94] It can also be wet spun into fibres like PVA. While PVA fibres have been found applicable to reinforce cement, chitosan fibres have been employed in

hydroxyapatite bone cement.[95] Chitosan can be used as a component in tissue scaffolds and cells may proliferate over chitosan based fibres.[96]

Chitosan is only soluble in water at pH < 6.[97] Its solubility in acetic acid increases with decreasing pH and it carries a positive charge in solution as pendant amine moieties are protonated. This property has been investigated for processes such as detoxifying water – the charge can enable chitosan to bind hazardous materials such as heavy metals and oils.[97] Chitin, chitosan and derivatives have been proposed as plant nutrients, growth stimulants and pesticides. Such mechanisms include stimulation of microbes which are better able to defend plants.[98]

2.4.2 Polymer physical characteristics

Despite their large size, polymers are capable of crystallizing into ordered structures. However, unlike smaller molecules, obtaining fully crystalline polymer materials is difficult.[99] In reality, those polymers which are able to form crystal structures do so only partially, and are known as semi-crystalline polymers. As discussed above, both chitosan and PVA are semi-crystalline materials. Crystal growth from the melt as well as from solution is discussed in this section. From the melt, entangled polymer chains begin to untangle and align as long as the temperature is both below the melting temperature (T_m) and above the glass transition temperature (T_g).[100] For solution crystallization, as evaporation occurs separated polymer coils begin to interact. In both situations, polymers align in favourable, folded chain crystals which then further begin to layer together (Figure 2.10). Below T_g molecular movement is frozen. The layered structures have a significant amount of amorphous content which then becomes incorporated into larger spherulites.[99] This means that the amorphous content is trapped in

this state and the polymer cannot fully crystallize – explaining why it is difficult to attain fully crystalline polymer materials.

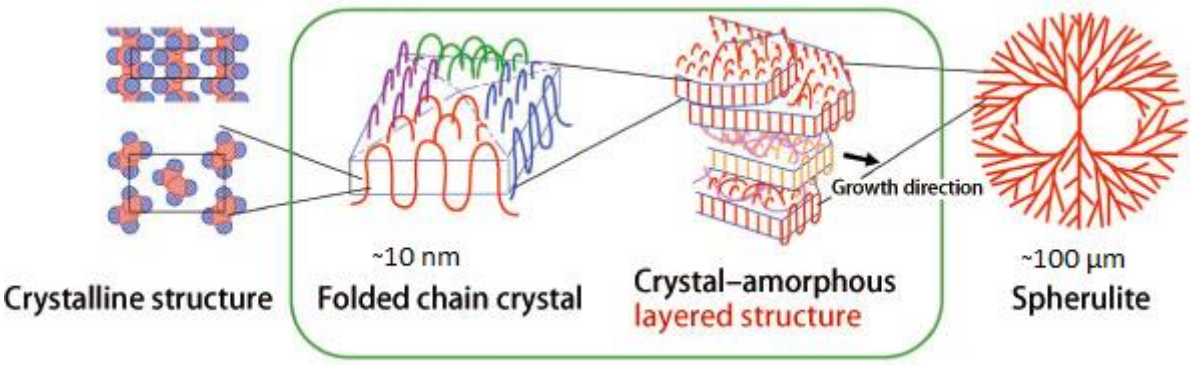

Figure 2.10 Hierachy of polymer crystal structures showing how ordered molecular structures lead to larger ordered spherulites. Image adapted from the web.[99]

Crystallinity is encouraged by slow cooling of the melt or by slow solvent evaporation. This ensures the entangled polymer chains have the opportunity to align before molecular movement is locked below the T_g or the solid polymer is formed from solution. Likewise, polymers with high molecular weight are more entangled and require longer times to crystallize.[101] The crystal structures of chitosan and PVA are presented in Figure 2.11. Crystallinity is favoured by intermolecular forces between polymer chains. The chitosan crystal structure is favourable due to intermolecular hydrogen bonding between oxygen atoms. The chitosan unit cell is comprised of 4 glucosamine moieties between which there are three hydrogen bonds – two intermolecular and one intramolecular.[102] For PVA, the chains are packed together via intermolecular hydrogen bonds.[103]

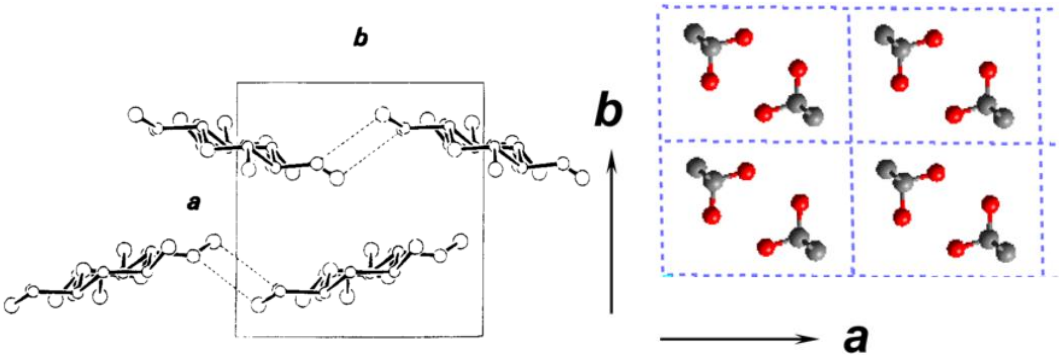

Figure 2.11 Crystal structures of dehydrated chitosan (left) and poly(vinyl alcohol) (right). Hydrogen atoms are omitted and in the case of the structure for chitosan, the dotted lines represent hydrogen bonding. Images adapted from Yui *et al* and Tashiro *et al* respectively.[102], [103]

The degree of crystallization of a polymer can be affected by its molecular weight. Relatively short polymer chains (i.e. low molecular weight) tend to more easily form crystals than more entangled longer chains. Molecular weight similarly affects many other physical properties of a polymer. Increasing molecular weight, in general, leads to an increase in mechanical properties of a polymer. The molecular weight, along with chain stiffness and nature of side groups, is a crucial factor governing the T_g of a polymer.[101] Intrinsic viscocity of a polymer is related to polymer molecular weight, according to the Mark-Houwink-Sakurada equation:[104]

$$[\eta] = KM^a \qquad (1)$$

Where η is intrinsic viscocity, K and a are constants determined by the solvent-solute system and temperature and where M is molecular weight.

2.5 Conclusions

This review has established the background and motivations for the coming chapters of this thesis – namely the need to improve intelligent design of agrochemicals in order to farming efficiency and food security. The range of

pesticides types and their typical formulations have been discussed – highlighting the loss mechanisms, including rain, which can reduce their efficacy. The review examined the methods that the agrochemical industry uses to formulate active ingredients to overcome these losses and highlighted two main routes for rainfastening of agrochemicals. Super-spreading surfactants are used to encourage the rapid absorption of active ingredients into the leaf so that they are not exposed to rain on the outer surface of the plant. However, another class of adjuvants, such as film forming polymers, which form difficult to dissolve deposits were also noted. It is the type of formulation that will be the focus of this work – with an emphasis on understanding why water-soluble polymers are able to exhibit rainfastness.

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Chapter 3 Physical characterisation and fluorescent labelling of polymers

3.1 Introduction

One of the key elements to this work was the determination of the underlying factors which govern rainfastness of water-soluble polymers. In this section, the physical properties of selected water-soluble polymers are characterised in a number of different techniques. The work focused mostly on 8 grades of poly(vinyl alcohol-*co*-vinyl acetate) (PVA) and 3 grades of chitosan, but some work was carried out using carboxymethyl cellulose, 3 samples of modified pectin and manufacturer labelled FITC-dextran. As discussed in the literature review, chitosan and PVA are in many ways analogous. They are semi-crystalline,[1], [2] linear polymers which are formed by deacetylation of a precursor.[3] Chitin and poly(vinyl acetate) are precursors respectively for chitosan and PVA.[4], [5] Both are difficult to dissolve in water as PVA requires heating and chitosan a pH of below 6. As discussed in chapter 1, part of the agrochemical delivery process is that the formulation is a solution which is deposited onto a plant surface. This fact has led us to focus on characterising these polymers as dried films and deposits.

Different grades of the same polymer may have vastly different physicochemical properties. Factors such as molecular weight, crystallinity, tacticity, polydispersity and copolymer composition all have interacting effects which determine the further properties of the polymer such as thermal/mechanical properties and solubility.[6] It was hypothesised that these properties would heavily influence the rainfastness of a polymer deposit on the leaf surface. In particular the molecular weight, crystallinity and solubility of the selected polymers are hypothesized to be

the main causes of rainfastness. This has driven the work in which these properties were characterised and in later chapters their influence on rainfastness is assessed. This section covers several analyses including; molecular weight by gel permeation chromatography; copolymer composition by proton nuclear magnetic resonance spectrometry; crystallinity by dynamic scanning calorimetry, X-ray diffraction or polarised light microscopy; swelling and solubility of cast polymer films by gravimetric analysis.

This section also highlights the procedures for the fluorescent labelling of the selected polymers as well as the characterisation of these labelled polymers. A key method for the determination of rainfastness in this work was fluorescence microscopy analysis of polymer deposits before and after rain washing. Fluorescent labelling is commonly carried out for a wide variety of applications,[7], [8] from *in vivo* visual diagnostics[8]–[10] and monitoring to retention studies[11] such as in the following chapters. Many studies have examined the mucoadhesion of water-soluble polymers to various tissues via fluorescence labelling and microscopy – and such methods informed aspects of this work.[12]–[14] The chemistry of fluorescent labelling is varied and depends on the reactivity of pendant groups of the polymer. This section will highlight the chemistry of three labelling methods used to label hydroxyl groups of PVA, amine groups of chitosan and carboxylic acid groups of several other polymers.

3.2 Materials

The polymers used in this work are listed in Table 3.1. Further materials used in this work are described in their relevant sections.

Table 3.1 Polymers used throughout this work are listed with the sample ID, supplier and any provided manufacturer specifications.

Sample ID	Polymer	Supplier	Manufacturer specifications	
			Composition	Molecular Weight (kDa)
PVA80	Poly(vinyl alcohol-*co*-vinyl acetate)	Sigma Aldrich	80% deacetylated	9
PVA88L	Poly(vinyl alcohol-*co*-vinyl acetate)	Alfa Aesar	88% deacetylated	'Low'
PVA88M	Poly(vinyl alcohol-4*co*-vinyl acetate)	Alfa Aesar	88% deacetylated	'Medium'
PVA88H	Poly(vinyl alcohol-*co*-vinyl acetate)	Alfa Aesar	88% deacetylated	'High'
PVA99L	Poly(vinyl alcohol-*co*-vinyl acetate)	Alfa Aesar	99% deacetylated	'Low'
PVA99M	Poly(vinyl alcohol-*co*-vinyl acetate)	Alfa Aesar	99% deacetylated	'Medium'
PVA99H	Poly(vinyl alcohol-*co*-vinyl acetate)	Alfa Aesar	99% deacetylated	'High'
PVA99VH	Poly(vinyl alcohol-*co*-vinyl acetate)	Sigma Aldrich	99% deacetylated	144
CSL	Chitosan	Sigma Aldrich	>75% deacetylated	50-190
CSM	Chitosan	Sigma Aldrich	75-85% deacetylated	'Medium'
CSH	Chitosan	Sigma Aldrich	>75% deacetylated	310-375
CMC	Sodium carboxymethyl cellulose	Sigma Aldrich	N/A	N/A
AP	Amidated pectin	Herbstreith & Fox	28% methylated 20% amidated 88% galacturonic acid	63
LMP	Low methyl ester pectin	Herbstreith & Fox	36% methylated 89% galacturonic acid	54
HMP	High methyl ester pectin	Herbstreith & Fox	59% methylated 80% galacturonic acid	67
FITC-DEX	FITC-labelled dextran	Sigma Aldrich	0.003-0.020 mol FITC per mol glucose	500

3.3 Fluorescent Labelling

3.3.1 Methods

Fluorescent labelling of poly(vinyl alcohol-*co*-vinyl acetate) was achieved using 5-(4,6-dichlorotriazinyl)aminofluorescein (5DTAF, Sigma Aldrich) as a fluorophore. 5DTAF is a derivative of fluorescein that is reactive towards hydroxyl groups.[15] To dissolve high molecular weight PVA, they must be constantly stirred at approximately 85 ^{o}C. A mass of fluorophore equivalent to react with 1% of PVA hydroxyl groups was added to a 10 mL solution (0.4% w/w) of PVA with the assumption that each fluorophore may only react with one hydroxyl moiety. The masses added were 3.9 mg for PVA80, 4.3 mg for the PVA-88 range and 4.8 mg for the PVA-99 range. A 0.05 M Na_2CO_3 (Sigma Aldrich) solution was added drop-wise until the fluorophore was seen to be dissolved, resulting in a solution of approximately pH 9. The mixture was stirred in the dark for 48 hours and then dialyzed for several days until pure, changing the water 3-4 times per day. Visking dialysis tubing (Medicell Membranes Ltd) with a molecular weight cut-off of 7 kDa was used; this size was to remove unlabelled 5DTAF as well as impurities such as sodium acetate.

Portions (1 mL) of purified and unpurified labelled PVA were added to two separate columns loaded with 20 g Sephadex G50 gel (Sigma Aldrich). The unpurified fluorescently-labelled PVA had a significant gap between large and small molecules eluting, indicative of unlabelled PVA. This was not the case with the pure solution of labelled PVA. In order to determine the level of labelling of each sample, calibration standards were prepared using a stock solution of PVA (90 kDa molecular weight) and 5DTAF which was serially diluted with 0.2% w/v

sodium carbonate. All samples were excited at 392 nm and intensity of emission was recorded at 420 nm using a FP-6200 Jasco fluorescent spectrometer. Intensity of samples of purified, labelled PVA were compared to the calibration curve which indicated the equivalent mass of free 5DTAF. From this was calculated the percentage of alcohol moieties labelled.

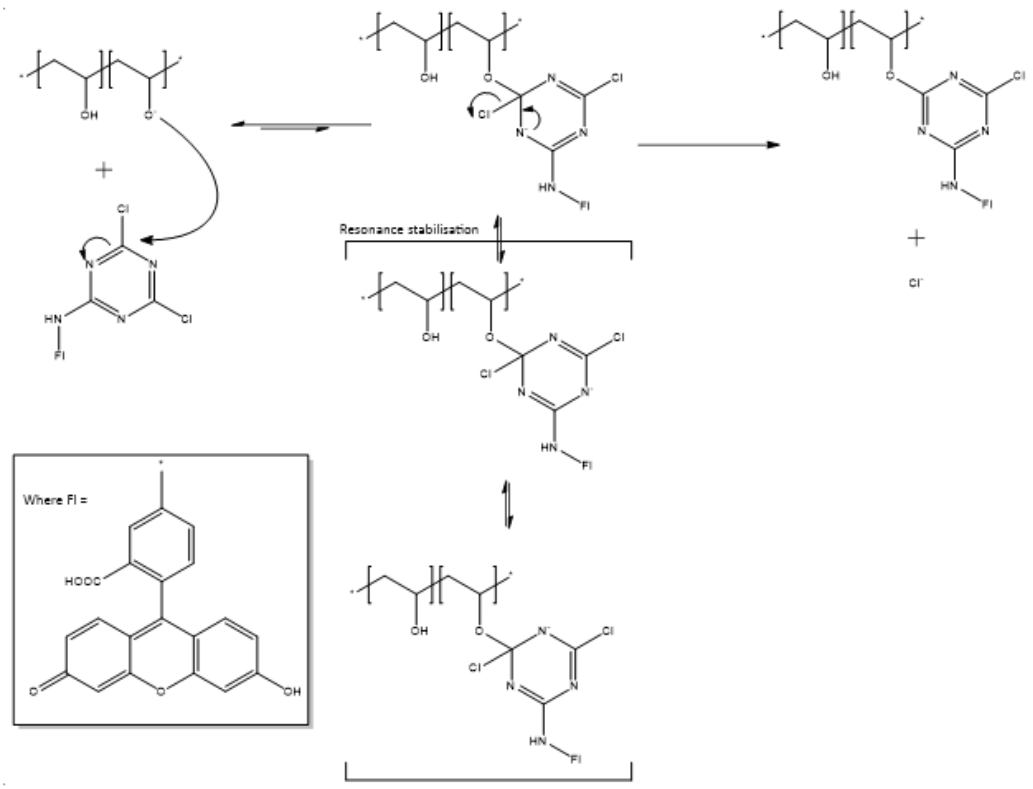
Chitosan was labelled using fluorescein isothiocyanate (FITC, Sigma Aldrich). Chitosan (1 g) was dissolved in 100 mL of acetic acid (0.2 M) and left to stir for 24 hours. FITC (100 mg) was dissolved in 50 mL of methanol and subsequently was added to the chitosan solution which was then stirred for 3 hours in the dark. The labelled chitosan was then precipitated in 1 L of sodium hydroxide (0.1 M) and filtered to obtain the impure labelled chitosan. The product was dissolved in acetic acid (0.2M) and dialysed using cellulose dialysis membrane with a molecular weight cut-off of 7 kDa against deionized water for several days to remove any unreacted FITC.

The remaining carbohydrate based polymers were labelled with 6-aminofluorescein (6AF, Sigma Aldrich). The reaction is mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma Aldrich) N-hydroxysulfosuccinimide sodium salt (sulfo-NHS, Sigma Aldrich). Polymers were dissolved in 2-(N-morpholino)ethanesulfonic acid (Sigma Aldrich) buffer of approximately pH 4 and left stirring for 24 hours. Then, one equivalent of EDC was added to the dissolved polymer followed by 2 equivalents of sulfo-NHS. To complete the reaction, one equivalent of 6AF is added and the mixture was stirred in the dark for 24 hours. The mixture was dialysed using dialysis membrane with a molecular weight cut-off of 7 kDa against deionized water for several days to

remove any unreacted 6AF. All labelled products were freeze dried and stored in a refrigerator.

3.3.2 Results of fluorescent labelling

The reaction between 5DTAF and PVA is more favourable when the solution pH is high;[15] however, this causes hydrolysis of the PVA. It was found that by dissolving partially hydrolysed PVA (PVA80, -88L, -88M and -88H) samples in 0.2M Na_2CO_3, the resulting labelled polymers were almost fully hydrolysed. By maintaining a lower pH, the partially hydrolysed grades of PVA were prevented from being fully hydrolysed. Some hydrolysis does occur though and the values roughly increase 5% for partially hydrolysed samples, but their partially hydrolysed character was preserved. Two slightly different reaction mechanisms have been proposed but involve the nucleophilic substitution of a PVA alkoxide with one of the chlorines of the 5DTAF triazine ring (Scheme 3.1). One route being via deprotonation of the hydroxyl group of PVA and the other being via the partial hydrolysis of acetate groups present to some degree in all PVA samples. Nucleophilic aromatic substitutions of chlorinated triazine rings are more favourable than with typical chlorinated benzenes.[16] This type of reaction is the basis for many reactive dyes than are used to functionalise cellulosic materials.[17], [18]



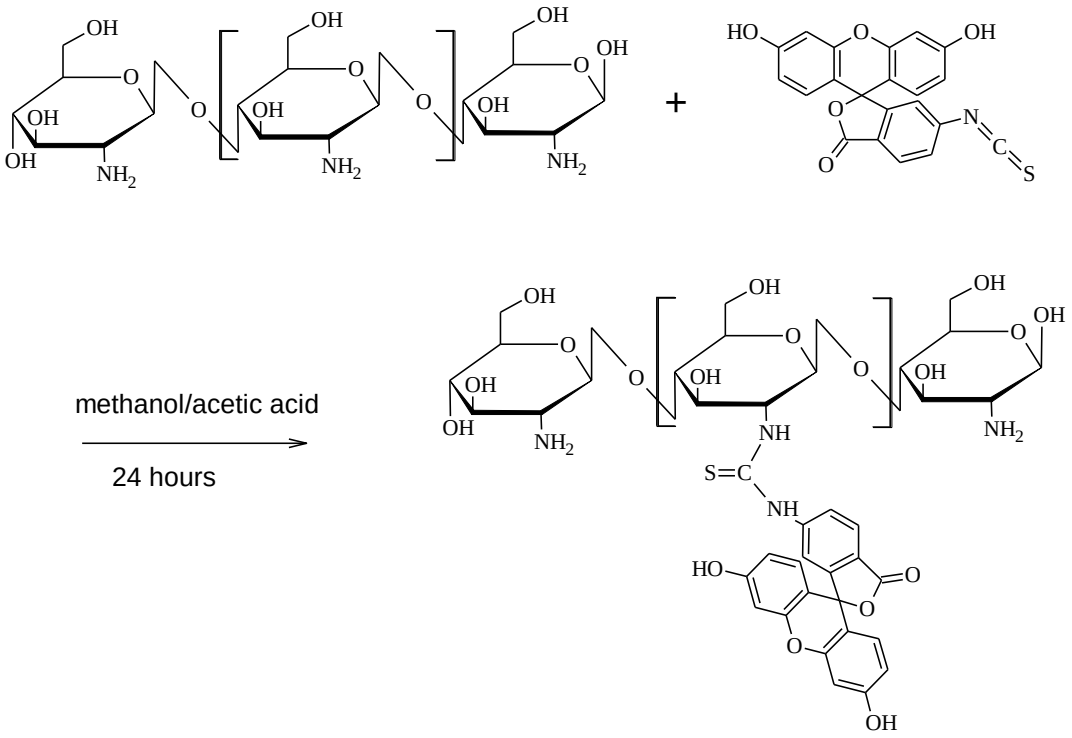
Scheme 3.1 Illustrates chemical structures of PVA and 5DTAF reactants, labelled PVA product as well as suggesting a potential route to stabilisation of an intermediate.

The low efficiency of the reaction can be attributed to the difficulty in forming PVA alkoxide via deprotonation of alcohol. Side reactions further decrease efficiency. These may be intramolecular reactions between 5DTAF molecules, the rapid hydrolysis of alkoxide by water, and competition between alkoxide and hydroxide as nucleophiles. Approximately 0.02% of PVA repeat units were labelled, and is consistent for each sample (Table 3.2). This degree of labelling is not particularly low when compared to the efficiency of other polymer and protein labelling reactions.[19] Ultimately the labelled polymers were not used quantitatively, and their fluorescence was used to determine the presence of the polymer qualitatively.

Table 3.2 Degree of labelling of PVA samples.

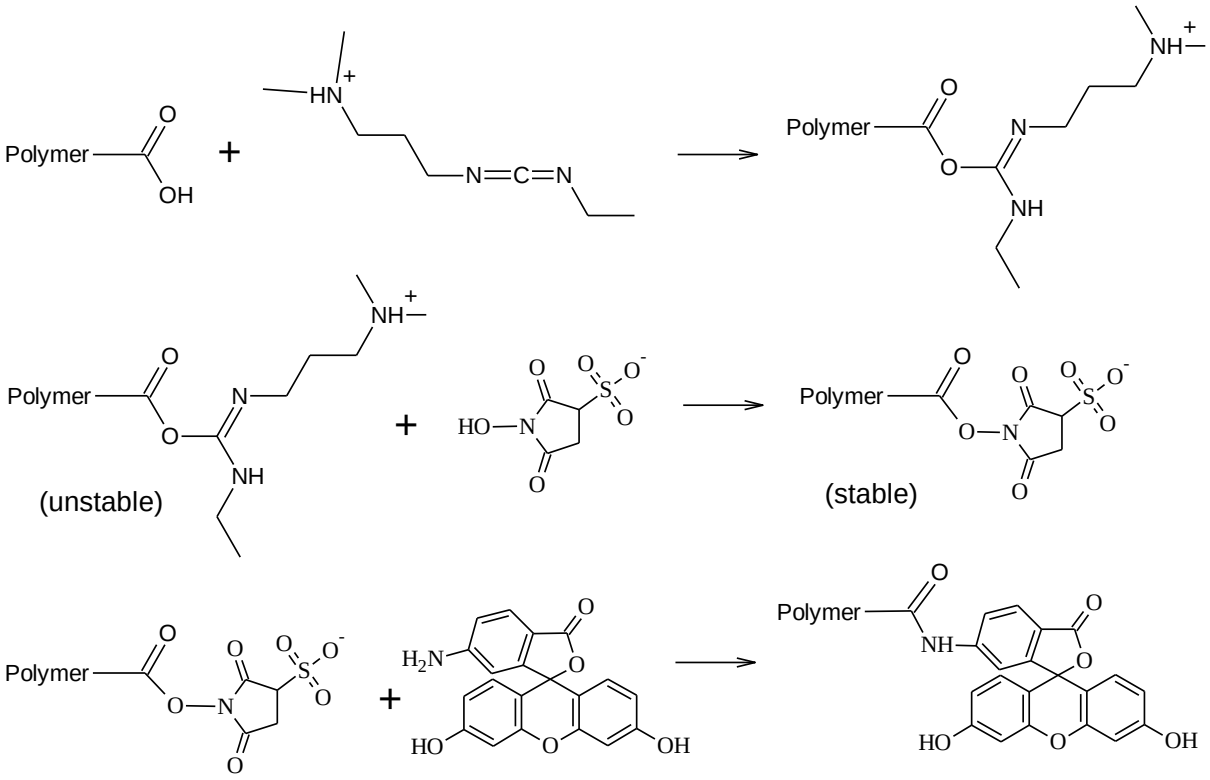
Sample ID	Equivalent [5DTAF] (mg/mL)	PVA-alcohol moieties labeled (%)
PVA80	0.009	0.023
PVA88L	0.008	0.018
PVA88M	0.007	0.016
PVA88H	0.006	0.013
PVA99L	0.010	0.021
PVA99M	0.007	0.015
PVA99H	0.008	0.016
PVA99VH	0.009	0.020

FITC is a long established and commonly used technique to fluorescently label polymers and proteins containing primary amine groups.[18], [20], [21] The amine group of chitosan reacts readily with the isothiocyanate group of the fluorescein derivative to form a thiourea link (Scheme 3.2).[22]



Scheme 3.2 Illustrates the chemical structures of chitosan, FITC and the labelled product.

The labelling of pectin and carboxymethyl cellulose proceeds via EDC coupling reaction between the carboxylic acid (carboxymethyl/galactaronic acid) and the amine of 6AF (Scheme 3.3). EDC reacts with the carboxylic acid to increase reactivity but is unstable in water and will quickly be hydrolysed. Sulfo-NHS replaces the EDC to form a more stable intermediate that is still reactive towards the amine of 6AF.



Scheme 3.3 Illustrates the sulfo-NHS mediated EDC coupling reaction to label carboxylic acid containing polymers with 6AF.

3.4 Physical characterisation methods

3.4.1 ^{1}H Nuclear magnetic resonance spectroscopy for polymer composition

Spectra for PVA and chitosan were recorded (Brüker 400MHz spectrometer) in order to characterize their copolymer composition or degree of (de)acetylation. For PVA, it is common to refer to the degree of deacetylation as the degree of hydrolysis when considering the copolymer composition between vinyl acetate and vinyl alcohol. For chitosan the degree of (de)acetylation indicates the ratio between the natural water-insoluble chitin and chitosan. PVA was dissolved in D_2O and chitosan was dissolved in D_2O acidified with trifluoroacetic acid (Sigma

Aldrich). The spectra were analyzed and peaks integrated using MestReNova Lite software (Mestrelab Research).

For PVA, peaks in the region 1.50 – 1.80 ppm are caused by protons of backbone $–CH_{2,}$ while those at 2.10 ppm are caused by protons of pendant acetate $–CH_3$ moieties. Peaks at 3.85 ppm are backbone –CH opposite acetate moieties and 4.05 ppm are backbone –CH opposite the hydroxyl moieties. (Figure 3.1). By integrating these peaks it is possible to determine the degree of hydrolysis (DoH) for PVA samples.

$$DoH = \left(1 - \frac{b}{d}\right) \times 100\ \% \qquad (1),$$

where *b* and *d* are the integral values for proton NMR peaks at 4.05 and 3.85 ppm, respectively.

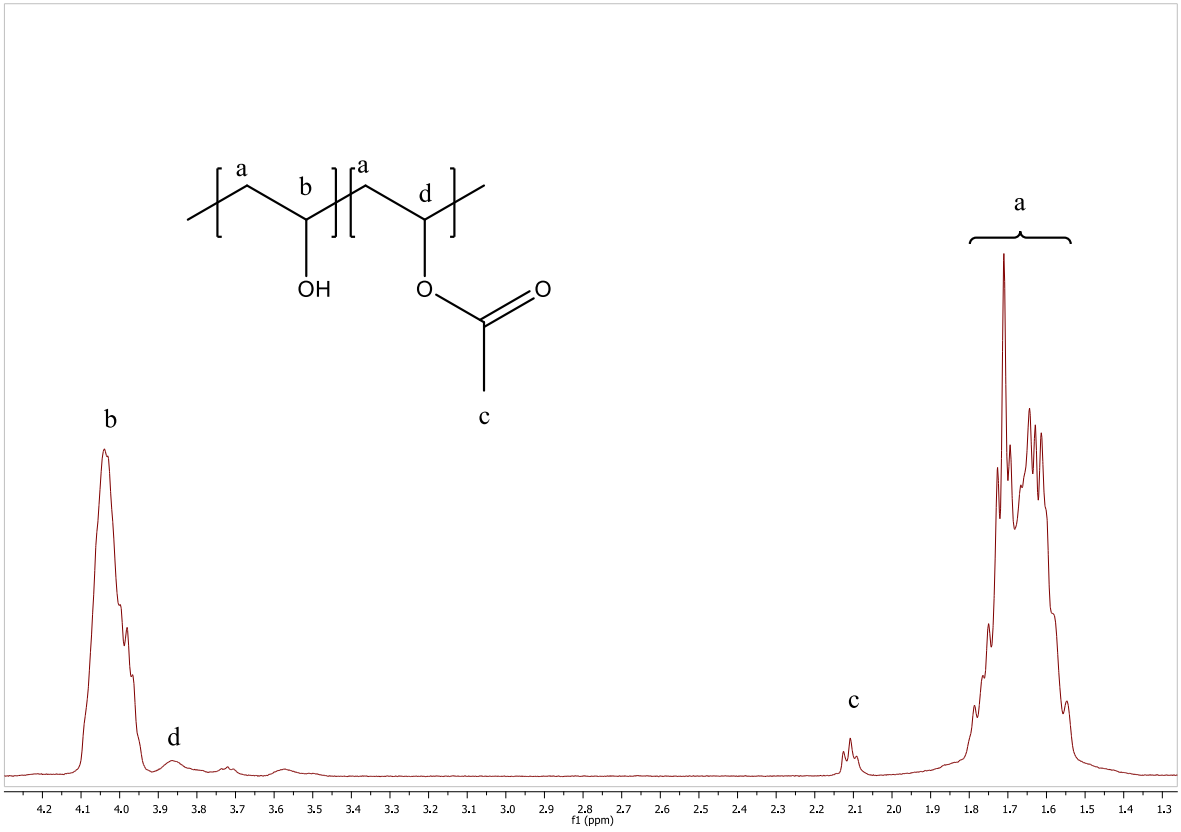


Figure 3.1 The labelled peaks and corresponding moieties of a typical PVA ^{1}H NMR spectrum.

For chitosan, peaks in the overlapped region of 3.25 – 4.00 ppm represent protons from H3-H6 on both the de- and acetylated moieties of chitosan as well as H2 from the acetylated moiety (Figure 3.2). Acetyl protons are present at 2.00 ppm. By integrating these peaks the fraction of acetylation is determined.

$$DA = \frac{(\frac{1}{3}Ac)}{\frac{1}{6}(H2, A; H3 \dots H6, \mathrm{A}, D)} \times 100 \qquad (2)$$

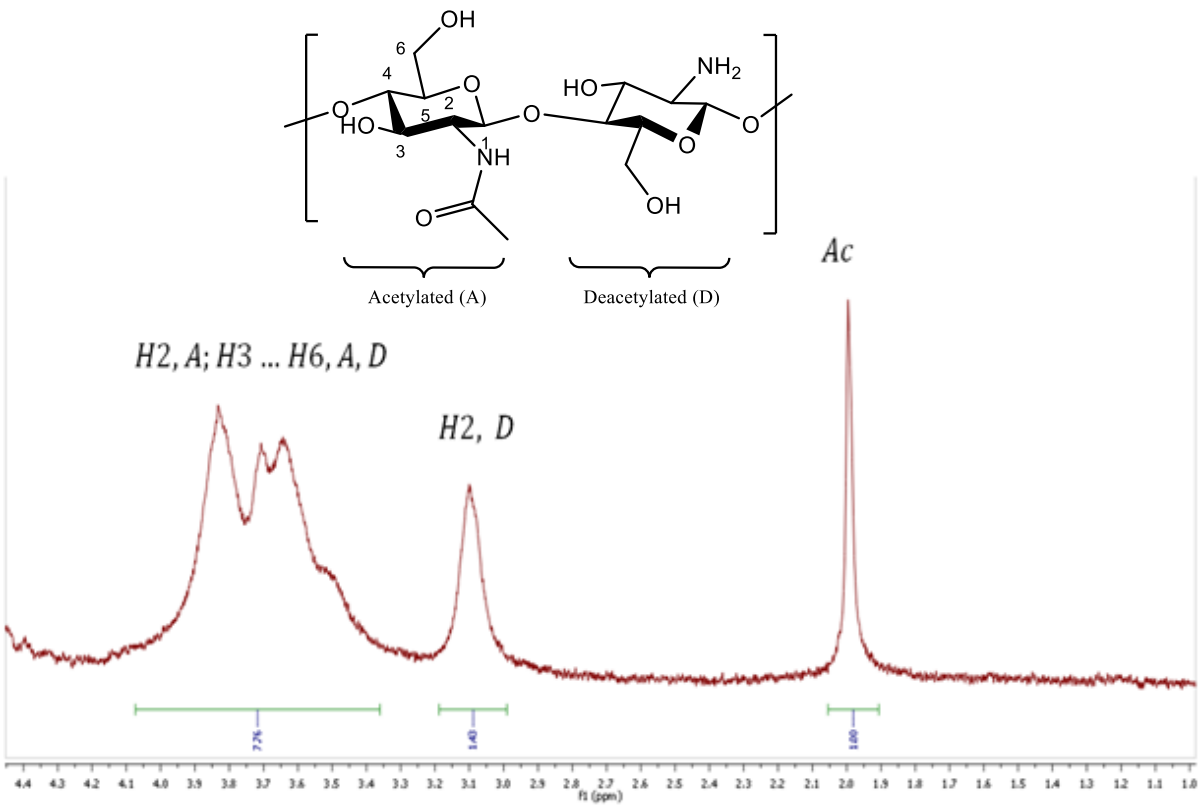


Figure 3.2 Exemplary assigned ^{1}H NMR spectrum of chitosan.

Tacticity of PVA was measured using NMR as well by measuring spectra of PVA dissolved in deuterated DMSO (Sigma Aldrich).[23], [24] In deuterium oxide, the hydroxyl protons are too labile to be detected. In DMSO this problem is overcome and the overall tacticity of the PVA samples can be determined by integrating the peaks responsible for different triads. In the context of PVA, isotacticity is when the hydroxyl moieties are all on the same side of the polymer backbone. Syndiotactic PVA indicates that the hydroxyl groups alternate and atactic PVA means that the hydroxyl groups are randomly situated. In practice, a mixture of tacticities is common. Peaks at 4.70, 4.55 and 4.30 ppm represent isotactic, heterotactic and syndiotactic triads respectively (Figure 3.3).

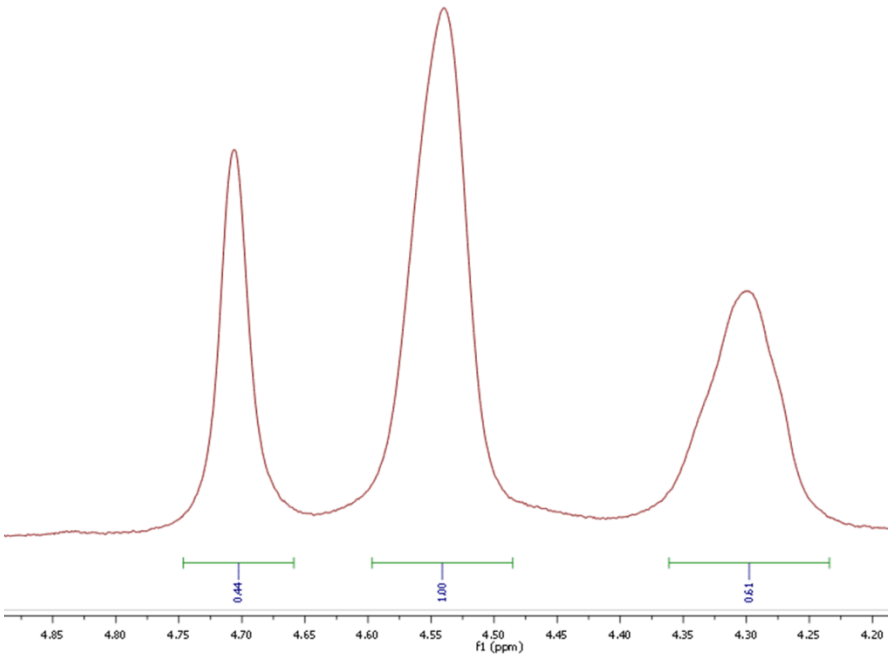


Figure 3.3 Exemplary hydroxyl proton spectrum of PVA in DMSO-d_6.

3.4.2 Gel permeation chromatography for polymer molecular weight

A Polymer Laboratories PL-GPC 50 PLUS instrument was used to determine the molecular weights of polymers described in this work. PVA samples were dissolved in a 0.05 M sodium nitrate solution to a concentration of 0.1 % w/w by heating to 90 °C. The 0.05 M sodium nitrate solution was used as the eluent as the samples were run through an aqueous column (Agilent PL Aquagel-OH Mixed-H 8μm) at a rate of 0.1 mL/min at room temperature. A set of standards of known molecular weight poly(ethylene glycol) were run in the same manner.

Aqueous 0.1 M solution of NaNO3, adjusted to pH 2.1 with trifuoroacetic acid was used as the mobile phase for chitosan samples. Pullulan standards were used to calibrate the instrument which was equipped with a refractive index detector. An Agilent PL Aquagel-OH (Mixed-H 8 mm) column was also used in this analysis with a flow rate of 0.5 mL/min at 30 ^{o}C.

PEG and pullulan standards were chosen for their similarity to PVA and chitosan respectively. Mark-Houwink parameters for PVA in the chosen eluent were available in the literature but not for the chosen chitosan solution. Thus the Mark-Houwink parameters were used to determine the molecular weight for PVA but not chitosan. Chitosan was simply compared to the standard molecular weight values of pullulan standards without any Mark-Houwink conversions. However, the obtained values were satisfactory and matched manufacturer specifications.

3.4.3 Swelling and solubility of cast polymer films

A gravimetric measurement was used to determine the swelling and solubility of certain solution cast polymer films. For PVA solutions were cast in plastic petri dishes and dried at room temperature to form films of approximately 1 g mass and 80-160 µm thickness. The films were initially weighed and then placed in 500 mL deionized water and their masses weighed periodically. The three temperatures used were 5, 15 and 25 °C which were controlled with a water bath, so as to mimic a moderate climatic range. Swelling degree was determined at each temperature and in triplicate.

$$SD = \frac{m - m_0}{m_0} \qquad (3),$$

where $\boldsymbol{m}$ is the mass of the film at time t and $\boldsymbol{m_0}$ is the mass of the initial dry film.

For chitosan, the swelling degree was determined in the same way but with the following exceptions. The mass of cast films was approximately 0.25 g and the films were swollen in 200 mL of phosphate buffer (pH 7). Measurements were made at 25 degrees °C only.

3.4.4 Crystallinity of polymers

X-ray diffraction (XRD) and dynamic scanning calorimetry (DSC) were used to estimate the crystallinity of certain solution cast polymer films, which were prepared as described in section 3.4.3. For PVA, XRD data was collected using a Brüker Nanostar on a wide angle X-ray scattering storage phosphor screen for 1 hour. The data was retrieved using a Fujifilm FLA-7000 reader and diffraction patterns were analyzed using ImageJ software (National Institutes of Health). Exemplary WAXS diffraction patterns for 4 PVA samples are provided (Figure 3.4). Crystallinity was determined by calculating the ratio of area under crystalline peaks to the total area under the diffractogram. However, the only PVA crystalline peaks used for the calculations were at approximately $2\theta = 21^{o}$. The peak at 21^{o} corresponds to the (101) plane of PVA, as accepted widely in the literature.[25], [26] However, the other peaks appearing in the diffractogram at 5, 45, 50 and 58^{o} do not appear in diffraction patterns for semi-crystalline PVA and were therefore not taken into consideration during calculations. The amorphous 'baseline' region under the curve was determined by eye and calculated using the area calculating function in ImageJ.

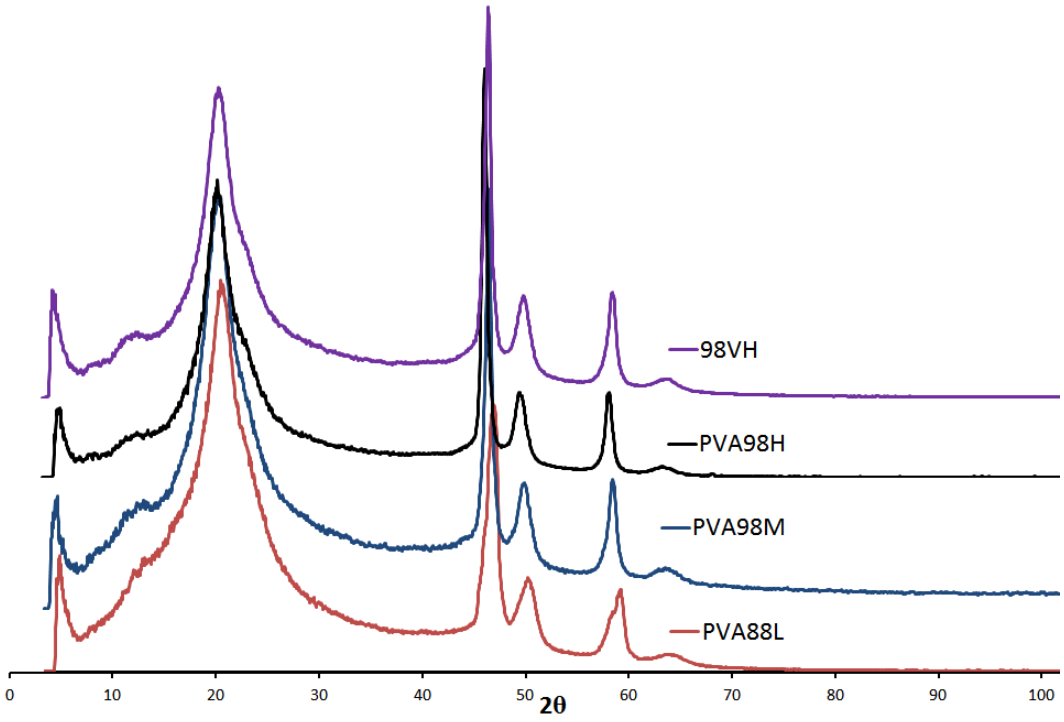


Figure 3.4 Exemplar wide angle X-ray scattering (WAXS) patterns of 4 PVA films.

Chitosan spectra were recorded using the same instrument. However, the spectra were inconclusive and the crystallinity was unable to be determined by this method (Figure 3.5). The spectra show significant amorphous content in the films cast from acetic acid solutions. Some sharper peaks, such as at $2\theta = 26^{o}$ for CSM and CSH, indicate the presence of at least some crystalline content.

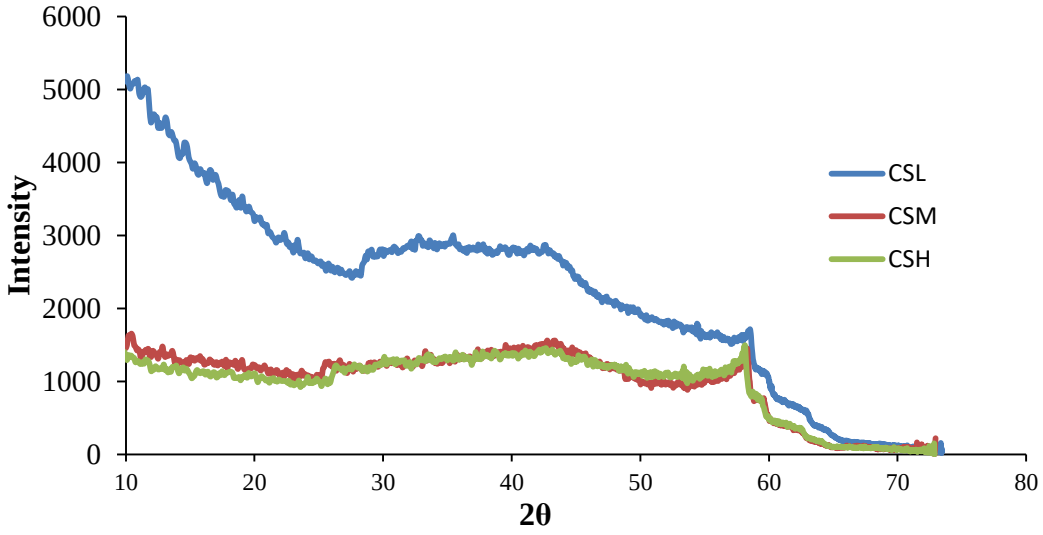


Figure 3.5 WAXS patterns of 3 chitosan films showing considerable amorphous content with some crystalline peaks.

DSC of PVA was performed using a TA Q2000 instrument and the temperature was ramped from 30 to 250 °C, cooled to 30 °C and again ramped up to 250 °C at a rate of 10 °C per minute. An exemplary DSC thermogram is shown in Figure 3.6. The enthalpy of fusion of the second heating was used to determine crystallinity.

$$\chi_c = \frac{\Delta H_m}{\Delta H_m^0 . \omega} \qquad (4),$$

where $\boldsymbol{\chi_c}$ is the crystallinity, ΔH_m is the enthalpy of fusion of the second heating, ΔH_m^0 is the enthalpy of fusion for 100% crystalline PVA from literature and ω the weight fraction of solid film content.[27] Thermogravimetric analysis was used to determine the water content of films, which was used to calculate their solid content.

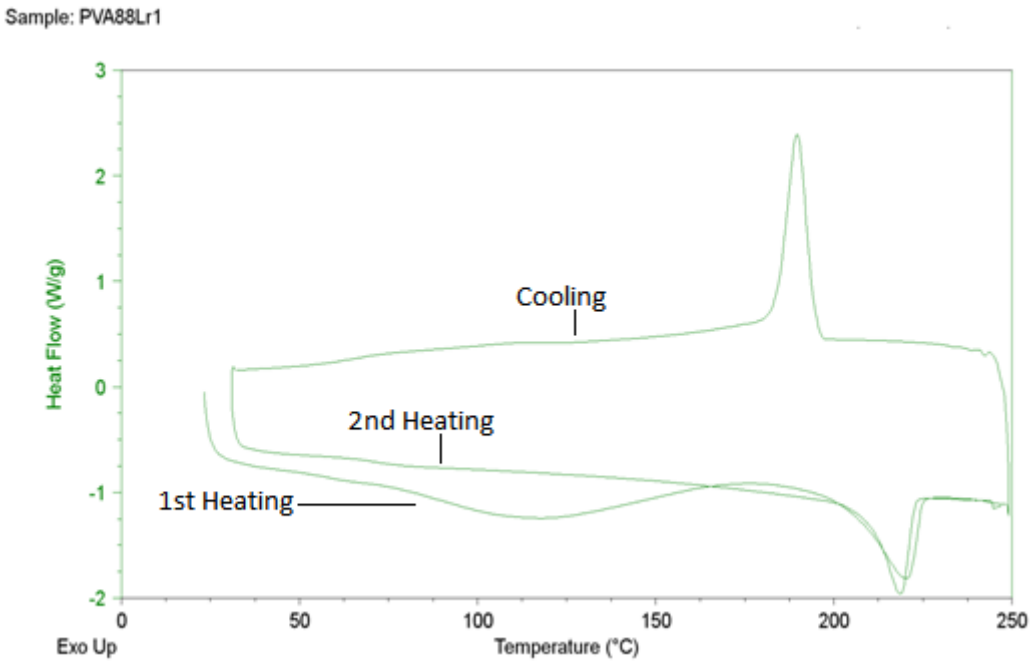


Figure 3.6 Exemplar Dynamic Scanning Calorimetry (DSC) thermograph of a PVA88L film, which was first heated 10°C per minute to 250°C, cooled by 10°C per minute until 35°C and ultimately heated to 250°C using the same ramping conditions.

3.4.5 Polarized light microscopy

In addition to the XRD and DSC measurements undertaken to determine crystallinity quantitatively, certain polymer films were examined using polarized light microscopy. A Leica DM2500 M fitted with polarized filters and a digital camera was used to acquire images of films which were solution cast at ambient room temperature conditions onto 2 different surfaces. A volume of 1 mL 4% PVA solution was pipetted onto both a glass slide and parafilm surface. Films cast on parafilm were detached and imaged on glass slides, as the technique requires light to be transmitted through the sample. Chitosan films were prepared as in section 3.4.3 and having been cast on plastic petri dishes; the films were first detached before measurement. Light passes through an initial polarizing filter before the sample, and through a second polarizer known as the analyzer, after the

sample. The analyzer is aligned to only allow light perpendicular to the vibrational direction of the light that the polarizer allows – no light may pass through both filters when no sample is present. Thus the field of view is completely dark when an isotropic sample such as glass is placed into the light path and contrast is only achieved with an anisotropic specimen.

3.5 Results and discussion

The results from the physical characterisation will be presented and discussed in this section. The results will be discussed in the context of our hypotheses regarding rainfastness of polymers. Further discussion of these results will take place in the subsequent chapters, with the added context of rainfastness results.

3.5.1 Polymer composition, molecular weight and crystallinity

Proton nuclear magnetic resonance spectroscopy (NMR) and gel permeation chromatography (GPC) were used to determine the copolymer compositions and molecular weights of poly(vinyl alcohol-*co*-vinyl acetate) and chitosan. Dynamic scanning calorimetry (DSC) and X-ray diffraction (XRD) were used to estimate the crystallinity of PVA. All of the results are presented in Table 3.3.

Table 3.3 Characteristics of fluorescently labelled and unlabelled polymer samples

Sample ID	Degree of deacetylation (%)		M_n (kDa)	M_w (kDa)	M_z (kDa)	M_p (kDa)	PDI (M_w/ M_n)	Crystallinity (%)	
	Labelled	Un-labelled						DSC	XRD
PVA80	84.7	79.1	N/A	9.0*	N/A	N/A	N/A	10.7	17.7
PVA88 L	91.9	85.3	17.4	20.3	24.6	10.6	1.17	39.2	43.6
PVA88 M	90.6	86.7	12.6	27.7	48.4	20.8	2.19	10.4	19.2
PVA88 H	90.8	86.2	15.0	33.1	56.3	27.8	2.20	10.0	18.1
PVA99 L	98.9	98.8	13.5	21.7	30.3	19.7	1.61	34.3	15.3
PVA99 M	98.8	98.6	32.4	51.3	71.3	59.0	1.58	30.0	28.0
PVA99 H	98.9	98.7	43.1	66.3	92.8	71.0	1.54	27.5	53.2
PVA99 VH	99.4	98.7	65.1	93.2	125.3	96.0	1.43	27.0	43.6
CSL	69.9	70.3	18.2	62.3	137.4	39.2	3.43	N/A	N/A
CSM	72.1	73.9	35.1	124.1	288.6	116.2	3.54	N/A	N/A
CSH	70.1	69.7	53.0	370.0	2414.7	128.0	6.98	N/A	N/A

*manufacturer specification

Calculations based on NMR spectra of both PVA and chitosan returned values for the degree of deacetylation. For all grades of PVA, manufacturer specifications were close to correct. As previously discussed, for the labelled samples, it was

found that carrying out the labelling reaction in 0.05M Na_2CO_3 produced PVA samples which were fully hydrolysed. However, to avoid hydrolysis the reaction procedure was adjusted to that described in section 3.3.1. The adjusted labelling reaction still leads to slight hydrolysis, but it was considered that the increases were insignificant. NMR spectra calculations for chitosan indicated that the degrees of deacetylation were close to those specified by the manufacturer. Chitosan samples were largely unaffected by the labelling procedure, likely due to the reaction not requiring any form of heating which might lead to further deacetylation. The NMR analysis of PVA tacticity showed that there was very little variation between samples (Table 3.4). Therefore, tacticity was no longer taken into consideration as a potential factor affecting the crystallinity, film properties or rainfastness of the PVA samples.

Table 3.4 Fraction of isotactic, heterotactic and syndiotactic triads in PVA as determined by proton NMR.

Sample ID	Triad fraction		
	Isotactic	Heterotactic	Syndiotactic
PVA80	0.203	0.521	0.276
PVA88L	0.203	0.521	0.276
PVA88M	0.204	0.524	0.272
PVA88H	0.202	0.518	0.280
PVA99L	0.215	0.488	0.298
PVA99M	0.217	0.493	0.291
PVA99H	0.217	0.493	0.291
PVA99VH	0.215	0.488	0.298

For the partially hydrolysed range of poly(vinyl alcohol-*co*-vinyl acetate) (PVA80, -88L, -88M and -88H) the molecular weight range was narrower than expected. The GPC determined molecular weight of 20.3 kDa for the 'low' molecular weight PVA was as expected but molecular weights of 27.7 kDa and 33.1 kDa for the 'medium' and 'high' molecular weight samples respectively were lower than expected. For these two samples, polydispersity (PDI) was above 2, indicating that both samples contained a disperse distribution of molecular weight grades. For the fully hydrolysed range of PVA (PVA99L, -99M, -99H, -99VH) calculated molecular weight was in a range between 21.7 to 93.2 kDa. For chitosan the molecular weights determined using pullulan calibration but without Mark-Houwink conversion were in good agreement with manufacturer specifications. Large PDI values are likely due to the biological nature of the polymer.[28]

Crystallinity of PVA films sometimes varied depending on the technique used but there was generally a good agreement between the results of both techniques. There are several reasons why these differences may have occurred. Firstly, during DSC analysis, portions of the polymer films are subjected to two heating cycles before a measurement is made compared to XRD where whole films are measured without any thermal treatment. Another reason is that for DSC, the value for 100% crystalline PVA is required to calculate crystallinity of an unknown sample and the value is taken from literature. Discrepancies in reported values lead to some uncertainty.[29] Consensus lies with values of 158 J/g for fully hydrolysed PVA and 139 J/g for partially hydrolysed PVA.[27], [30]–[32]

Polymers of low molecular weight typically have higher crystallinity. This is observed for PVA88L, which has relatively high crystallinity. This is

compounded by the fact that it is quite monodisperse. A high proportion of the molecules will be of very similar small size, meaning that high crystallinity is quite favourable. PVA88M and -88H show low crystallinity despite relatively low molecular weight. This is likely due to their observed PDI values of greater than 2.0. The PVA-99 range of samples generally showed higher crystallinity than the partially hydrolysed range, despite higher values for molecular weight. This is likely due to the acetate pendant groups of the partially hydrolysed PVA grades disrupting packing of molecules. Without these bulkier pendant groups, the hydroxyl groups of the fully hydrolysed PVA are able to form strong hydrogen bonds between each other (Figure 3.7).

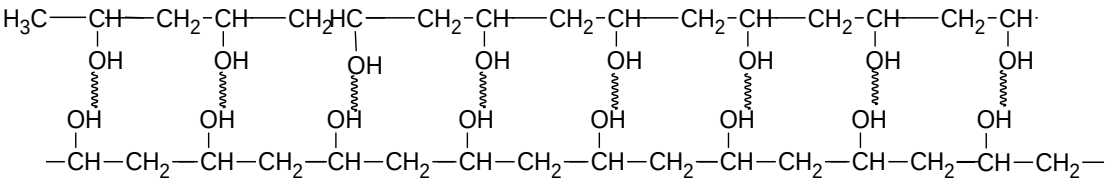


Figure 3.7 Alignment of PVA chains without acetate moieties highlighting the potential for strong intermolecular hydrogen bonding in fully hydrolysed PVA samples.

The crystallinity of chitosan films was not unable to be determined from the WAXS spectra. However, some crystalline character was observed in the same sharp peak for each sample. Generally, the crystallinity of chitosan is proportional to degree of acetylation.[33] As in PVA, a higher proportion of bulky acetyl groups is likely to disrupt formation of organised crystalline regions. As all three chitosan samples were 70% deacetylated, the crystallinity of the samples was likely to be similar as well. Both inter- and intra-molecular hydrogen bonds which encourage order would also be disrupted due to acetate moieties.

3.5.2 Film solubility

Swelling data for solution cast PVA films are presented in Figure 3.8. Each graph represents the change over time in swelling degree of a particular sample in water at 5, 15 and 25 ^{o}C. These temperatures were used in order to approximate different climatic conditions. Positive swelling degrees indicate swelling while values below zero show a loss of mass and therefore dissolution. Some plots show samples dissolve within an hour while some do not dissolve after 24 hours. The effect of temperature is clear. At lower temperatures, samples universally took longer to reach peak swelling degree and took longer to dissolve than those at higher temperatures. This highlights that this small temperature range does have an effect on the polymer dissolution; however, it is not a drastic change. Therefore, only ambient temperature and humidity was used for analysis of rainfastness later in this work. Those polymers which do not dissolve are likely to be rainfast when deposited on leaf surfaces. It could be theorized that the swelling and solubility parameters are most relevant to rainfastness in the first hour as this simulates a dry deposit being subjected to rain

Beyond the effect of temperature, there is a distinct difference in results between films of different molecular weights. All films formed with partially hydrolyzed PVA (PVA80, PVA88L, PVA88M and PVA88H) with molecular weight ranging between 9-33 kDa, dissolve within 20 minutes, while the films of fully hydrolyzed samples (PVA99M, PVA99H and PVA99VH) with molecular weight ranging between 52-93 kDa, do not dissolve even after 24 hours. This difference may be attributed to degree of hydrolysis as well, but the fully hydrolyzed sample of PVA99L with molecular weight of approximately 22 kDa also is seen to break apart, if not dissolve, within 20 minutes.

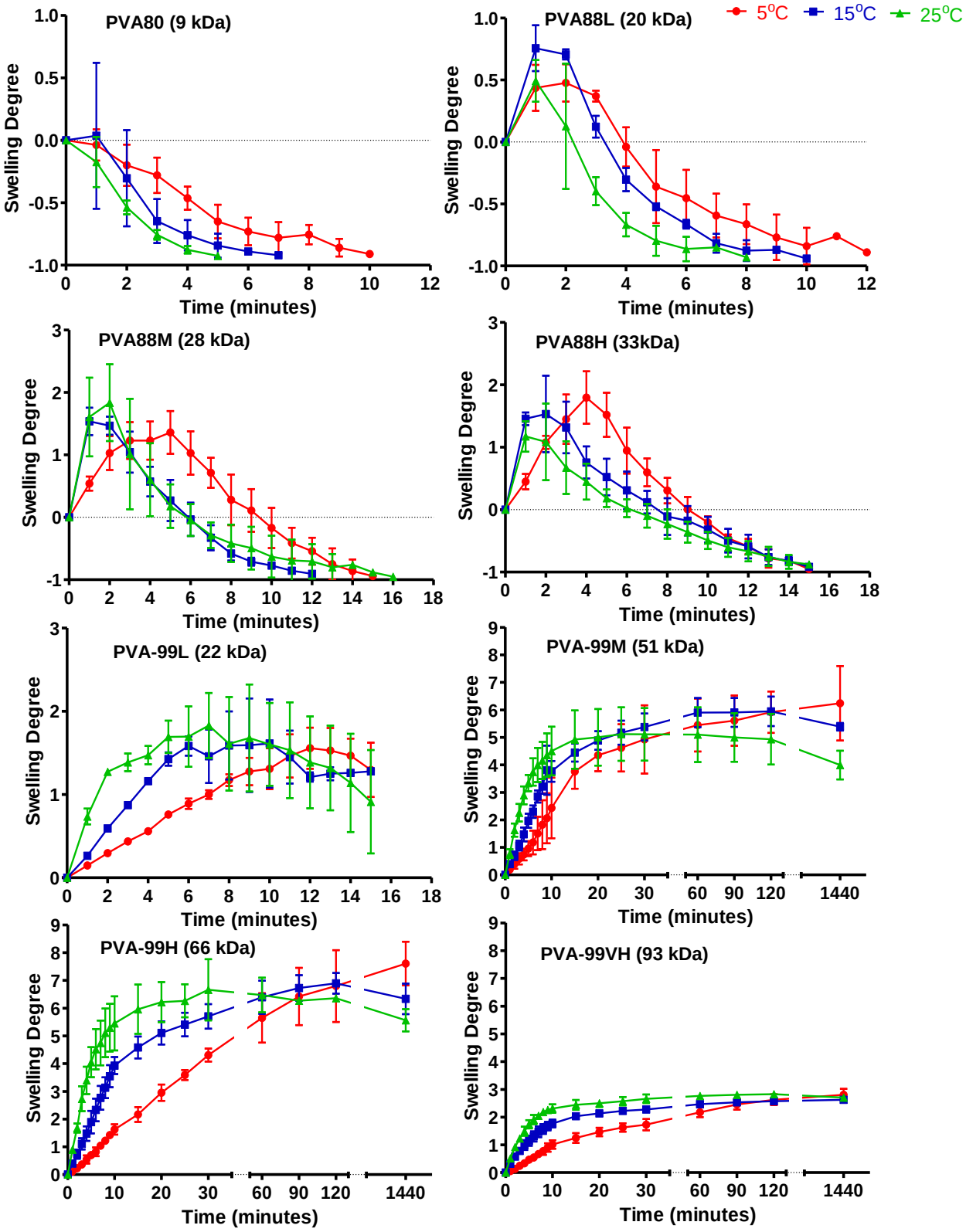


Figure 3.8 Swelling and dissolution of PVA films in water at 5, 15 and 25 °C. Each experiment was performed in triplicate and the data are presented as mean values ± standard deviation.

It was observed that PVA99L dissolved differently to other samples. Instead of a gradual dissolution it begins to mechanically break apart, making the process of weighing the sample much more difficult and a larger degree of error can be seen in the results when compared with others. After 15 minutes, although the sample is not dissolved, it is so broken apart that weighing the sample is not feasible. The anomalous behaviour is not apparent with any partially hydrolyzed samples of a similar molecular weight or any of the fully hydrolyzed samples of higher molecular weight. Therefore this mechanism of dissolution can be attributed to a combination of the low molecular weight and full degree of hydrolysis of the sample.

Priest discussed at length the swelling and dissolution of cast PVA films in water.[34] These discussions highlighted the prevalence of crystalline and amorphous regions of the polymer, with the crystalline regions acting as anchors for the polymer to resist water dissolution. Priest on the conditions of preparation of solid PVA from solution:

When a solid polyvinyl alcohol prepared by evaporation is reintroduced into liquid water at a given temperature, it imbibes water to an extent which depends on the number of cross-linking ordered regions which resist disintegration by hydration at that temperature. The size distribution of regions of crystallization depends on the method of preparation. Thus, it can be predicted that swelling values obtained from films prepared employing different conditions would vary widely.

The general trend of results is that PVA films of high molecular weight and high degree of hydrolysis resist water dissolution most effectively. It was already

discussed that high degree of hydrolysis of PVA is likely to be the driving force behind higher degree of crystallinity as well. However, results for the samples (PVA88L and PVA99L) indicate that a relatively high crystallinity does not prevent dissolution. This could be due to a lack of high molecular weight chain polymers connecting crystalline areas of the polymer and thus water is more easily able to dissolve and break apart any structured polymer regions. The crystalline regions act as an anchor for the polymer and the amorphous regions are able to link these regions together – thus acting as a cross-linked polymer. The fact that PVA99VH (92 kDa) has a much lower maximum swelling degree of approximately 2-3, than PVA99M and PVA99H (50 kDa and 66 kDa respectively and maximum swelling degree of approximately 6-7) shows that at high molecular weight the penetration of water into the bulk polymer becomes more difficult. It is also predicted that there will be some differences between the dissolution of PVA on leaf surfaces and the dissolution/swelling observed for cast films due to differences in size and thickness.

Further investigation of the swelling of high molecular weight PVA was achieved by examining the kinetics of diffusion of water into the polymer. For PVA99VH, the initial diffusion of water into the polymer (at 15 ^{o}C) was shown to be Fickian, as exemplified by linearity in the initial slope of a mass versus square-root of time plot (Figure 3.9). Therefore the diffusion distance is proportional to the square-root of time. However, for the other swelling polymers, PVA99M and 99H, the plot at the same temperature did not show linearity. In fact, the mass change at this temperature was proportional to time. As exemplified by the initial slope in those figures plots from figure 3.8. This type of diffusion has been described as opposed to Fickian, and is a process by which a sharp penetration front is

observed in the polymer which advances at a constant rate – or in other words the diffusion distance is directly proportional to time.[35]

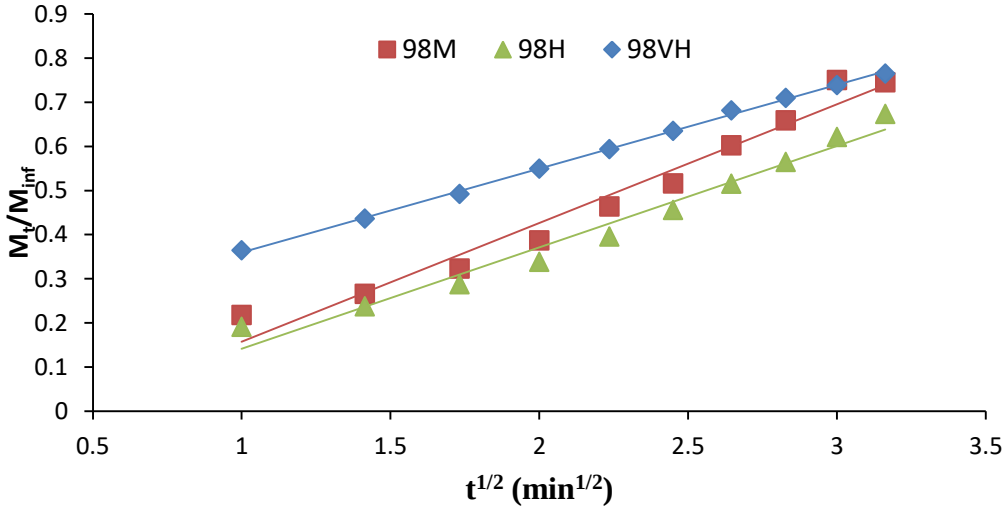


Figure 3.9 Kinetics of mass uptake for the initial swelling of 3 PVA films at 15 °C.

The diffusion coefficient of water into PVA99VH was estimated from the following equation.

$$\ln\left(1-\frac{M_t}{M_\infty}\right)=\ln\left(\frac{8}{\pi^2}\right)-\frac{D\pi^2 t}{4L^2} \qquad (5),$$

Where M_t is mass at time t and M_∞ mass of swollen polymer at equilibrium, D is the diffusion coefficient and L is half of the film width. Based on film thickness of between 80-160 µm the diffusion coefficient of water in PVA99VH at 15 °C was between 1.44-5.75 x10^{-8} cm^2s^{-1}. This value is slightly lower than the range of 1x10^{-5} and 3x10^{-7} cm^2s^{-1} found in literature.[36] This lower value of diffusivity for water swelling into PVA99VH may indicate that the sample will be particularly rainfast on a leaf surface.

The ‘low’ molecular weight chitosan used in this study was of relatively high molecular weight. CSL and CSM of molecular weights 62.3 kDa and 124.1 kDa respectively showed similar behaviour in pH 7 phosphate buffer. They reached swelling degrees comparable to PVA99VH (Figure 3.10). All forms of chitosan were swollen by water very quickly, and in the case of CSL maximum swelling degree was reached after 1 minute. CSH continues to swell steadily over 24 hours. Swelling at lower temperatures would likely reduce the rate of swelling and the maximum swelling degree, and vice versa for higher temperatures, as was the case for PVA. Hydrogen bonds can form between sheets of chitosan via water molecules if the chitosan is hydrated.[37] Potentially, as deposits and films of chitosan are rehydrated by water, they may be stabilised by these hydrogen bonds between sheets.

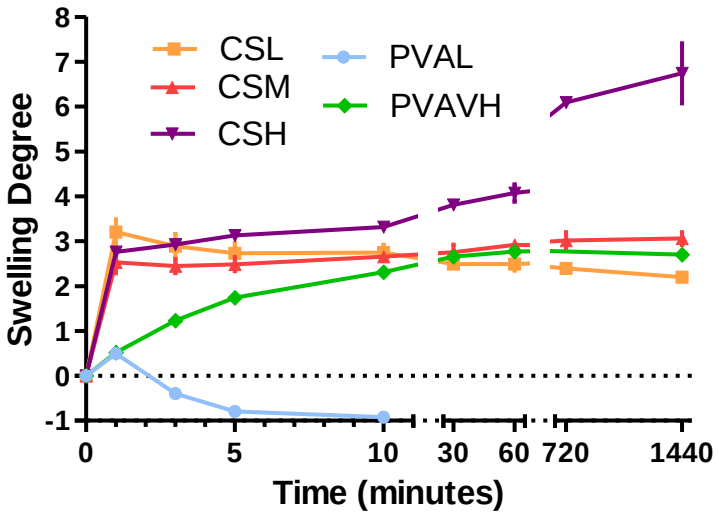


Figure 3.10 Swelling behaviour of chitosan films in pH 7 phosphate buffer at 25 °C. PVA films included for comparison. Data are shown as mean values (n = 3) ± standard deviation.

In the literature, a chitosan sample of 1300 kDa swollen under the same conditions reached a swelling degree of only 0.25 after 1 hour.[38] This may

indicate that as the molecular weight of chitosan increases, the ability of water to penetrate into the polymer bulk phase is reduced. However, this is not observed in our results, which may be as a result of alternative analysis methods, although similar thickness samples were used. In another study, chitosan reached a swelling degree of approximately 5.2 after 2 hours which is in line with our findings, the thickness of samples used was again similar to our experiment.[39]

Chitosan that was placed into a water bath of deionised water rapidly swelled to a much greater extent than described above. Over the course of 24 hours the films break apart and it becomes difficult to measure the change in mass accurately. After 24 hours, CSH in DI water had a swelling degree of ~60 (not shown) compared to ~7 in pH 7 phosphate buffer. Although the pH of the water is 7.7 at the start of the experiment, it rapidly decreases to below pH 6. This is likely due to entrapped acetic acid being released from the chitosan films.

3.5.3 Polarised light microscopy

Images obtained from polarised light microscopy show higher contrast, brighter images when a sample is anisotropic. Figure 3.10 indicates that PVA samples of higher molecular weight show greater anisotropic character than those of low molecular weight, particularly at the edge of the sample. The phenomenon observed at the edge of films is due to the polymer particles being deposited via capillary flow to the edge of the film as the solution evaporates.[40] This well-known ‘coffee ring effect’ causes aggregation of polymer material at the edge of the film, inducing order and thus, anisotropic character. It is evident that, for PVA, greater order is achieved when films are formed from samples of higher molecular weights. It is theorized that the longer polymer chains in these samples are better able to form ordered structures, such as lamellae. The longer and more

ordered chains may lead to better long range order, forming spherulites as the solvent evaporates.

Polarized light microscope images show good agreement with measured crystallinities of the polymer films, with the exception of PVA88L. Low molecular weight polymers did not produce anisotropic films and showed little crystallinity via DSC or WAXS measurements. The exception of PVA88L, with approximately 40% crystallinity via DSC and WAXS, did not produce anisotropic films either. Conversely, high molecular weight PVA with generally higher values of crystallinity did produce anisotropic films. Almost all of the films exhibited differences in anisotropy between different regions of the film. These regions could also be found at the edges of the films. Lack of order in certain regions could be the cause of enhanced water solubility – where these regions offer routes to water penetration into the film. This could be a potential route to losses when deposits are subjected to rain.

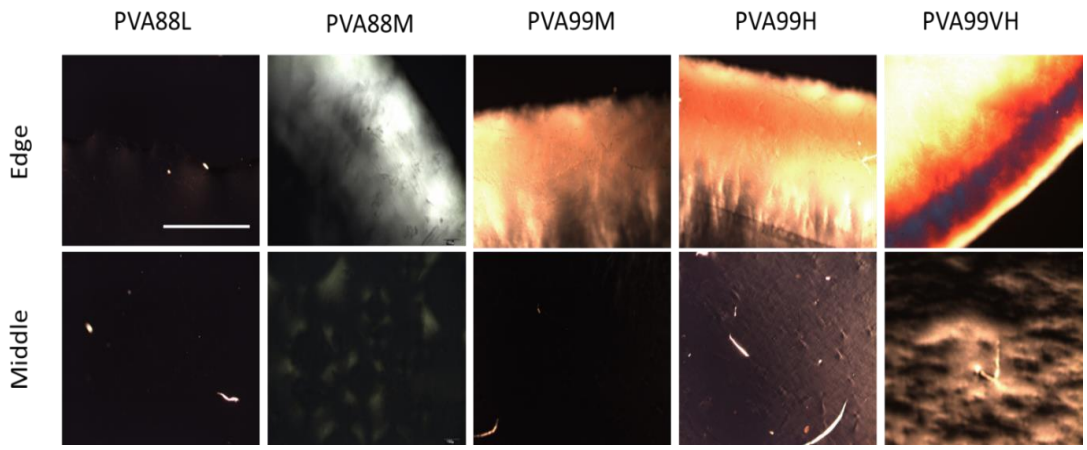


Figure 3.11 Polarized light microscope images from 5 PVA films cast on parafilm but detached and viewed on glass slides; both the edge and middle of the films are shown. Higher contrast indicates a higher degree of anisotropy. Molecular weight increases from left to right and scale bar equals 1 mm.

Chitosan was similarly imaged using polarised light (not shown) and films were observed to exhibit the same behaviour. The anisotropic character of chitosan films increased with molecular weight, and their anisotropic character was similar to the grades of high molecular weight PVA.

3.5 Conclusions

PVA and chitosan have been characterised extensively to determine molecular weight, polymer composition, crystallinity and properties of dried films. This has been achieved with a variety of experimental methods. The discussion focused on how these properties relate, such as how high crystallinity and molecular weight of PVA both contributed to low solubility of PVA99VH films in solution. The following chapters will relate these physical characteristics to rainfastness, with the ultimate aim of understanding which properties of polymers most influence rainfastness in the context of crop protection. To this end, fluorescent labelling of these polymers for use in fluorescent microscopy studies has been outlined in this chapter. Some characterisation of these fluorescently labelled polymers was carried out in order to check that their composition was not greatly affected by the labelling process. It was confirmed by NMR that for PVA and chitosan labelling, which are the main focus of this work, that very little change in composition occurred.

3.6 References

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Chapter 4 Rainfastness of water-soluble polymers: Methods and analysis

4.1 Introduction

The work so far has highlighted the physical properties of poly(vinyl alcohol) (PVA) and chitosan thought to be relevant to rainfastness. PVA, chitosan and a selection of other water soluble polymers were fluorescently labelled for microscopy imaging purposes. This chapter highlights the development of novel methods for determining the rainfastness of deposits from those water-soluble polymers on leaf surfaces. Firstly, a lab scale method was developed using *Vicia faba* leaves and a simple washing method. The method was scaled up to incorporate simulated rain available via a 'raintower' at Syngenta's Jealott's Hill International Research Centre (Bracknell, UK). The discussion in this chapter focuses on how the physical properties of the polymers, also established in the previous chapter, impacted these results. In addition, further characterisation of polymer interactions with a surface are discussed in the context of rainfastness. These characterisations comprised contact angle goniometry and analysis of deposit dimensions on the leaf surface by various forms of microscopy, including cryo-scanning electron and confocal microscopy.

The methods for assessing rainfastness centred on a microscopy technique which involved the sequential imaging and washing of fluorescently labelled polymer deposits on leaf surfaces. ImageJ software was used to quantify these images to produce wash-off profiles which illustrated the rainfastness of each polymer. The difference between the lab and raintower scale methods are discussed, as well as the impact of the washing method on the rainfastness of polymer deposits.

As already highlighted in the literature review, rainfastness is a term to describe how an agrochemical formulation retains during rainfall. To date, rainfastness of polymers has not been extensively studied in the literature. The few existing studies have focused on topics such the effect of rain on copper fungicide wash-off.[1], [2] Other studies have looked at the retention of droplets on leaf surfaces, rather than the retention of already dry deposits.[3]–[7] These studies often focus on the effect of surfactants at improving retention of sprayed droplets.[8] Other environmental factors may also be countered by rainfast adjuvants, for example, a formulation which resists wash-off by rain is likely to resist wash-off by dew. If a deposit retains well on a leaf surface, then this may prevent losses due to physical abrasion by wind or workers walking past plants in the field. This is relevant for the safety of workers and reducing their exposure to potentially harmful materials. However, the focus of this project is rainfall and therefore other conditions like the abrasion have not been studied.

4.2 Developing methods to measure rainfastness

The following section outlines the method for measuring rainfastness using fluorescence microscopy. Real leaves from plants were used to be as accurate as possible. Polymers were fluorescently labelled and characterised (see chapter 3) and all other materials are described in their relevant sections.

4.2.1 Plant surface and materials

Preliminary work went into establishing *Vicia faba* (faba bean or field bean) plants as the main source of leaves for this work. Several plants were considered, including wheat, rapeseed, tomato, banana and soy bean. All are commercially

relevant crops but grown and cultivated in very different conditions.[9] Each offered different surface characteristics but ultimately field bean proved the least challenging to work with. Banana, wheat and rapeseed leaves are extremely hard to wet due to nature of their waxy surfaces.[10], [11] This proved challenging for the application of a regular droplet. Soybean and tomato are less hydrophobic but have an abundance of long trichomes which also make deposition of droplets difficult.[12] Field bean plants are relatively hydrophilic and have a less dense distribution of small trichomes – they proved by far the easiest plant to work with in the following experiments.[13]

Field bean plants used in this work were supplied by Syngenta UK Ltd. The plants were grown under controlled conditions and used at approximately growth stage (GS) 18 – 21, although some plants exhibited signs of entering the flowering growth stage. Growth stage is a term from the BBCH growth scale, a resource which is used to classify the various stages of plant growth (Table 4.1).[14]–[16] GS18 for field bean describes plants towards the end of the leaf development stage where at least 8 leaves are unfolded and GS21 describes the stage of the plant where the first side shoot is detected. Only leaves from leaf position 3 (LP3) were selected to be used. In general, leaf position refers to the position of leaves on the plant, LP1 being the first set of leaves from the bottom of the plant, LP2 being the second set and so on. Each plant pot contained on average 3 plants, each with 2 leaves of LP3. Evidence indicates that surface properties of leaves vary with age and leaf position – wettability of soybean leaves was shown to be affected by growth stage.[17] While wettability of the leaves is unlikely to directly impact on the rainfastness of a dry deposit, it could influence the droplet characteristics on the leaf and thus the dimensions of the dry deposit.

Table 4.1 An excerpt from the BBCH-scale handbook describing the first three growth stages for *Vicia faba*.[15]

Code	Description
	Principle growth stage 0: Germination
GS00	Dry Seed
GS01	Beginning of seed imbibition
…	
	Principal growth stage 1: Leaf development
…	
GS10	Pair of scale leaves visible
GS11	First leaf unfolded
GS12	Two leaves unfolded
GS13	Three leaves unfolded
…	Continuous until
GS19	9 or more leaves unfolded
	Principle growth stage 2: Formation of side shoots
GS20	No side shoots
GS21	Beginning of side shot development: first side shoot detectable
GS22	2 side shoots detectable
GS23	3 side shoots detectable
…	Continuous until
GS29	End of side shoot development: 9 or more side shoots detectable
GS30	**Principal growth stage 3: Stem elongation**
GS40	*Principle growth stage 4: Booting *not observed in *Vicia faba*
GS50	**Principal growth stage 5: Inflorescence emergence**
GS60	**Principal growth stage 6: Flowering**
GS70	**Principal growth stage 7: Development of fruit**
GS80	**Principal growth stage 8: Ripening**
GS90	**Principal growth stage 9: Senescence**
GS99	Harvested product

4.2.2 Washing and imaging methods

Each of the fluorescently labelled polymers (chapter 3) was dissolved in either DI water or 0.2 M acetic acid (chitosan) to a concentration of 0.4% w/w unless otherwise stated. Droplets (0.2 µL) of the labelled polymer solutions were placed on the adaxial leaf surface using a microliter syringe (Gastight fitted with a 0.2 µL dispenser, Hamilton Company) and allowed to dry. The leaves were only detached from the plant stem shortly before measurements were taken in order to maintain their condition. Careful steps were taken to avoid touching or in any way affecting the leaf surface during the whole process.

In the lab scale technique leaves were fixed to glass slide with sticky tape. The deposit was imaged (Figure 4.1) under a fluorescent microscope (Leica MZ10 F, fitted with an 'ET GFP' filter, camera and fibre optic light source) and then washed with 1 mL of DI water so as to imitate rain. The glass slide with leaf attached was clamped at a roughly 45^{o} angle, but this angle was not measured. The 1 mL wash was dispensed dropwise onto the deposit using a burette held directly above. The deposit was then sequentially imaged and washed until it was seen to be removed, or until a change in fluorescent coverage was no longer detected – resulting in a series of images which depict the wash-off behaviour of each PVA sample (Figure 4.2). These profiles usually consisted of 11 images.

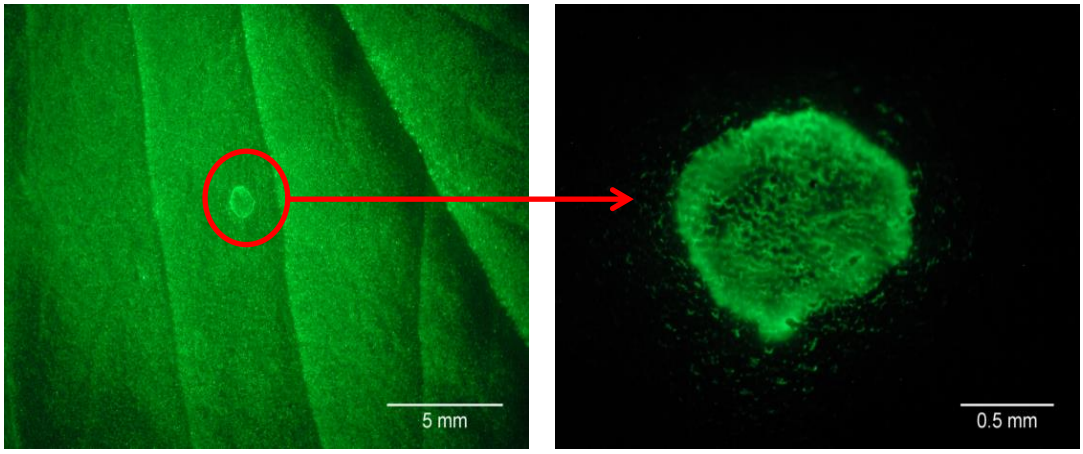

Figure 4.1 Fluorescent microscopy image of the *Vicia faba* leaf surface with a deposit illuminated by white light (left) and a zoomed image of a fluorescing deposit to be analysed by ImageJ software (right).

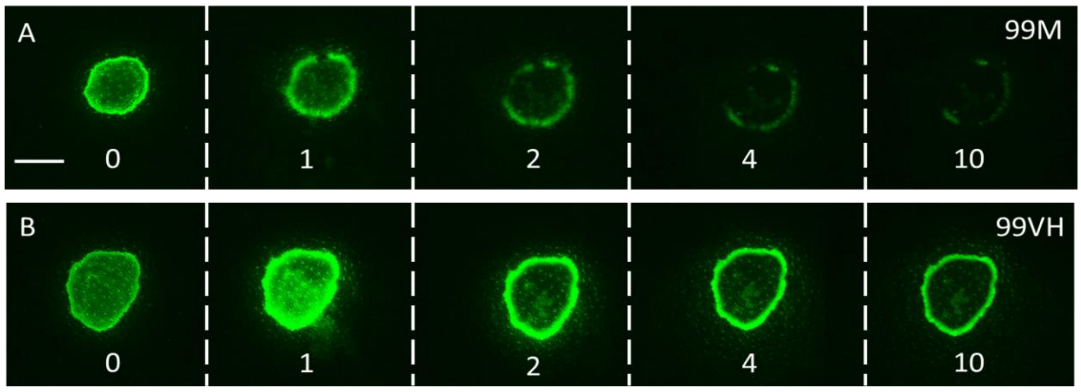

Figure 4.2 Unprocessed images of PVA99M (A) and PVA99VH (B) deposits on *Vicia faba* leaf surfaces at various washing stages. The number indicates the amount of washes of 1 mL DI water, and where 0 washes indicates the initial dry deposit. Scale bar equals 1 mm.

The basic principle of the raintower method is the same as described above, with the main change being the washing method. Leaves were lightly stuck (so as to be easily removed and replaced) to flexible wooden boards. These boards were clamped at an angle and placed on rotating platforms under the rain source. The rotating platforms ensured the leaves were not just subjected to rain in one particular area of the rain path. The rain was achieved by pumping water through nozzles near the ceiling and filtering this rain with shutters. Both the flow rate of the pump and the shutter opening can be adjusted in order to tune the droplet size and intensity of the rain. The result is a system that is capable of mimicking a number of rain conditions. In nature, low rainfall intensities are characterized by small droplet sizes.[18] In order to achieve this, the flow rate of the water must be high, but the shutter opening minimal. Conversely, high intensity natural rain tends to be comprised of large droplets. Therefore the flow rate is kept low and the shutters are opened much more. Two sets of conditions were selected in order to represent rainfall of medium and high intensities. The intensity of 10 mm/h of rain was achieved with approximate flow rate of 2800 L/H water and a shutter

opening of 25 mm. A flow rate of 2300 L/h and shutter opening of 55 mm provided the high intensity rain of 30 mm/h. During experimentation calibrations were made three times per day using graduated rain gauges and adjustments were made to keep the intensities consistent. Instead of a volume of 1 mL of water being used as a wash as at lab scale the deposits were exposed to a timed period of rain, termed a 'rain event'. Both washing methods are illustrated in Figure 4.3.

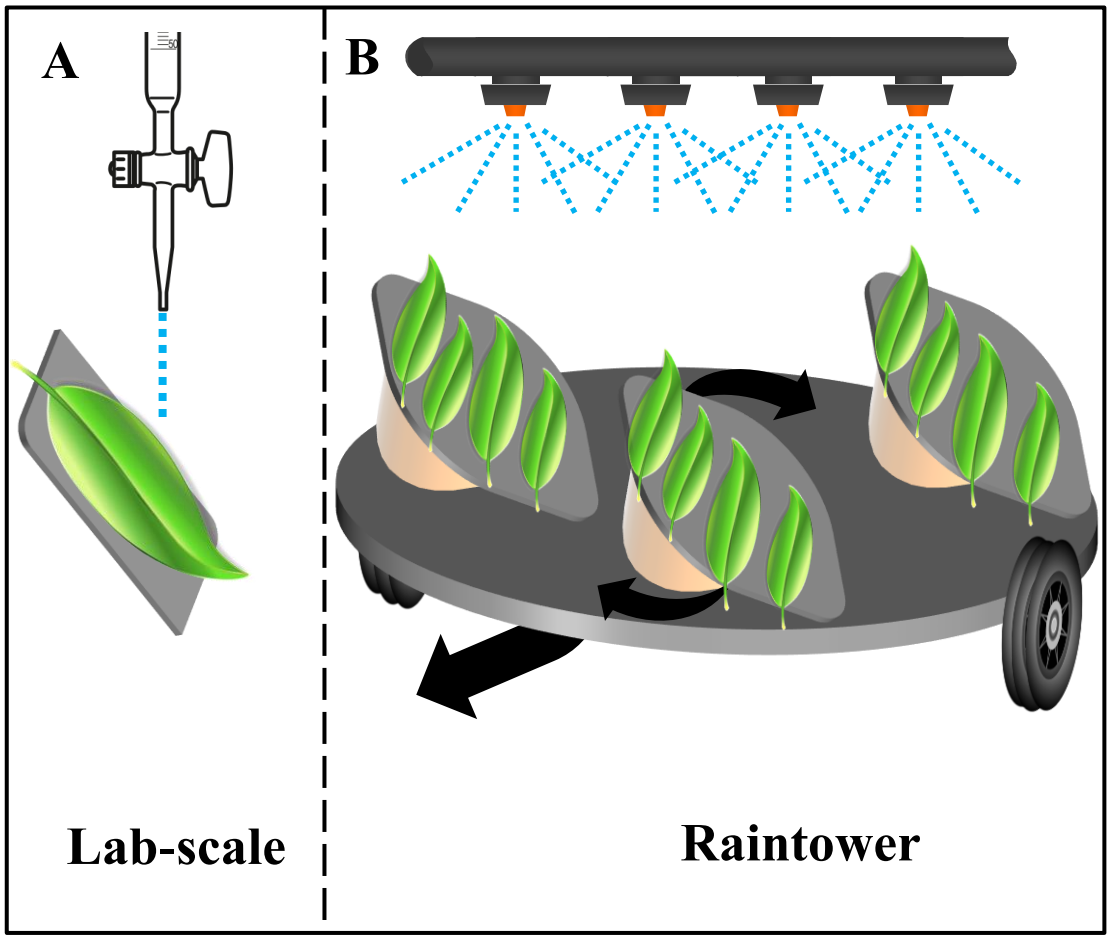

Figure 4.3 Basic illustration of the two washing methods for testing rainfastness of deposits on leaf surfaces.

4.2.3 ImageJ analysis

Sequential imaging and washing builds a series of images which depict the wash-off of a fluorescent deposit from a surface. ImageJ software was used to analyse

the images by determining the coverage of the fluorescent polymer deposit. The software determines if each pixel in the image is over an adjustable brightness threshold and a macro can be written to automate the analysis of a large number of images. The analysis macro also produces a ‘false’ image of the processed deposit. This image indicates with binary colouring which pixels were above and below the threshold brightness. This is one way to check that the software is analysing images properly. The brightness threshold can be adjusted to detect a weakly fluorescing polymer, but should be kept consistent when processing a series of images. The images in a series must be of the same resolution and taken using the same exposure and software settings.

The fluorescence intensity was explored as a way to quantify the exact mass of the polymer on the leaf before and after washing but this was unfruitful. This was due to the fact that the droplets dried into annular deposits via Marangoni flow.[19] This ‘coffee ring’ effect means that the fluorescence in this area is over-saturated which means intensity could not be correlated with the exact quantity of the polymer. Therefore, the fluorescence is used to determine if there is still polymer coverage in an area after a wash. The first image of the dry deposit was taken as the value for ‘100% coverage’ and the subsequent images were quantified as a percentage with regard to the initial dry deposit. Plotting the quantified coverage with the number of washes produces a wash-off profile of each deposit. Exemplary profiles illustrate two samples which retain to different degrees over 10 washes (Figure 4.4).

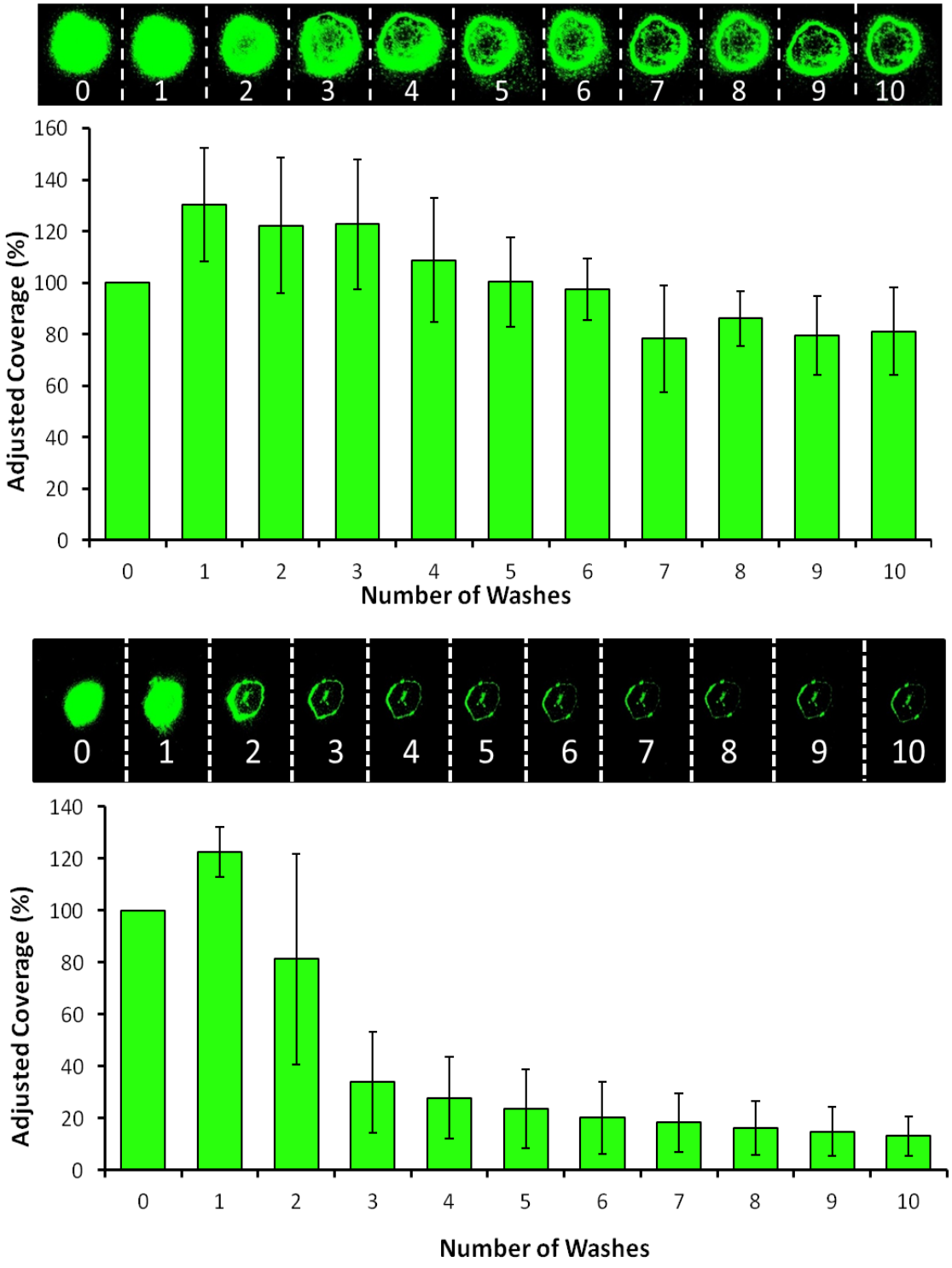

Figure 4.4 Exemplary wash-off profiles of two different fluorescently labelled polymer deposits with corresponding processed 'false' images from the corresponding series. Error bars represent standard deviation, n=3.

4.3 Lab-scale results

Images obtained via the lab-scale washing were analysed using ImageJ and their results are presented in this section. The range of fluorescently labelled polymers is listed in Chapter 3. Results from fluorescently labelled carboxymethyl cellulose, amidated pectin, high methylated pectin, low methylated pectin and dextran are presented in Figure 4.5. These polymers all had significant losses after one wash and therefore only contain one washing result in the graph. Images from these experiments show a complete loss of fluorescence after one wash, with an occasional small region of retention which is subsequently removed on the next wash.

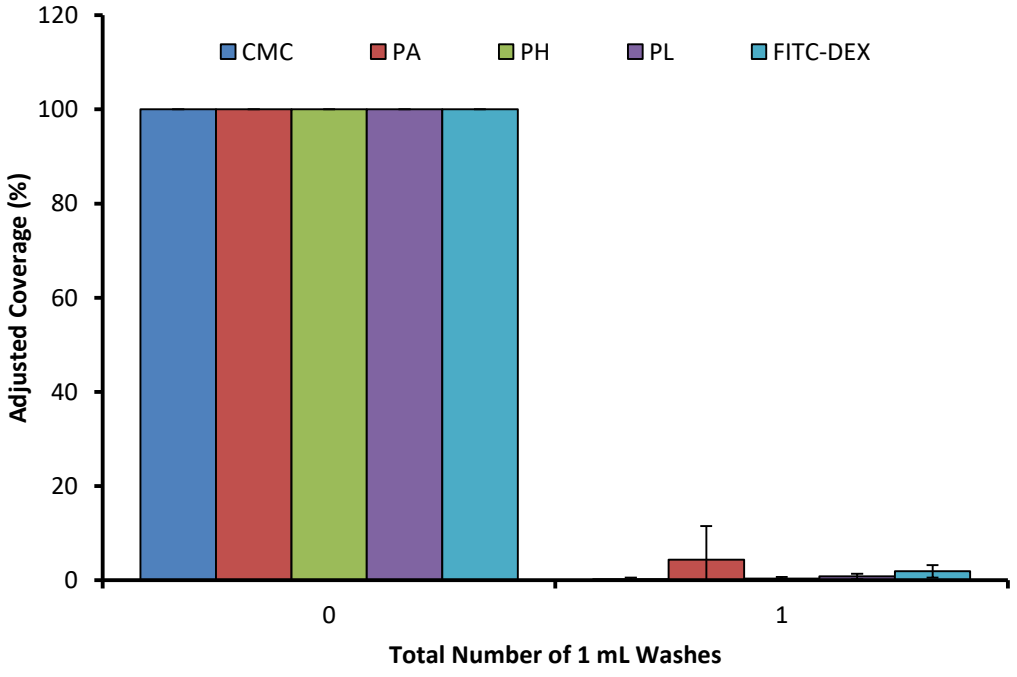

Figure 4.5 Lab-scale wash-off profiles for 5 fluorescently labelled polymer samples – 'carboxymethyl cellulose (CMC), amidated pectin (PA), high methylated pectic (PH), low methylated pectin (PL) and dextran (FITC-DEX). Droplets (0.2 µL, 0.4% w/w) were allowed to dry on leaves and imaged prior to sequential washing (1 mL) and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Mean values are reported (n=3) ± standard deviation.

Results from analysis of all 8 PVA samples are grouped in Figure 4.6. These samples exhibited different behaviour when washed – with low molecular weight and partially hydrolysed grades of PVA being washed off easily. PVA99M, 99H and 99VH were still retained to some degree after 10 washes. Some spreading is observed in initial images after washing, indicated by values of coverage above 100%.

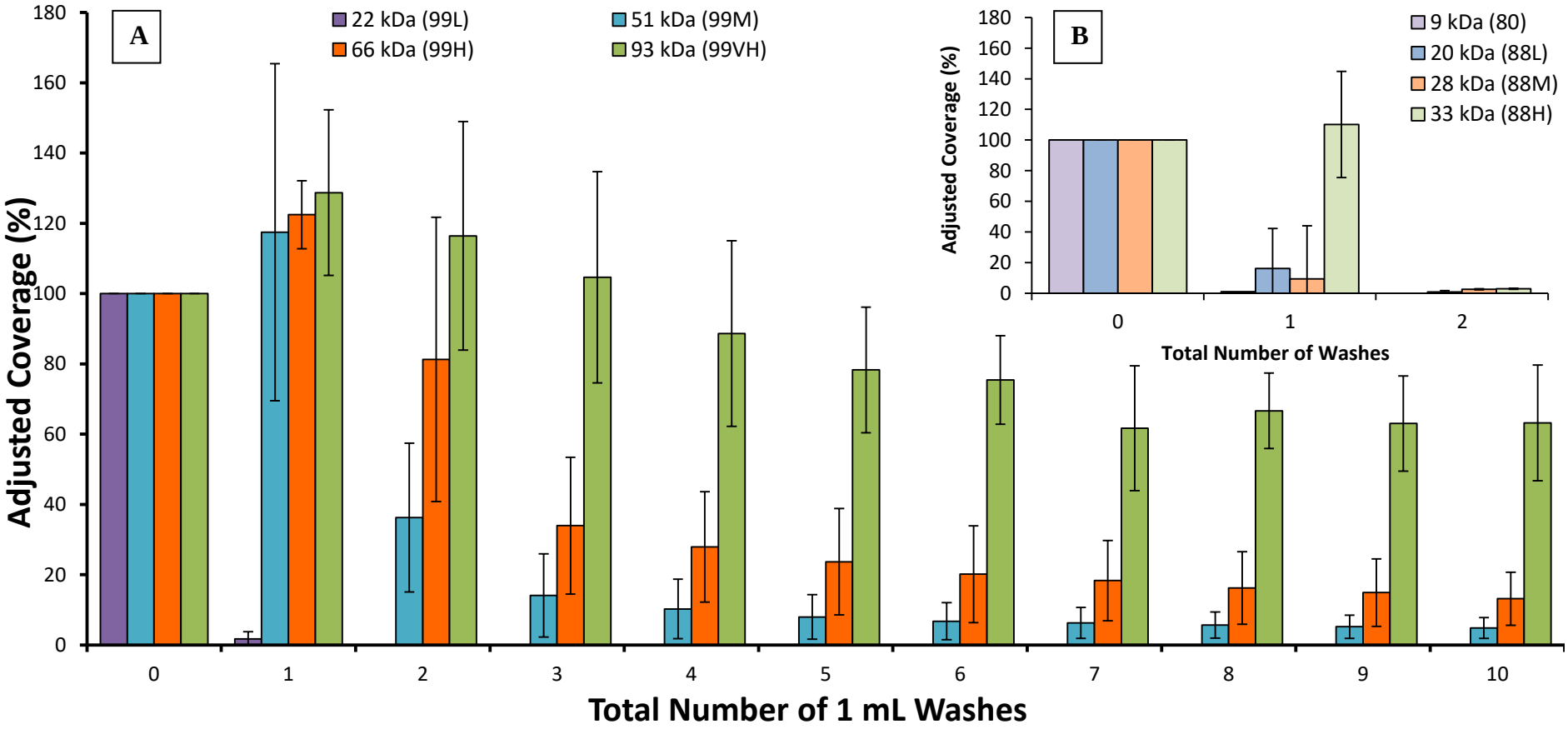

Figure 4.6 Lab-scale wash-off profiles for all 8 fluorescently labelled PVA samples – 'A' showing the 4 fully hydrolysed samples while 'B', inset, shows the 4 partially hydrolysed samples. Droplets (0.2 µL, 0.4% w/w) were allowed to dry on leaves and imaged prior to sequential washing (1 mL) and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

Results from the wash-off of chitosan (Figure 4.7) comprise the final graph presented in this section. Two samples of PVA, from the previous figure, have been included in the results for comparative purposes. All three samples of chitosan retain, even after 10 washes. Initial spreading is minimal compared to that observed in PVA.

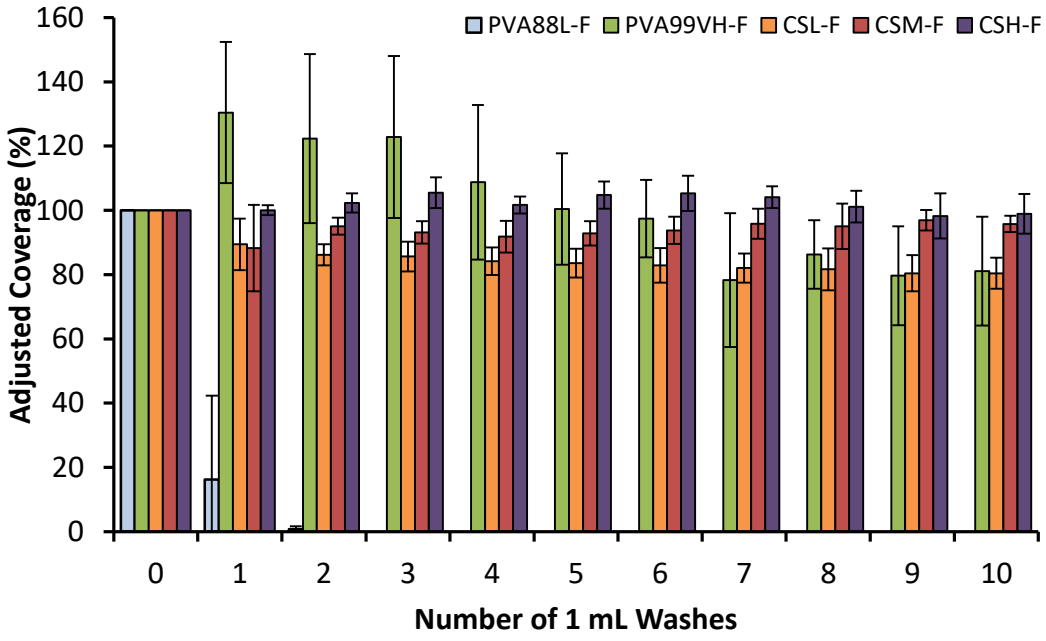

Figure 4.7 Lab-scale wash-off profiles for 5 fluorescently labelled polymers. Droplets of polymer formulations were allowed to dry on leaves and imaged prior to sequential washing and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

4.3.1 Lab-scale discussion

The discussion will focus on the results obtained from PVA and chitosan washes (Figures 4.6 and 4.7), as CMC, PA, PH, PL and FITC-DEX polymers measured did not show retention (Figure 4.5). However, why they did not retain is essentially due to their water solubility. All samples readily dissolved in water and were readily removed upon lab-scale washing. Higher molecular weight grades of

high methylated pectin that require heating to dissolve in water do exist, and these would offer potential as rainfast polymers.[20]

The increase in coverage observed for several PVA samples after the initial washes can be attributed to spreading beyond the initial deposit dimensions. The spreading may be a result of the most water-soluble portions of the deposit beginning to be washed away. Soluble grades of PVA, such as PVA80, 88L, 88M and 99L, do not show spreading at all – they are washed off completely rather than partially. The spreading is noticeable in the captured images, some of which are presented in Figures 4.2 and 4.4. The spreading occurs consistently for those PVA grades of higher molecular weight (PVA99H and –VH) and less consistently for others. For PVA99M – a medium molecular weight – the spreading contributes to large error bars for the initial coverage values. In some captured images the spreading is evident, while in others the spreading has already occurred and the coverage has already been lost. This is an indication of the effect of PVA molecular weight characteristics on PVA wash-off.

The samples with the lowest molecular weights washed off very readily. For the 5 lowest molecular weight PVA samples, almost 100% of coverage is lost by the 2nd or 3rd wash. However, for the 3 remaining samples (PVA99M, PVA99H, and PVA99VH) with the highest molecular weights; significant coverage is retained after up to 10 washes. After approximately 3-5 washes a ‘tenacious’ amount of the deposit was observed that was only gradually removed during the remaining washes. There is a threshold molecular weight in the region of 33-52 kDa over which PVA starts to become more resistant to wash-off. Alternatively this threshold could be described as the molecular weight over which PVA is rainfast. Over this threshold molecular weight rainfastness correlates with molecular

weight. This relationship between molecular weight and coverage is evident at all wash numbers and is plotted for 2 and 10 washes (Figure 4.8).

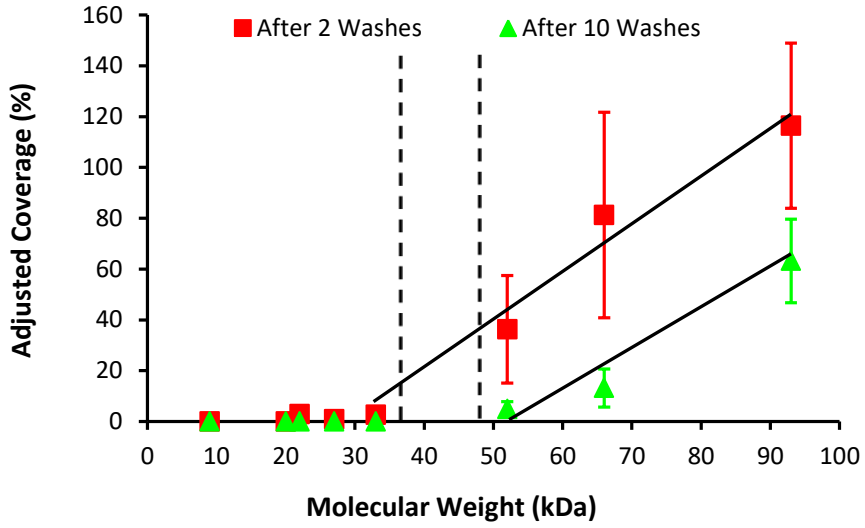

Figure 4.8 Adjusted coverage of fluorescently labelled PVA deposits of different molecular weights on leaves after 2 and 10 washes, the dashed lines indicating the threshold region of molecular over which samples are rainfast.

Lab-scale results for fluorescently labelled chitosan showed that three grades of chitosan (CSL, CSM and CSH) resisted wash-off excellently (Figure 4.7). After 10 washes the coverage of initial deposits changed very little, performing to the same level as high molecular weight PVA. Although supplied as 'low' molecular weight – at 62 kDa the lowest molecular weight chitosan sample tested still exceeds the established PVA threshold. However, this PVA threshold cannot be considered to be particularly relevant to chitosan as chitosan is generally available at much higher molecular weight values.[21] While the differences between samples are less pronounced, the trend in results for chitosan suggests that the molecular weight dependence is still relevant for this class of polymer in the region of 62-370 kDa. A curve for the dependence of rainfastness on molecular

weight is proposed in Figure 4.9. The dashed line is a speculative assessment of the coverage for lower molecular weight grades of chitosan. This is based on the fact that only chitosan of oligomeric size is water-soluble, therefore it is reasonable to expect that even very low molecular weight chitosan would still be rainfast.[22]

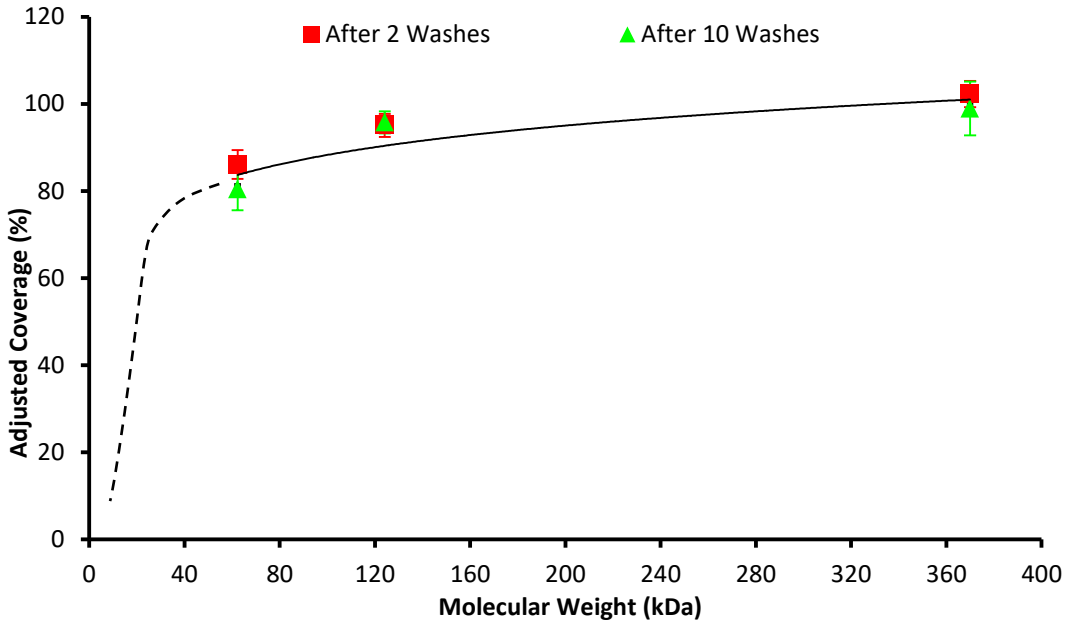

Figure 4.9 Adjusted coverage of fluorescently labelled chitosan deposits of 3 molecular weights on leaves after 2 and 10 washes, the dashed line illustrates potential coverage for lower molecular weights.

4.4 Raintower scale results

Results from raintower analysis of PVA samples washed with rain of 10 mm/h intensity are grouped in Figure 4.10. These samples exhibited behaviour similar to that when washed at the lab-scale – with low molecular weight and partially hydrolysed grades of PVA being washed off easily. PVA99M, 99H and 99VH were still retained to some degree after 10 washes. Some spreading is observed in initial images after washing, indicated by values of coverage above 100%.

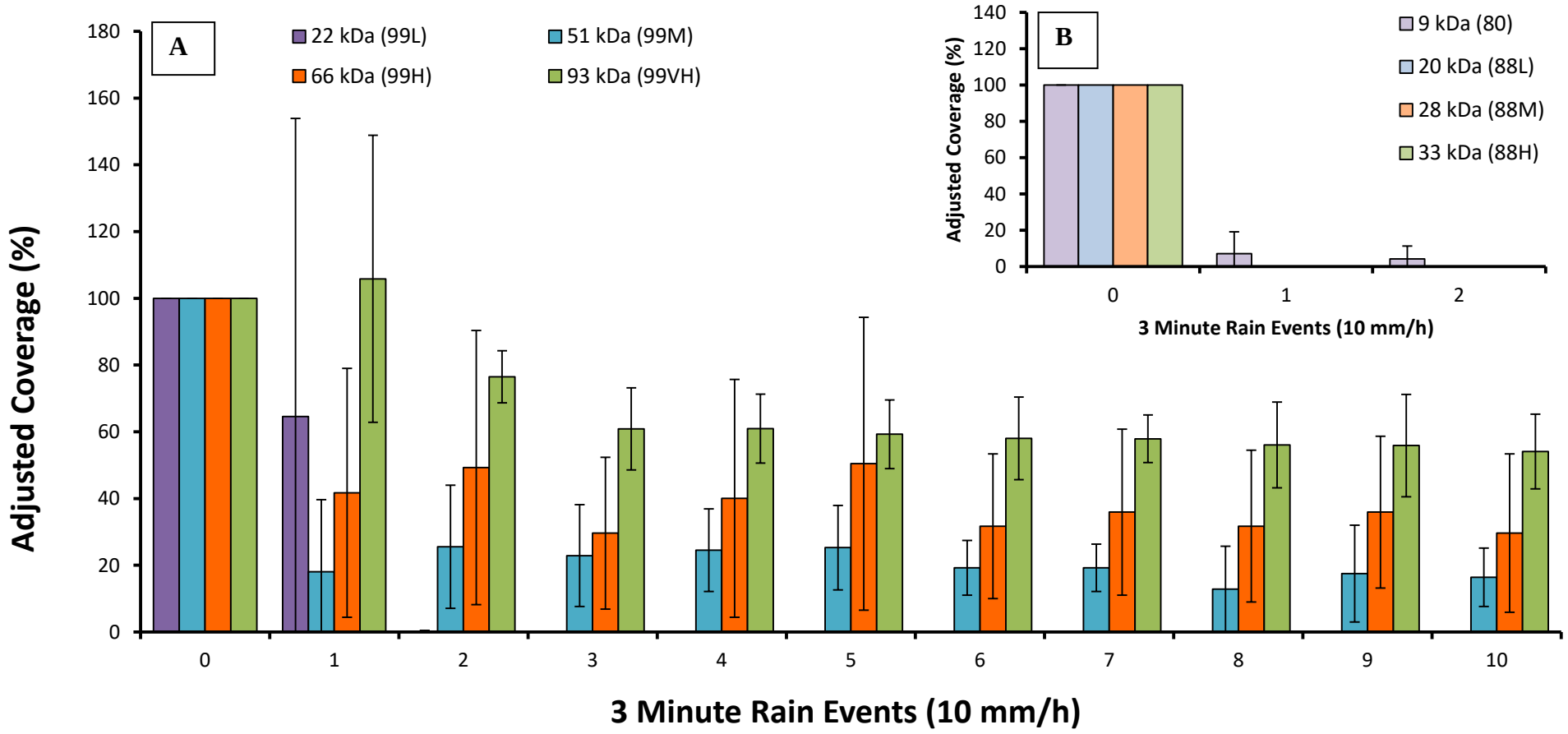

Figure 4.10 Raintower wash-off profiles for all 8 fluorescently labelled PVA samples – 'A' showing the 4 fully hydrolysed samples while 'B', inset, shows the 4 partially hydrolysed samples. Droplets (0.2 µL, 0.4% w/w) were allowed to dry on leaves and imaged prior to sequential rain washes and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

PVA samples that did not show retention were not carried over into the wash-off analysis by high intensity rain washing. Figure 4.11 illustrates the analysis of images from washing PVA88L, 99L, 99M, 99H and 99VH with 30 mm/h intensity rain.

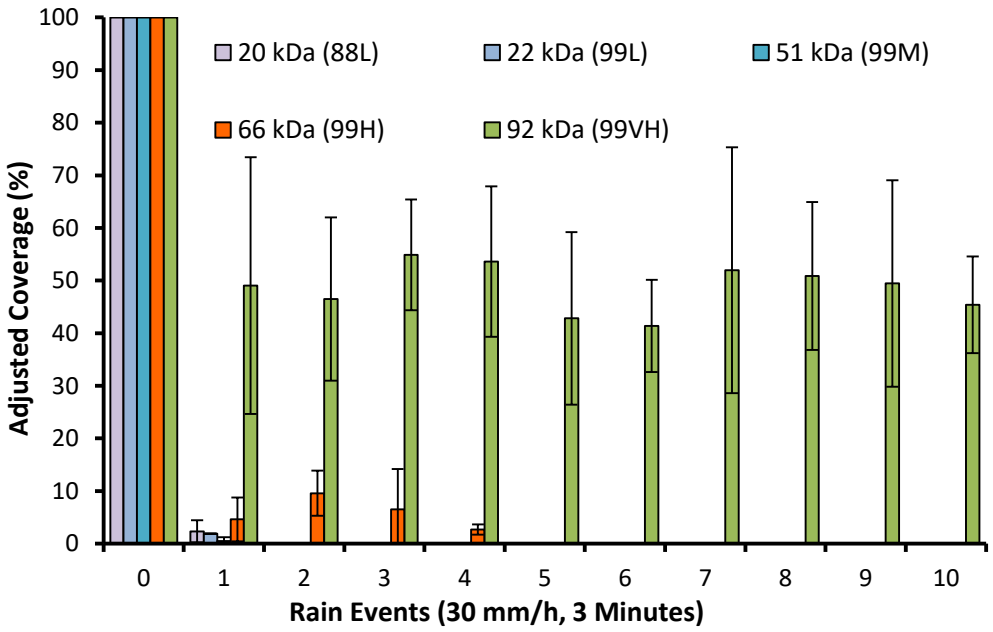

Figure 4.11 Raintower wash-off profiles for 5 selected fluorescently labelled PVA samples. Droplets (0.2 µL, 0.4% w/w) were allowed to dry on leaves and imaged prior to sequential rain washes and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

Results from both rain intensities for low and medium molecular weight chitosan (Figure 4.12) comprise the final graph presented in this section. PVA88L and -99VH, from the previous figure, have been included in the results for comparative purposes.

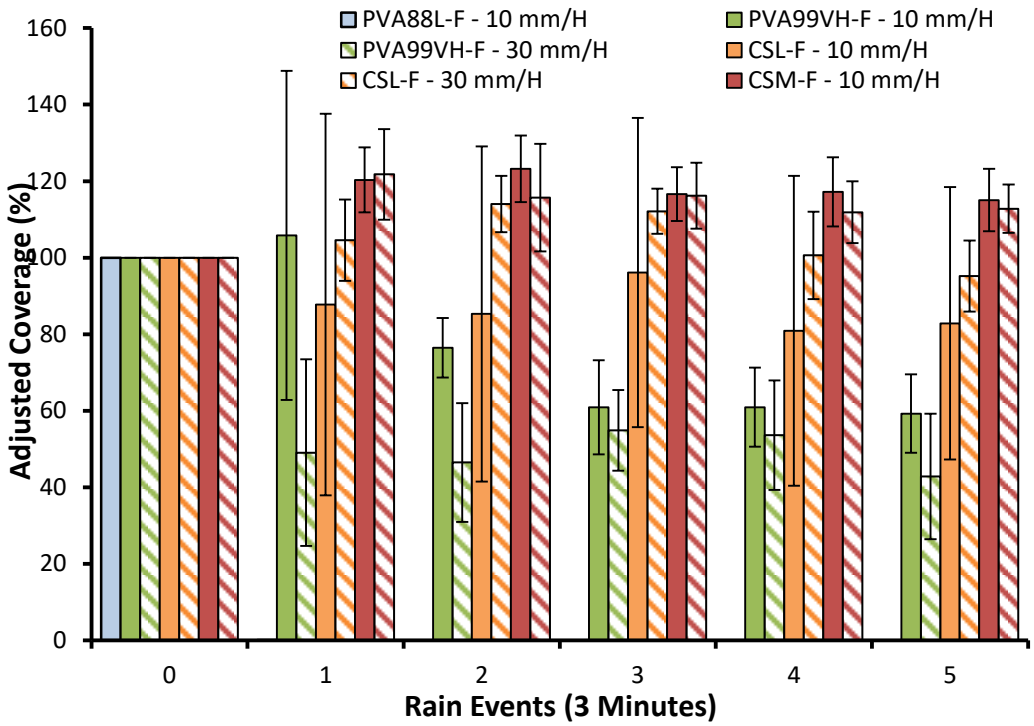

Figure 4.12 Raintower wash-off profiles for 4 fluorescently labelled polymers. Droplets of polymer formulations were allowed to dry on leaves and imaged prior to sequential rain washing and re-imaging. Some polymers were washed with two different rain intensities. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

4.4.1 Raintower Discussion

As described in section 4.2.2, two rain intensities were generated to wash leaves – 10 and 30 mm/h. These rain intensities correspond to moderate and heavy rainfall, respectively.[23] For PVA, results from washing deposits with moderate rain (Figure 4.10) show a similar trend to those results gathered at the lab scale (Figure 4.6). All samples, except the three of highest molecular weight, were washed off by the 2nd or 3rd wash and molecular weight dependence was observed in the three samples that showed retention. Instead of quantifying the coverage against number of distinct 1 mL washes, the experiments have been performed using 3 minute rain events as the washing method. Using this method there was more

variability in the results, as illustrated with larger standard deviation values than at lab-scale, particularly with the sample of PVA99H.

The results reach a plateau level of coverage sooner than they do for lab-scale results and there is almost no spreading observed. While the molecular weight trend is the same between raintower and lab-scale methods, the values of coverage after raintower washing were generally lower than after lab-scale washing. These factors highlight differences in the washing method between the lab-scale and raintower scale experiments. Impacting velocity of raindrops is higher for droplets falling for longer. In the lab-scale method, the droplets fall from a very small distance (< 10 cm) while in the raintower the droplets fall from a height of approximately 10 meters. This suggests that the energy that the droplets impact the leaf and deposit has some effect on their retention.

When increasing the rain intensity to 30 mm/h several samples which showed no resistance to 10 mm/h rain were disregarded. As a consequence, the PVA results comprise 5 samples (Figure 4.11). This heavy rain intensity was able to remove all but one sample of PVA after 5 washes. The best performing PVA was again PVA99VH, which was able to resist wash-off for up to 10 washes. Rain at this higher intensity is comprised of larger droplets, which reach higher terminal velocity than smaller droplets produced from lower intensity rain.[18] Accordingly, the coverage values of PVA99VH deposits are lower than previously measured when washing with low intensity rain or lab-scale washing. Also, more variance in the results is observed indicating that the deposits are not consistently able to retain when subjected to this intensity of rain. This evidence, along with the fact that PVA99M and PVA99H were washed-off readily by the heavy intensity rain, suggests that an important factor for removing the PVA by

washing is the intensity of the rainfall, rather than the volume or rain exposure time.

The chitosan samples (Figure 4.12) both showed excellent ability to retain and exceed the coverage of the most resistant PVA grades. Chitosan with the lowest molecular weight (62 kDa) showed better performance than the best performing PVA sample. Interestingly, the increase in intensity of rain does not make a significant difference in retention of chitosan samples. This perhaps highlights that chitosan deposits are significantly better at retaining on leaves than PVA and that a higher intensity rain may be required to remove chitosan deposits.

4.5 Other characterisation of polymer solutions and deposits on the surface

Some additional characterisation was undertaken beyond those presented in the previous chapter. These observations were made on either the leaf surface or parafilm. Both fluorescently labelled and unlabelled PVA deposits and solutions were characterised on field bean surfaces via methods including scanning electron microscopy and contact angle goniometry. Deposits of labelled PVA were characterised on parafilm. Polymer deposit dimensions are also discussed in this section and the characterised properties are analysed in the context of the polymer rainfastness described above.

4.5.1 Contact angles and deposit dimensions

Static contact angles of droplets of PVA solutions on *Vicia faba* leaf surfaces were characterised with an Attension Theta Lite goniometer. It was expected that PVA of lower molecular weight would more easily wet leaf surfaces, via

decreased surface tension, than PVA of higher molecular weight.[24] It was hypothesised that this property would lead to a larger deposit on the leaf surface as the droplet dried – a larger surface area subject to washing would be one reason for the poor rainfastness of low molecular weight polymers. Although PVA solutions were better able to wet leaf surface better than DI water, there was no significant difference between the grades of PVA (Figure 4.13).

Contact angles between sessile drops and PVA film surfaces were also measured. The fully hydrolysed films were more easily wettable than partially hydrolysed films. Although water has a better affinity for the fully hydrolysed films, likely due to less hydrophobic acetate moieties; this evidently does not have an impact on the rainfastness of deposits on leaf surfaces.

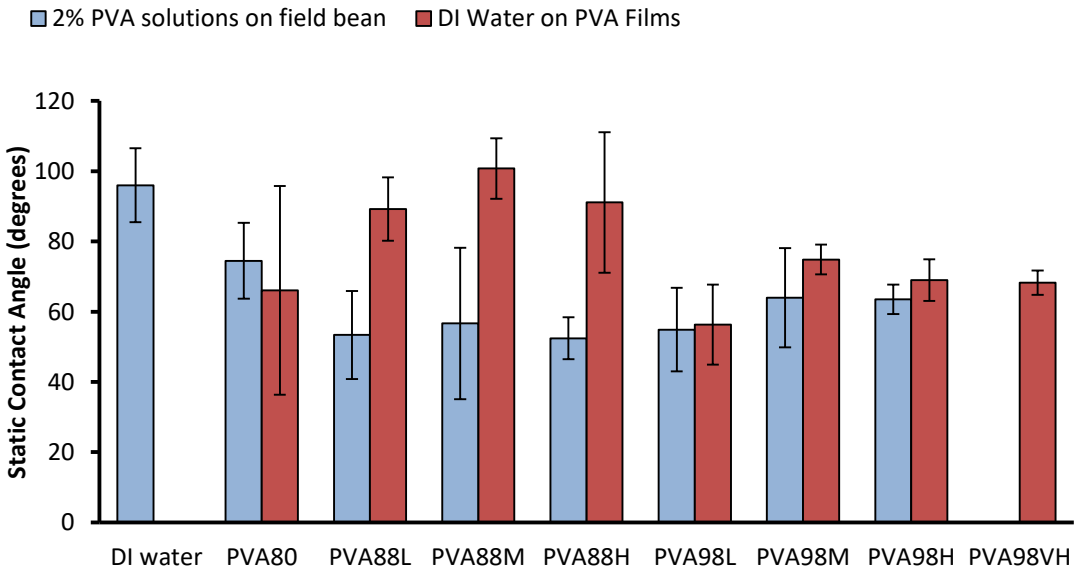

Figure 4.13 Contact angles between sessile droplets of PVA solutions on *Vicia faba* leaf surfaces and DI water on PVA films.

ImageJ was used to examine the dimensions of the deposits on leaf surfaces (Table 4.2). Those deposits measured were those washed at the lab-scale and each

deposit was formed from the drying of a 0.2 µL droplet of 0.4 % w/w polymer. The results show that deposits formed were approximately 1 mm in diameter. As with the contact angle of PVA solutions on leaf surfaces, no trend was found to exist between molecular weight and the size of PVA deposits. It can be concluded that the sizes of the deposits were not greatly influenced by the polymer properties and that the size did not greatly affect rainfastness either.

Table 4.2 Average diameter of fluorescently labelled deposits on *Vicia faba* leaf surfaces determined via ImageJ, where n=3 ± standard deviation.

Sample ID	Average diameter of dry deposits (mm)
PVA80	1.02 ±0.04
PVA88L	1.00 ±0.04
PVA88M	0.95 ±0.10
PVA88H	1.08 ±0.05
PVA99L	1.18 ±0.07
PVA99M	0.99 ±0.05
PVA99H	1.06 ±0.09
PVA99VH	0.95 ±0.06
CSL	1.20 ±0.16
CSM	1.18 ±0.13
CSH	1.11 ±0.07
CMC	1.16 ±0.11
AP	0.91 ±0.01
HMP	0.91 ±0.13
LMP	0.97 ±0.10
FITC-DEX	1.07 ±0.06

4.5.2 Observations from microscopy (cryo-SEM, confocal, fluorescence)

Cryo-SEM micrographs were obtained using a FEI Quanta FEG 600 Scanning Electron Microscope equipped with a Quorum PP2000T cryo-stage. The micrographs highlight the *Vicia faba* leaf architecture and the annulus of an

unlabelled PVA deposit (Figure 4.14). The image shows that the polymer aggregates at the edge of the deposit in an annulus highlighted by the arrows. The annulus is able to cover details of the leaf surface – making them barely visible. Conversely, the leaf area of the inner deposit is only thinly covered by the polymer film and the leaf architecture is still visible.

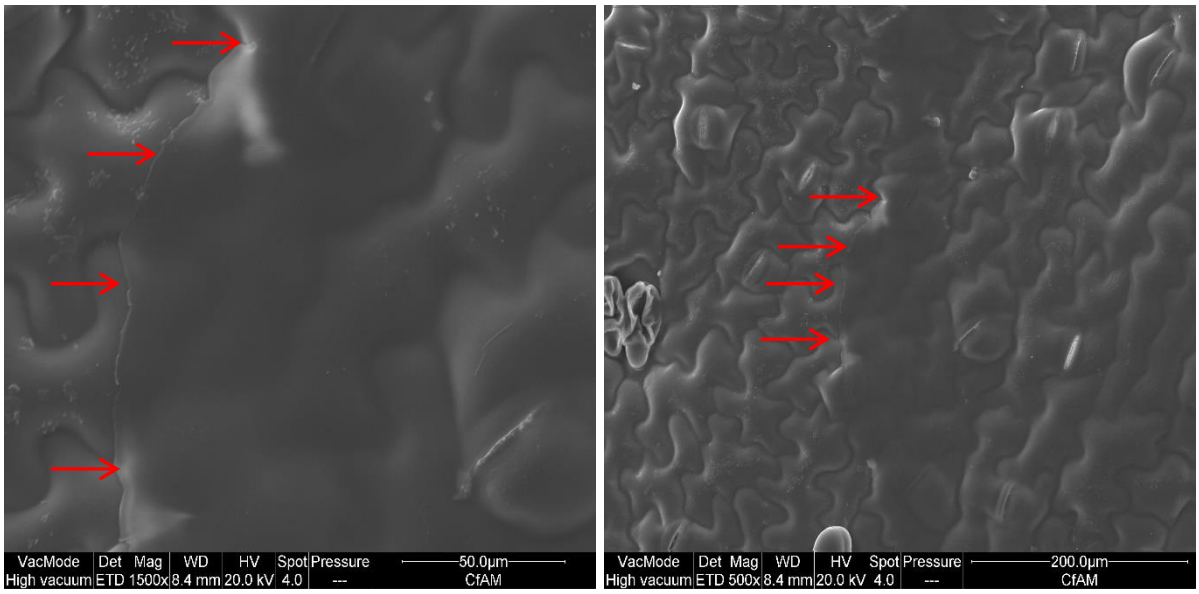

Figure 4.14 Cryo-SEM images of a PVA deposits on a *Vicia faba* leaf surface, red lines indicate the outer edge of the annulus at two different magnifications and they are in the same position on both images.

Further characterisation of the PVA deposits on the surface can be gained by fluorescence microscopy of PVA deposits on a parafilm surface (Figure 4.15). The parafilm surface was explored as an alternative for the leaf – this was of interest as plants of the correct growth stage were not always available and required several weeks to grow. While it is not a bad substitute in terms of hydrophobicity, it lacks the complicated surface architecture of a real leaf. The deposits produced from drying droplets are fairly uniform in dimensions and form the same annulus shape as on the leaf surface. The image shows 3 washes of

labelled PVA99H. The first wash clearly shows the middle of the deposit initially dissolving. The second shows the mostly dissolved middle deposit and the third wash shows that the entire middle of the deposit has been removed, leaving only the annulus. The detail and uniformity of the dissolution available in these images is not observed in the images obtained from leaf washing. The image from the third wash shows a small part of the annulus missing. As discussed in the previous chapter polarised light microscopy showed that certain areas of the edge of PVA or chitosan films could be less anisotropic than others. This could mean that these regions are more susceptible to being washed off than other areas of the annulus, which could be the cause of the losses observed in 3rd wash below.

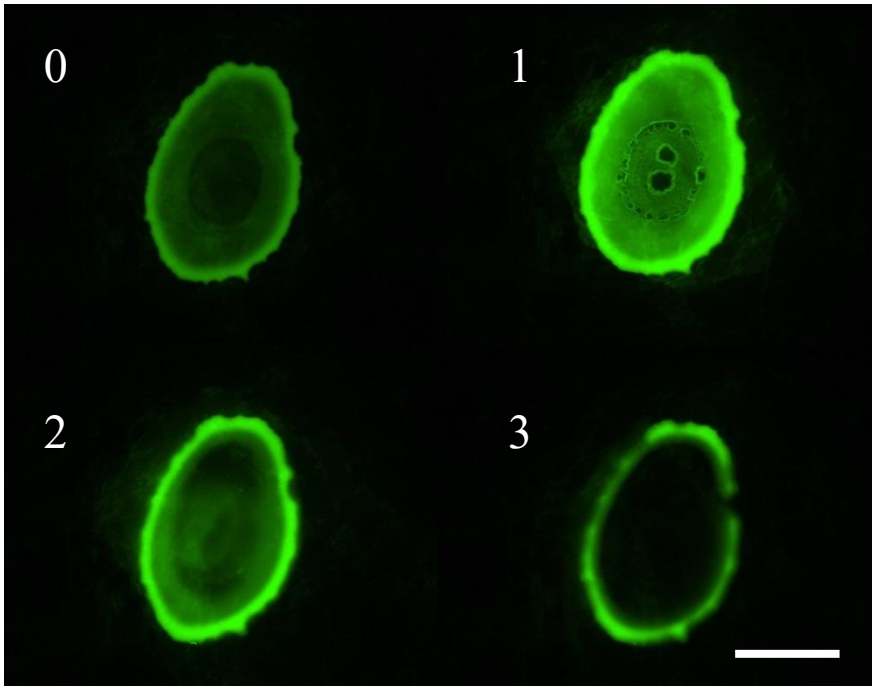

Figure 4.15 A fluorescently labelled PVA deposit being washed off of parafilm, illustrating the gradient in fluorescence between the annulus and centre as well as the mechanism of dissolution from the inside out. Scale bar = 0.5mm.

Confocal microscopy (Nikon A1+ confocal fluorescence microscope) was used to gain further information on the deposit characteristics. Obtained images of a section of a labelled PVA deposit again highlight the concentration of material in

the annulus compared to the rest of the deposit (Figure 4.16). The thickness of the annulus was shown to be approximately 10 µm and there was some evidence that some polymer had penetrated into the leaf from the annulus. From the images, the surface of the leaf can be determined from the top layer of fluorescence.

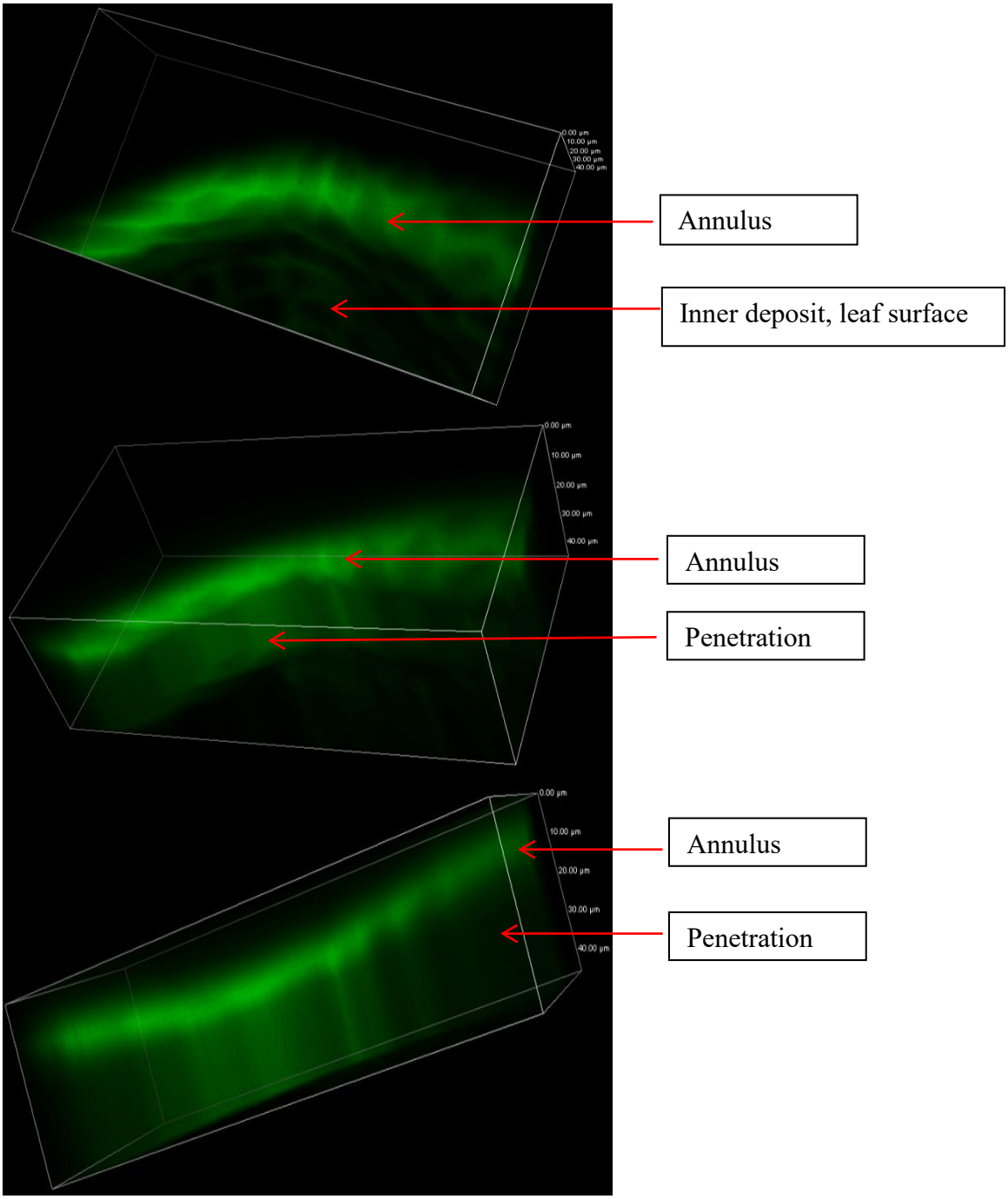

Figure 4.16 Fluorescence confocal images of the same fluorescently labelled deposit from 3 angles, highlighting the depth of annulus fluorescence.

4.6 Further discussion of polymer rainfastness

A large amount of discussion will focus on PVA, as this was the polymer most extensively characterized in this work. Results from swelling of PVA films (figure 3.7) correlate with all PVA deposit wash-off profiles. When compared to films or labelled deposits of PVA99M, PVA99H and PVA99VH; films and labelled deposits of PVA80, PVA88L, PVA88M, PVA88H and PVA99L are much more easily dissolved in water and washed-off of leaves. The fact that the films of PVA99M, PVA99H and especially PVA99VH resist water infiltration to such a degree provides an explanation as to the source of its excellent rainfastness performance. Although partially hydrolyzed samples of PVA contain hydrophobic acetate moieties, the disruption to intra- and intermolecular hydrogen bonding between alcohol moieties reduces water resistance of these samples. The additional acetate moiety also makes the formation of crystalline regions more difficult, thus decreasing water resistance.

Typical polymer dissolution models state that as solvent begins to penetrate the polymer bulk phase, the polymer surface is transformed from a glassy to rubbery state, at which point stress may cause the polymer to crack and break apart.[25], [26] These polymer dissolution models for amorphous polymers state that there are continuous layers between pure polymer and pure solvent where dissolution is driven by solvent diffusing towards the pure polymer, with chain disentanglement occurring towards the pure solvent.[25] The ‘infiltration layer’ consists of the solvent initially entering into normally occurring fissures and holes in the polymer surface. As more water enters, the polymer swells to a greater degree, with the regions of the polymer closest to the solvent eventually becoming a liquid polymer solution. It has previously been reported that polymer films below a

threshold molecular weight do not exhibit the gel layer and are likely to crack apart rather than swell and dissolve, as was observed for PVA99L.[26] It was also reported that films of higher molecular weight swell more. This is not observed in the results of chapter 3, with 93 kDa PVA99VH swelling much less than 51 and 66 kDa PVA99M and PVA99H. This difference is most likely due to a combination of high molecular weight and high crystallinity in this particular sample.

As highlighted by chapter 3 crystallinity results, PVA is a semi-crystalline polymer with a high degree of amorphous character. With the exception of PVA88L, the samples with the best retention after 10 washes and that resisted dissolution by water as films had the highest degrees of crystallinity. As previously discussed, the three samples which retained well on the leaf surface (PVA99M, PVA99H and PVA99VH) during lab scale and low intensity rain washes showed a tenacious amount of coverage which remained almost constant between the 5^{th} and 10^{th} washes. This behavior could be the result of crystalline portions remaining attached to the leaf. The flexible amorphous domains and rigid, insoluble crystalline domains of such samples may combine to provide the ideal properties to resist physical detachment from the leaf during more rigorous washing.

PVA88L has a much higher degree of crystallinity than other partially hydrolyzed samples, comparable to the high molecular weight, fully hydrolyzed samples. It also had an unusually low polydispersity, leading to the assumption that the degree of crystallinity observed was due to the abundance of similarly sized polymer chains being able to form ordered regions in spite of the unfavorable bulky acetate moiety. This suggests that a high degree of crystallinity alone is not

the key factor for rainfastness. Previously it was shown that coverage of PVA deposits does not vary much with continued washings after the second or third wash, either at lab scale or raintower scale. By defining rainfastness as the coverage still on the leaf after second wash, it is possible to compare this quantifiable value with molecular weight and crystallinity (Figure 4.17). This highlights that a combination of high molecular weight and a relatively high degree of crystallinity are key factors for rainfastness performance of PVA.

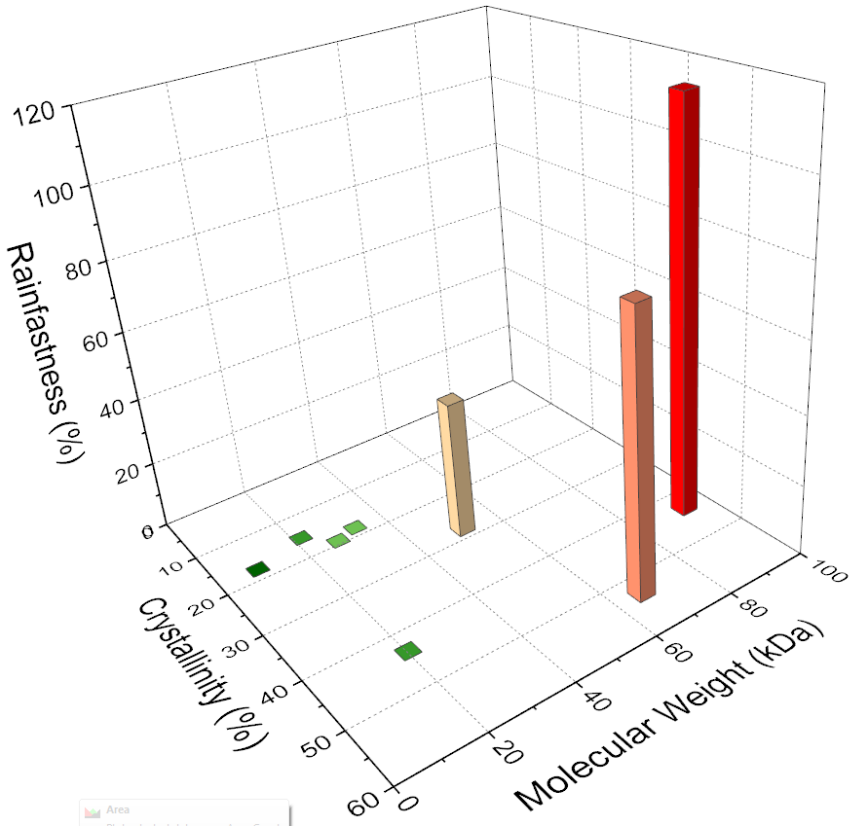

Figure 4.17 Lab-scale rainfastness of fluorescently labelled PVA deposits as a function of polymer molecular weight and crystallinity.

Fully hydrolysed samples of PVA above a threshold molecular weight retained well, while no partially hydrolysed samples above the threshold were tested. Therefore it is difficult to conclude the effects of hydrolysis degree on rainfastness

of PVA. It is likely that higher molecular weight grades of partially hydrolysed PVA will be rainfast due to their lack of solubility in water at ambient conditions. However, due to the decreased crystallinity of partially hydrolysed samples, they are unlikely to show improved retention of fully hydrolysed samples.

The formation of an annulus, or the prevalence of the 'coffee ring affect', occurred for each of the PVA samples. The phenomenon of droplets forming this pattern has been attributed to capillary flow, driven by the droplet evapouration.[27] Interestingly the annulus is less prevalent for chitosan deposits, and in some cases is not obvious at all (Figure 4.18). These samples universally show the greatest rainfastness. In solution, chitosan is protonated and carries a positive charge, which perhaps changes the dynamic of this drying process. It is possible that charged chitosan particles repel and are less likely to aggregate in annular deposits. The fact that these deposits are evenly distributed could potentially explain their improved properties of retention. Another factor to consider is that the charge of chitosan particles could interact with the charge of a leaf surface. Leaves of a different bean species were shown to exhibit a charge under natural conditions, which could be either be positive or negative depending on the conditions.[28]

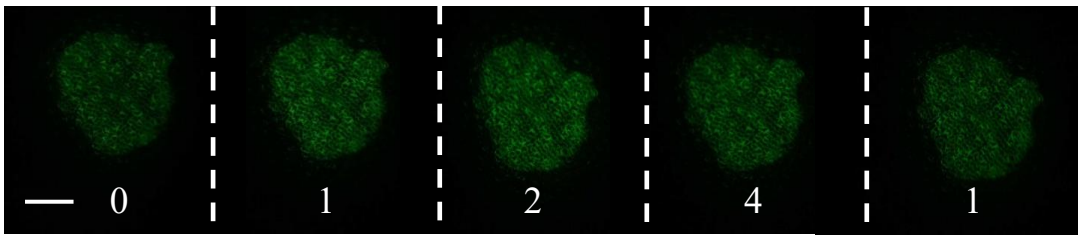

Figure 4.18 Exemplar unprocessed images of a fluorescently labelled chitosan deposit on a *Vicia faba* leaf surface at various washing stages. Scale bar = 0.5 mm.

Chitosan was rainfast in both lab scale and raintower tests. As with PVA the results correlated with the behavior of chitosan films in water, which did not dissolve pH 7 phosphate buffer (Figure 3.8). Unlike with the films in DI water, which tended to break apart, the deposits were very resistant to washing with DI water. Although not shown above, chitosan when washed with acetic acid was removed from leaves immediately. This perhaps indicates that chitosan adjuvants would be much less effective in areas that experience any type of acidic rain.[29] Despite the excellent potential exhibited by chitosan as a rainfast adjuvant, it is limited to use in formulations with a pH < 6. An additional drawback is cost of obtaining chitosan via the relatively expensive process of chitin deacetylation.[30] These issues could pose potential problems for widespread industrial use.[31]

4.7 Conclusions

Novel methods for quantifying the rainfastness of deposits of fluorescently labelled polymers have been established using artificially generated rain and smaller lab-scale washing. The methods can be used to test any fluorescently labelled compounds and could be useful tools towards more intelligent design of rainfast formulations. The raintower method has been a worthwhile validation of the lab-scale washing method and allows the conclusion that the lab-scale method is a good estimation of moderate rain conditions. The methods established enable future studies to measure the performance of other polymers. Moreover, incorporating active ingredients into the experimental design could indicate if the polymers are effective at improving the rainfastness of agrochemical formulations. The existence of a critical molecular weight under which PVA is

not rainfast and over which the rainfast scales with molecular weight has been demonstrated. It has been shown that high molecular weight PVA with a high degree of crystallinity is more difficult to wash off of *Vicia faba* leaves. Chitosan of a moderate molecular weight (62 kDa) is an even better rainfast polymer, usually retaining to a greater degree than the highest molecular weight PVA. The grades of PVA and chitosan that are rainfast all require stimuli to dissolve in water – either acidic conditions or heat. Wash-off results for chitosan and PVA correlate with the behavior of films submerged in water, while polymers which readily dissolve in water at ambient conditions show no rainfastness.

4.8 References

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Chapter 5 Rainfastening of azoxystrobin with water-soluble polymers

5.1 Introduction

The previous chapter established which polymers were rainfast on leaf surfaces, as well as some properties which are likely to be responsible for the rainfastness. This chapter explores the rainfastening effects of those polymers on a real world active ingredient. It was hypothesised that those polymers which retained on leaf surfaces would be able to increase the retention of an active ingredient, however, until this point it has not been tested. We adapt the fluorescence microscopy methods established in the previous chapter to follow the active ingredient on the leaf surface, rather than the polymer. To do this, a fluorescently active pesticide was chosen as the model agrochemical and formulated with unlabelled polymer solutions. The model compound chosen was Azoxystrobin, which is a broad spectrum fungicide.[1]–[5] Its widespread use and fluorescence activity makes it an ideal model agrochemical to develop and evaluate rainfast formulations.

Bond (De Sangosse), commercially marketed as a droplet retention and rainfastness agent, was analysed to act as a commercial control.[6] The performance of Bond would give a further idea of how microscopy analyses of coverage can be compared to the rainfastness. It would also allow comparisons between PVA/chitosan treatments and a commercial adjuvant. Bond is comprised of 45% styrene-butadiene copolymer and 10% alcohol alkoxylate surfactant. An understanding of its mechanism of action can be gained from patent literature.[7] The surfactant stabilises the emulsion of styrene-butadiene particles in water which ‘fuse’ upon drying to form a porous film. This film is able to resist

dissolution by water and improve the retention of any active ingredient formulated with it. Hydrophobic active ingredients may have an affinity for the hydrophobic environment of the surfactant-stabilised latex particles.[8] This is an analogous mechanism to that of the water-soluble polymers being examined in this work, which also form films that are difficult to dissolve. Bond is often included in rainfastness studies in the literature, and comparisons are often made in the context of results achieved using Bond.[9]–[13]

In addition to the microscopy analysis, which is not a 'mass balanced' method for determining the rainfastness of polymers, we employ a mass spectrometry method for determining rainfastness quantitatively. The exact amount of azoxystrobin left on leaves after rain washing was analysed by high performance liquid chromatography tandem mass spectrometry. Microscopy methods were employed alongside this LC-MS/MS method, in order to further validate the methods established in the previous chapter.

LC-MS/MS is a powerful technique able to quantify the concentration of azoxystrobin in complex mixtures which contain contaminants from the leaf.[14] High performance liquid chromatography enables the processing of a large number of samples in a short period of time. In mass spectrometry, the samples are ionised and their mass to charge ratios (m/z) are detected by the analyser. In tandem mass spectrometry (MS/MS), distinct ions based on m/z from the first detection are further ionised into daughter fragments. The daughter fragments can be produced from collision-induced dissociation, higher energy collision dissociation, electron-transfer dissociation and electron-capture dissociation.[15] The fragments are again separated by mass spectrometry and detected using a

second analyser (Figure 5.1). The quantitation of a species, such as azoxystrobin, is made simple as the fragmentations are available in the literature.[16]

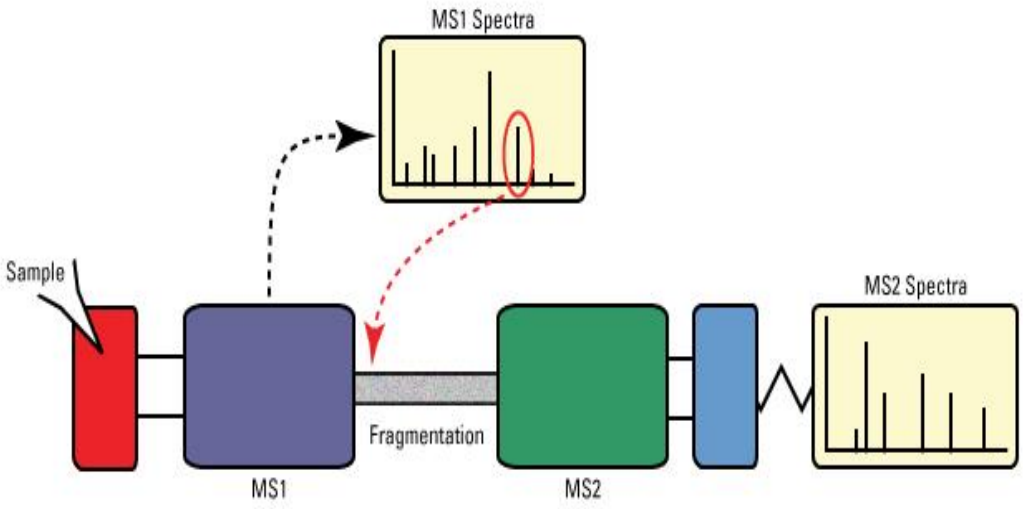

Figure 5.1 Basic schematic of the tandem mass spectrometry method. Figure taken from the web.[17]

5.2 Methods and materials

In the following sections, several formulations were analysed via fluorescent microscopy methods established in chapter 4 and a 'spot and wash' method to be described below. Azoxystrobin is added to solutions of water-soluble polymers and a list of the formulations is available in Table 5.1. Azoxystrobin is insoluble in water and therefore is added as a pre-formulated 'millbase' provided by Syngenta. 50% by weight, the azoxystrobin is milled into particles and suspended in water using xantham gum and a surfactant. Bond (provided by De Sangosse), an adjuvant containing '45% styrene butadiene copolymer and 10% alcohol alkoxylate', was used as a commercial control and similarly formulated with azoxystrobin.

Table 5.1 Details of the polymer-azoxystrobin treatments

Treatment ID	Adjuvant/ polymer details	Concentration of polymer (% w/w)	Concentration of Azoxystrobin (% w/w)
AZ	See materials section above	-	1.0
BOND-AZ	See materials section above	0.15	1.0
CSL-AZ	62 kDa chitosan	0.40	1.0
CSM-AZ	124 kDa chitosan	0.40	1.0
CSH-AZ	370 kDa chitosan	0.40	1.0
PVAL-AZ	20 kDa poly(vinyl alcohol)	0.40	1.0
PVAM-AZ	51.3 kDa poly(vinyl alcohol)	0.40	1.0
PVAH-AZ	66.3 kDa poly(vinyl alcohol)	0.40	1.0
PVAVH-AZ	93 kDa poly(vinyl alcohol)	0.40	1.0

5.2.1 Microscopy analysis of polymer-azoxystrobin formulations

The fluorescent microscopy methods described in chapter 4 were adapted to study the wash-off of azoxystrobin. Both lab-scale and raintower methods were employed to study the fluorescently active fungicide. Instead of using the ImageJ analysis to follow the fluorescence of labelled polymers, it was used to follow the pesticide. The experiments employed *Vicia faba* leaves of the same growth stage and leaf position as described in chapter 4. Bond, a 'spreader/sticker' type adjuvant was used as a commercial control and according to specifications. The manufacturer states that the maximum recommended concentration of the active ingredients should not exceed 0.15% w/w with respect to the active ingredients that make up Bond.

5.2.2 Liquid chromatography tandem mass spectrometry method (spot and wash)

A quantitative measure of azoxystrobin retention on leaf surfaces is possible via a 'spot and wash' method involving liquid chromatography and mass spectrometry analysis. For a typical azoxystrobin treatment a number of leaves were dosed with an azoxystrobin-polymer mixture. On each leaf 10 droplets (0.2 µL) of a treatment with known concentration (Table 5.1) were deposited using a microliter syringe and allowed to dry for a period of time (usually one hour). The 10 droplets were placed apart from each other so that they did not interact or conglomerate. Apart from this, the droplets were placed randomly apart on the adaxial leaf surface.

One set of leaves was washed with acetonitrile without undergoing a rain wash. This was carried out in order to determine the effectiveness of the acetonitrile treatment at recovering the full amount of azoxystrobin on the leaf surface. The azoxystrobin recovery was performed by putting the leaf in a falcon tube with 10 mL of acetonitrile and vigorously shaking the tube. The other sets of leaves were placed under the rain (10 mm/h) for the required amount of time for a rain event. Additional control samples were generated by spiking the same volume of acetonitrile with 10 droplets of an azoxystrobin treatment in order to determine the reliability of the dosage method. The dosage, recovery and generation of control samples are illustrated in Figure 5.2.

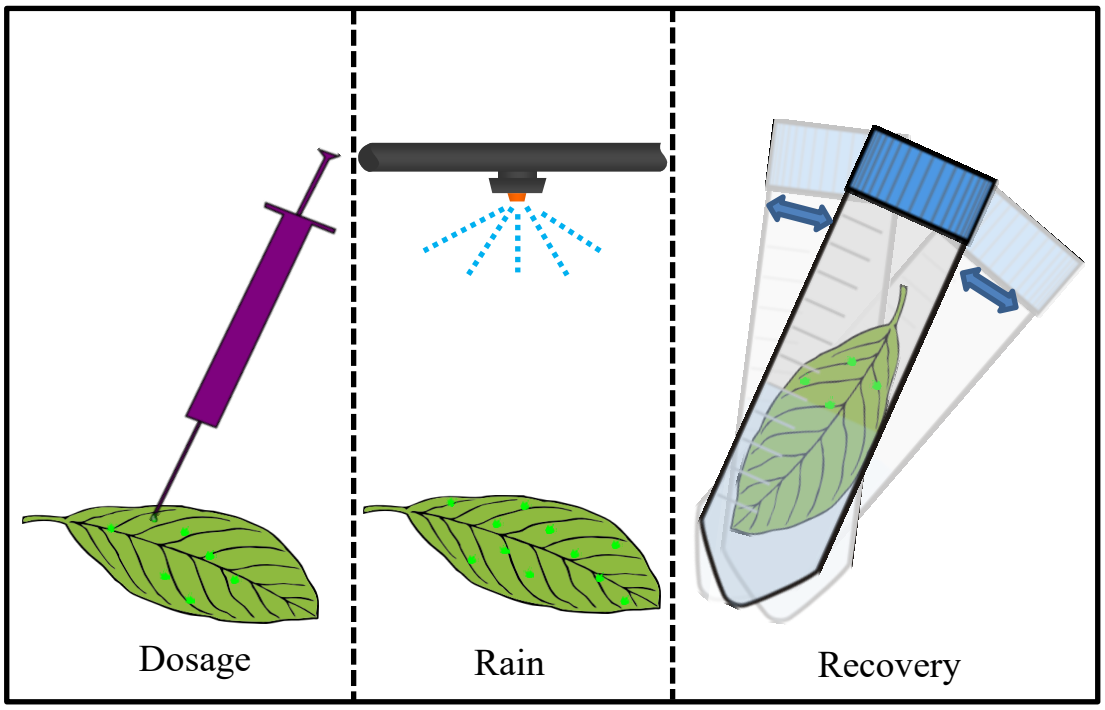

Figure 5.2 Basic illustration of the spot and wash method – the formulations spotted onto leaves are recovered with acetonitrile. Control samples are generated by dosing directly into acetonitrile.

Ultra-high pressure liquid chromatography (UPLC) was carried out using an Agilent Technologies 1290 Infinity instrument. It was fitted with a Thermoscientific triple stage quadrupole Quantum Ultra tandem mass spectrometer detector (LC-MS/MS). The system was running with a pressure of 361 bar using an Acquity UPLC ethylene bridged hybrid C18 column and mobile phase of acetonitrile with 0.2% formic acid. The mass spectrometer detectors were able to analyze the parent ion (m/z = 404.2) and three fragments (m/z = 156.2, 172.2, 183.3). The daughter fragment of m/z = 156.2 was used for the actual quantitation of azoxystrobin, but calibration of the instrument was performed using each fragmentation. Samples were analysed in a random order to eliminate any potential error from drift in the detector. Further to this, several

untreated leaves were washed with acetonitrile and analysed – contaminants from leaf washings were not detected as azoxystrobin fragments.

5.3 Azoxystrobin microscopy analysis: Results and discussion

5.3.1 Lab-scale washing of polymer-azoxystrobin formulations

The wash-off profiles for PVA-azoxystrobin formulations are presented in Figure 5.3. This figure includes the three high molecular weight samples which showed rainfastness alone and a low molecular weight PVA which did not exhibit rainfastness alone. The azoxystrobin was tested alone and when formulated with Bond, a commercial control. As in previous wash-off profiles all samples tested reached a stable level of coverage after 4-5 washes. The azoxystrobin control exhibited a respectable degree of rainfastness alone. This result was not unexpected as azoxystrobin is poorly-soluble in water. The highest molecular weight PVA (92 kDa) and Bond did not significantly increase the rainfastness of azoxystrobin in the lab-scale test.

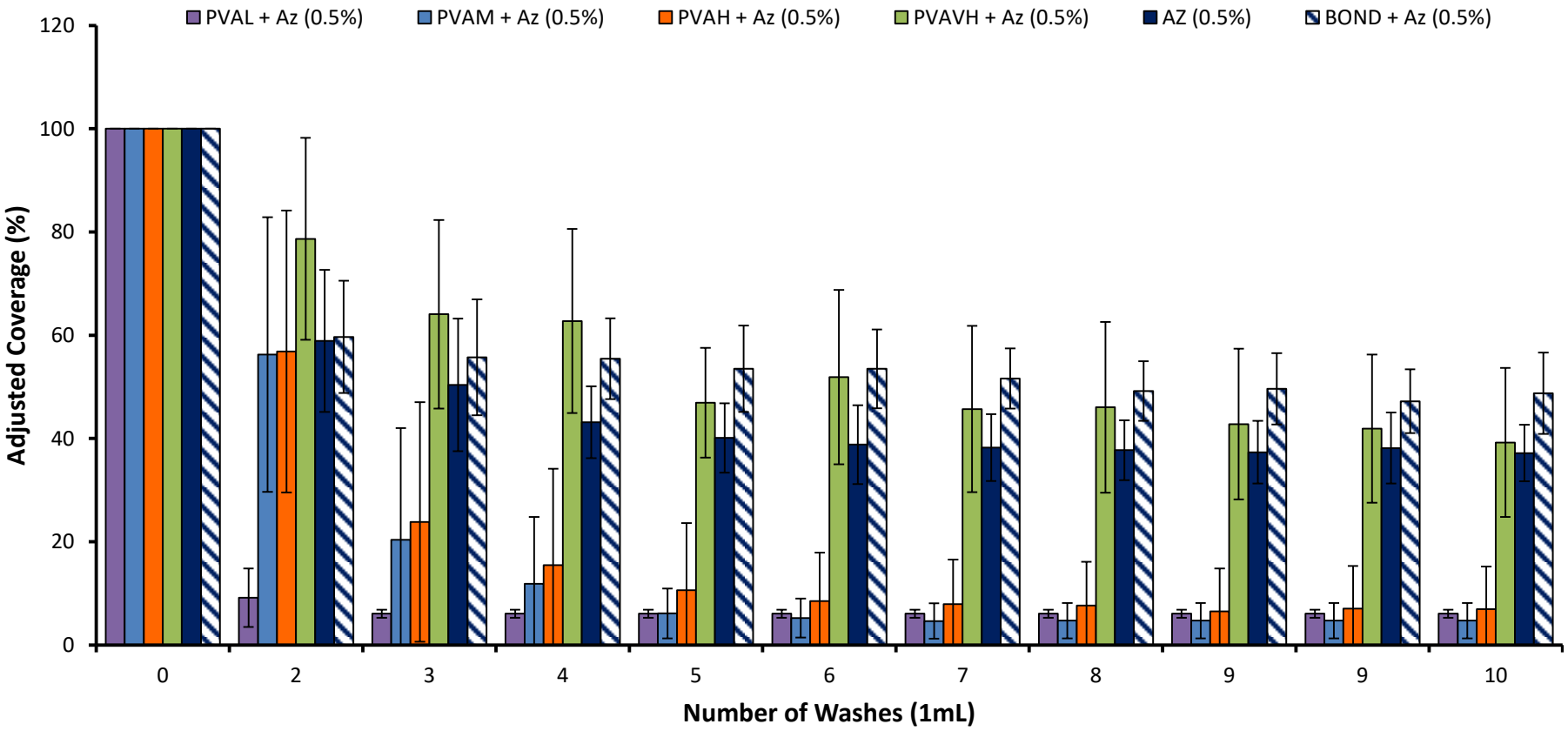

Figure 5.3 Lab-scale wash-off profiles for 6 azoxystrobin formulations. Droplets of treatments were allowed to dry on leaves and imaged to measure fluorescently active azoxystrobin coverage prior to sequential washing and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

Non-rainfast low molecular weight PVA (20 kDa, PVAL) decreased the measured coverage of azoxystrobin compared to the control. Potentially, this is due to the PVAL enhancing the solubility of azoxystrobin. In chapter 4 the contact angles of PVA solutions on leaf surfaces showed that low molecular weight PVA was unable to wet the leaf surface better than high molecular weight PVA. This leads to the conclusion that PVAL is not acting as a surfactant any more than high molecular weight PVA is. Most likely the loss of azoxystrobin retention is from the fact that azoxystrobin aggregates with PVA in the annulus (Figure 5.4). Therefore, when the highly soluble PVA is dissolved and dislodged, the azoxystrobin is removed with it. The figure shows that the low molecular weight PVA forms a more significant annular deposit than the azoxystrobin or bond-azoxystrobin deposit alone. As this grade of PVA does not resist water dissolution well, the azoxystrobin is readily removed along with the PVA.

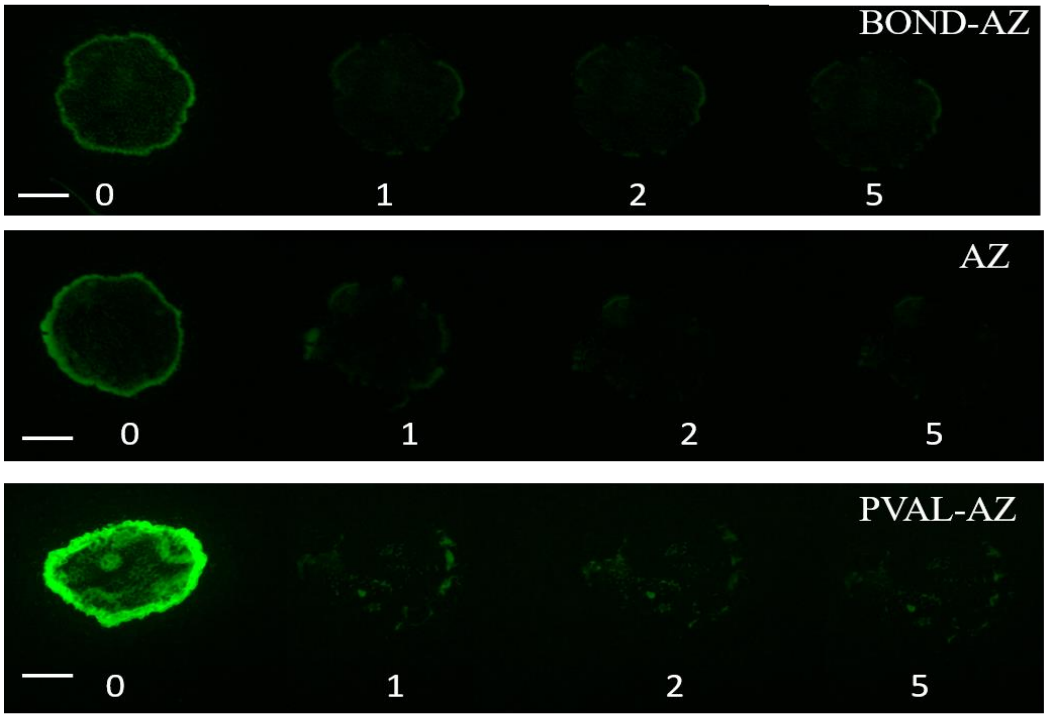

Figure 5. 4 Deposits of azoxystrobin formulations, before and after being exposed to lab scale washings. The scale bar equals 0.5mm.

PVAM and PVAH, which showed some degree of rainfastness alone, also decreased the measured coverage of azoxystrobin. Lab-scale coverage of fluorescently labelled PVAM and PVAH was reduced to 20% or below after 10 washes, so the result from formulation with azoxystrobin was not unexpected. It is likely the reduced coverage compared to the control was caused by the same factors that affect the reduced coverage of azoxystrobin when formulated with PVAL. The fact that PVA forms annular deposits reduces the retention of azoxystrobin unless the PVA is able to resist dissolution very well – as in the case of high molecular weight (PVAVH, 92 kDa).

A chitosan sample was tested using the lab-scale method and results are presented in Figure 5.5, along with the control samples of azoxystrobin and Bond. Two concentrations of medium molecular weight chitosan (124 kDa, CSM) were formulated with azoxystrobin. CSM was able to dramatically improve the retention of azoxystrobin over the control, Bond and the highest molecular weight PVA formulations. Formulating azoxystrobin with 0.4% w/w CSM improved the coverage after 10 washes to approximately 90%. This continues on from results in the previous chapter where fluorescenly labelled chitosan deposits proved to have excellent retention on the leaf surface. Interestingly, reducing the concentration of CSM in the chitosan-azoxystrobin formulation did not reduce the retention by much. Despite a reduction in the concentration tenfold, the coverage of azoxystrobin after 10 washes was only reduced to 75%.

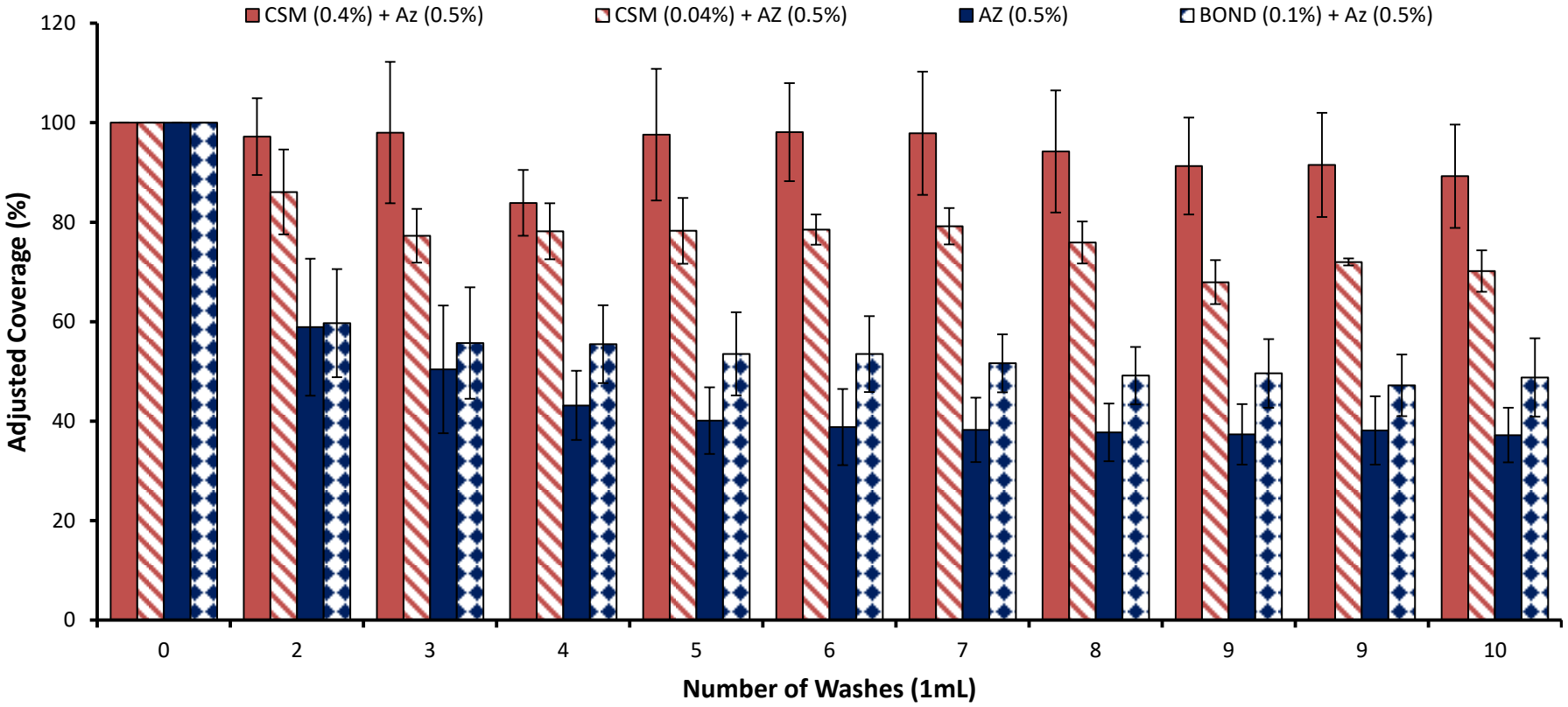

Figure 5.5 Lab scale wash-off profiles for 4 azoxystrobin formulations. Droplets of treatments were allowed to dry on leaves and imaged to measure fluorescently active azoxystrobin coverage prior to sequential washing and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

5.3.2 Raintower washing of polymer-azoxystrobin formulations

Raintower washing was used to examine selected polymer-azoxystrobin formulations and results are presented in Figure 5.6. Non-rainfast PVAL, rainfast PVAVH and CSM were tested as well as the control samples of azoxystrobin and Bond-AZ. Treatments with ten-fold reduced concentration of both PVAVH and CSM formulations were measured in order to further understand the effects of concentration on the adjuvancy of these polymers. Control samples of azoxystrobin and the Bond formulation performed as they did at the lab-scale.

While the formulation containing the lower concentration of CSM performed as it did at the lab-scale, the formulation with the higher concentration of CSM did not significantly improve the coverage over this lower concentration. Another discrepancy between methods was the result of PVAVH, which performed similarly to chitosan in this method, while at the lab scale it was unable to improve retention of azoxystrobin compared to the control. A tenfold lower concentration of PVAVH exhibited drastically reduced rainfastening of azoxystrobin over the higher concentration. Reducing the concentration of PVA in the formulation reduces its effectiveness far more than is the case than with chitosan. Despite discrepancies, the rainfast polymers established in the previous chapter do either enhance or do not hinder the rainfastness of azoxystrobin. Those samples which were proven not to be rainfast in chapter 4 are unable to improve the retention of azoxystrobin, as would be expected from previous results.

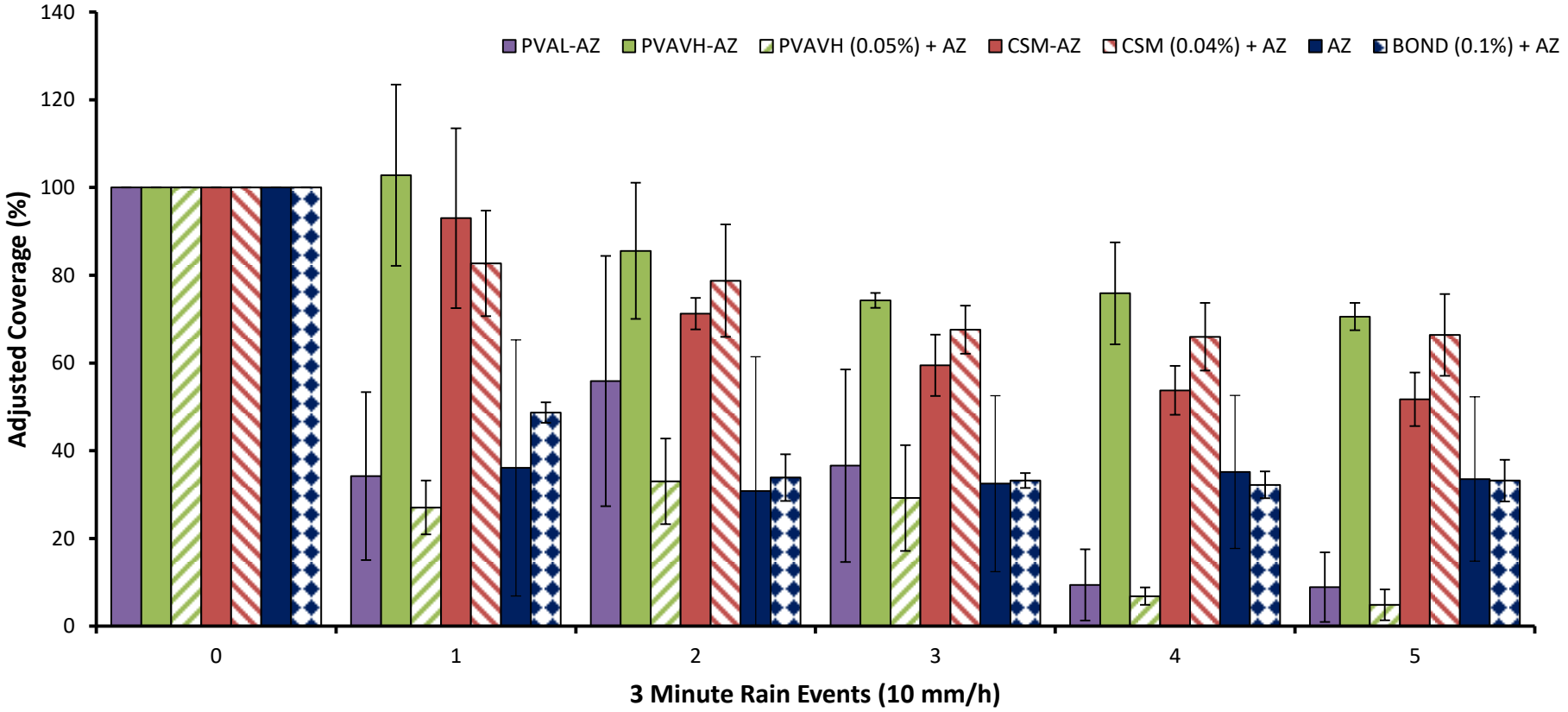

Figure 5.6 Raintower scale wash-off profiles for 4 azoxystrobin formulations. Droplets of treatments were allowed to dry on leaves and imaged to measure fluorescently active azoxystrobin coverage prior to sequential washing and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

5.4 Quantifying azoxystrobin on a leaf surface with liquid chromatography mass spectrometry ('spot and wash')

This method was undertaken in order to be a quantitative analysis of the exact loss of azoxystrobin from the leaf surface after washing. Therefore, the mass spectrometry instrument was carefully calibrated to ensure the confidence of the obtained values. Calibration standards of azoxystrobin in acetonitrile were prepared and measured starting with the lowest concentration. Concentrations of 0.1 and 5 ppm were measured in triplicate and the calibration standards were measured before and after a series of unknown samples. There were no discrepancies between the calibration curve measured before and after the unknown samples – ensuring no detector drift. The unknown samples were also measured in a randomised order. The instrument detects a signal from 4 azoxystrobin transitions – the parent ion and three daughter fragmentations. A calibration curve was established for each fragmentation (Figure 5.7) and m/z = 156.2 was used to quantify azoxystrobin concentration. Untreated leaves were washed with acetonitrile to generate leaf washings which were measured in order to check that no contaminants were analysed as azoxystrobin fragments during LC-MS/MS.

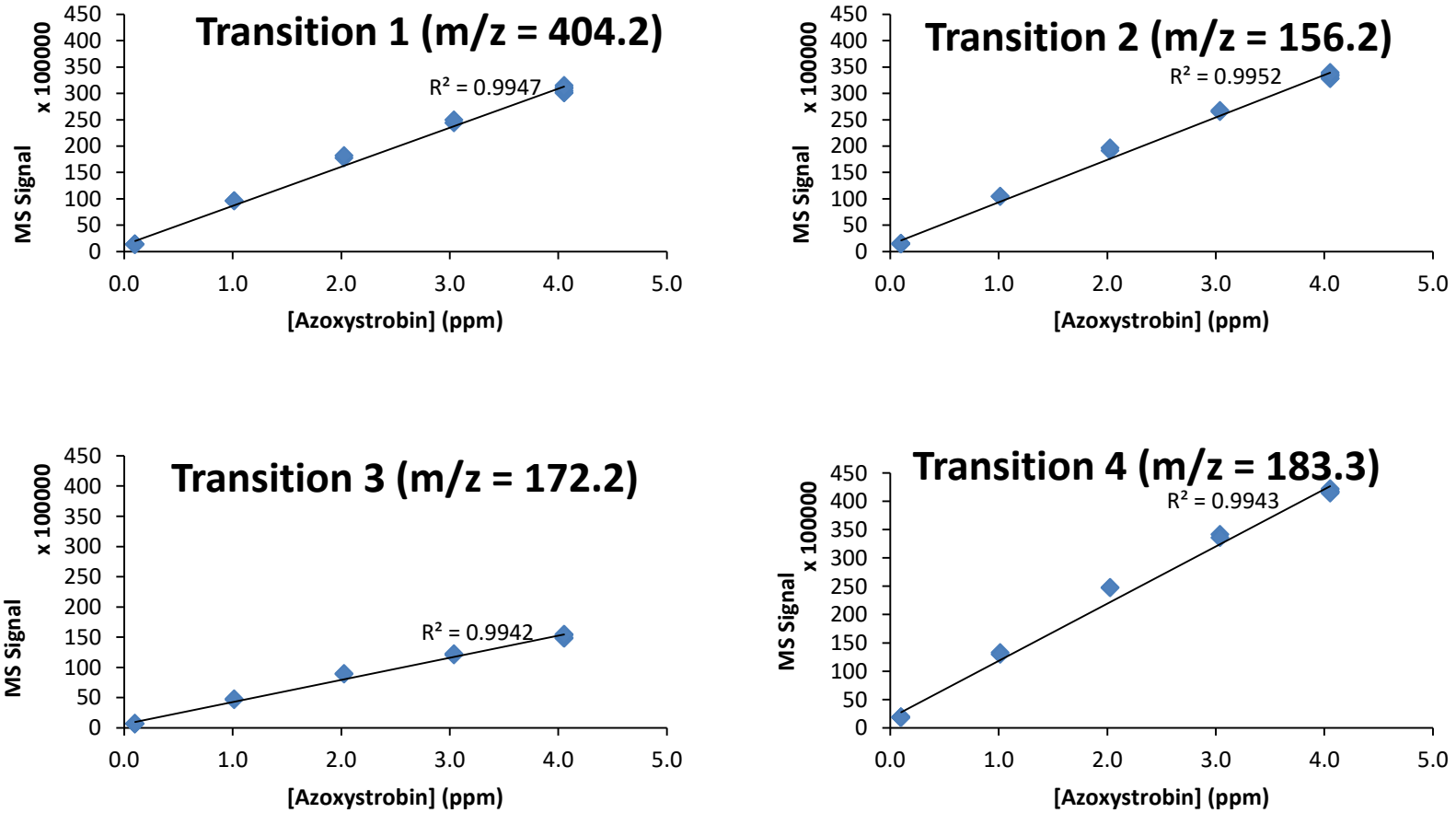

Figure 5.7 Mass spectrometry signal vs. azoxystrobin concentration calibration curves for 4 azoxystrobin fragments, where transitions 2-4 are daughter fragments of transition 1. The lowest and highest concentrations were each measured three times, and are included on the curves.

5.4.1 Spot and wash results and discussion

In the initial spot and wash experiment, 4 formulations of PVA, 3 formulations of chitosan, a control azoxystrobin formulation and a Bond-azoxystrobin formulation were tested. Acetonitrile (10 mL portions) was used to recover azoxystrobin from leaf surfaces before and after rain washing. Figure 5.8 reports the azoxystrobin concentration detected in these acetonitrile samples.

The samples representing the initial bar in a colour coded series (denoted by 01-10 in the sample ID) were produced by dosing azoxystrobin directly into acetonitrile – these samples represent the maximum amount of azoxystrobin recoverable from a leaf. The second bar in a series (denoted by 11-20 in the sample ID) represents the azoxystrobin recovered from a leaf after 1 hour of drying but before rain washing. This was in order to identify any issues recovering the azoxystrobin from the leaf surface, such as poor solubility of polymers in acetonitrile or pentration of azoxystrobin into the leaf.

The difference between the concentration of azoxystrobin in the control acetonitrile and the concentration from pre-rain leaf washings was largely insignificant. There were no significant differences between these values for all samples except PVAL where the dose of azoxystrobin in acetonitrile was lower than expected. All other differences were not significantly different and can be attributed to variance in the syringe operation. The third bar in a series (denoted by 21-30 in the sample ID) represents the remaining azoxystrobin recovered by acetonitrile after 1 hour of rain washing. Lower concentrations were detected for formulations which do not retain after rain washing.

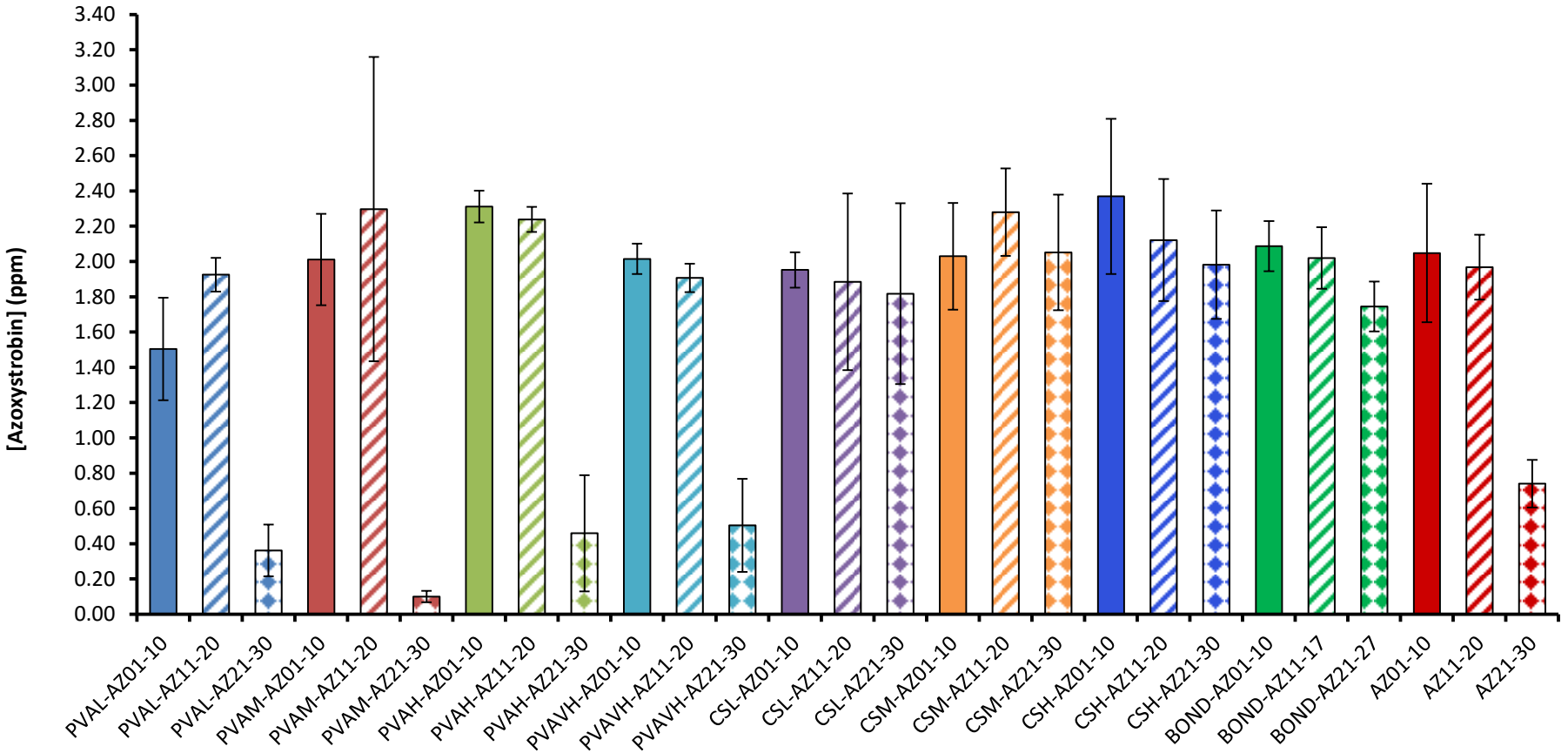

Figure 5.8 Concentration of azoxystrobin in acetonitrile where the first bar in a series (01-10, filled bars) represents the concentration of azoxystrobin dosed directly into acetonitrile, the second bar in a series (11-20, striped bars) represents the azoxystrobin recovered from leaving a treatment to dry on a leaf for one hour and the third bar (21-30, chequered bars) represents the azoxystrobin left on a leaf after being subjected to one hour drying and one hour under rain. Mean values reported (n=10) ± standard deviation.

Figure 5.9 illustrates the percentage of azoxystrobin recovered from leaves after one hour of rain washing and was generated from the data in Figure 5.8. The azoxystrobin recovered from the leaf after washing was reported as a percentage of the azoxystrobin recovered after 1 hour of drying but before washing. Approximately 40% of the control azoxystrobin treatment retained after one hour of 10 mm/h intensity rain. This value matched very closely to the approximately 40% azoxystrobin coverage measured via microscopy methods.

All three samples of chitosan improved the retention of azoxystrobin to approximately 100%, i.e. chitosan was able to completely rainfasten azoxystrobin in this experiment. Results from both chitosan and the azoxystrobin control were consistent with the coverage values measured in the previous microscopy experiments in section 5.3. No PVA formulation was able to improve the rainfastness of azoxystrobin. Particularly surprising were the results for the highest molecular weight PVA, which had improved the coverage retention in the raintower scale microscopy method – but not the lab scale. Bond significantly increased the retention of azoxystrobin to the same degree as chitosan, which was not observed at the lab-scale or raintower scale.

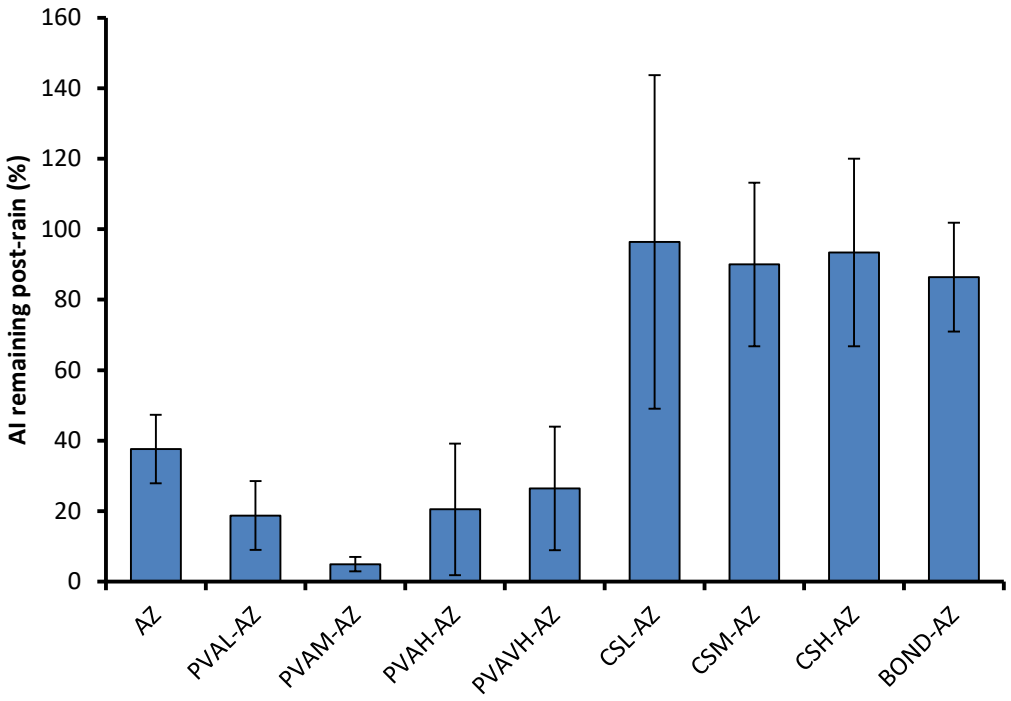

Figure 5.9 Percentage of azoxystrobin treatments on leaves after one hour of rain (10 mm/h) as determined via LC-MS/MS. Results reported as mean values (n=10) ± 95% confidence intervals.

These results do highlight some inconsistencies in the methods – namely that high molecular weight PVA did not show the efficacy that it did during microscopy analysis. One reason for the difference is potentially due to the rain-washing method. In this experiment, leaves are washed only once and for a continuous 60 minutes. In the lab-scale and raintower methods the leaves are subjected to smaller but more numerous rain events. It is possible that a continuous rain event is more detrimental to PVA rainfastness than multiple smaller rain events. In order to probe the result from PVA treatments further, the experiment was repeated using 10, 20 and 30-minute rain events.

5.4.2 Further ‘spot and wash’ analysis with accompanying fluorescence microscopy

Figure 5.10 represents azoxystrobin recovered from leaf surfaces after 10, 20 and 30 minutes of rain. It was hypothesised that the results would illustrate the loss of azoxystrobin retention with increased length of rain event and correlate with results achieved from the previous spot and wash method using 1 hour of rain. The results show that the length of time that treatments were exposed to rain did not impact the retention of azoxystrobin, with the exception of low molecular weight PVA. This grade of PVA showed significant loss of retention – 25% of the dosed azoxystrobin was recovered after 10 minutes of rain washing while only 15% was recovered after 30 minutes. However, the other treatments examined, including three high molecular weight grades of PVA, showed the same azoxystrobin retention after 30 minutes of rain as they did after 10 minutes of rain.

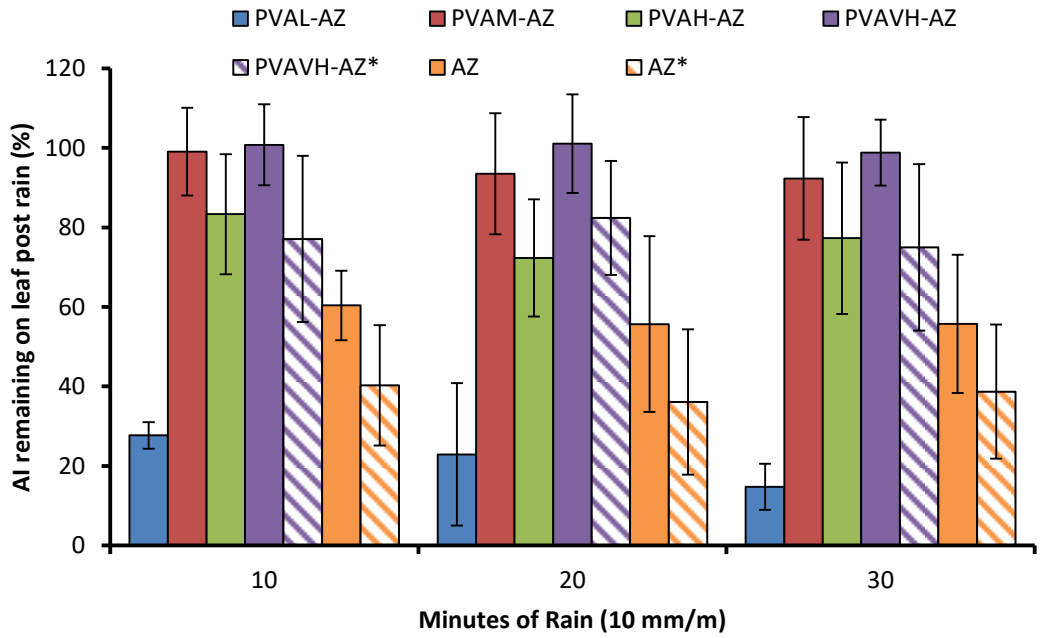

Figure 5.10 Percentage of azoxystrobin treatments recovered from leaves after 10, 20 and 30 minutes of rain as determined via LC-MS/MS. Sample IDs with asterisks were dried for 1 hour as opposed to 12 hour drying times for the rest of the treatments. Results reported as mean values (n=5) ± 95% confidence intervals.

As well as altering the length of rain event, the treatments were dried for longer, in order to establish if this had been the cause of poor PVA retention in the previous spot and wash test. Drying time of the deposits was increased to 12 hours, although PVAVH and the azoxystrobin control treatments were examined after both 1 hour and 12 hours of drying. The previous spot and wash experiment was undertaken with a drying time of 1 hour. Prior to this, in all microscopy experiments discussed in this work, drying time was not carefully controlled. Therefore, the results of this experiment were used to elucidate any benefits to longer drying time on the rainfastness of formulations.

Comparing firstly the difference between the samples dried for 12 hours in this experiment and the samples dried for 1 hour in the previous experiment: Except for the low molecular weight PVA treatment and the azoxystrobin control, increasing the drying time to 12 hours markedly improved the azoxystrobin

recovered from leaf surfaces. This can be rationalised in a number of ways. It is possible that increased drying time directly increased retention – longer drying times increase crystallinity of polymer films, which potentially become more water resistant.[18] However, this is somewhat refuted by the fact that PVAVH dried for only 1 hour in this experiment exhibited much improved retention as well. Alternatively, as the maximum rain event in this experiment was 30 minutes, and the rain event in the previous experiment was 60 minutes, between this time a large amount of the azoxystrobin retention may be lost. This does not seem likely given the fact that there were barely any changes to the azoxystrobin recovered between 10 and 30-minute rain events. Finally, the most likely explanation is that the discrepancies were due to uncontrollable factors, such as variance in the leaf surface characteristics in the leaves used. Despite efforts to ensure all plants are grown under the same conditions, it is not unreasonable to expect differences to arise.[19]

To accompany this spot and wash experiment a microscopy analysis was performed using plants from the same batch (Figure 5.11). This microscopy analysis was performed using 10, 20 and 30-minute rain events in order to directly compare the microscopy and mass spectrometry results for rainfastness. Deposits were also dried for 12 hours so as to match the conditions of the previous spot and wash method. Aside from these changes, the method is the same as the raintower method used in section 5.3.2. A sample of PVAVH was washed for 1 hour to further understand the result observed in the first spot and wash experiment where only approximately 30% of azoxystrobin was recovered after 1 hour of rain washing (Figure 5.9). These microscopy results show that PVAVH-azoxystrobin

treatments still maintained approximately 50% coverage after 30 minutes of rain washing, which did not change significantly after 60 minutes.

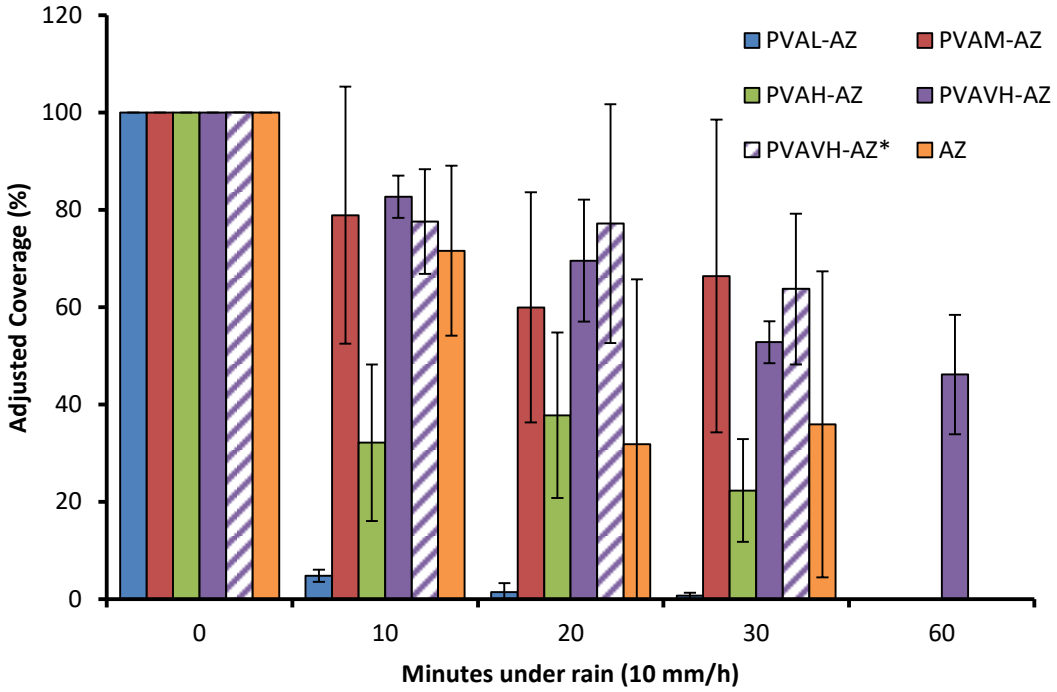

Figure 5.11 Raintower scale wash-off profiles for 5 azoxystrobin formulations. Droplets of treatments were allowed to dry on leaves and imaged to measure fluorescently active azoxystrobin coverage prior to sequential washing and re-imaging. Image analysis was used to quantify coverage and by adjusting the coverage value of dry deposits to represent 100% coverage. Results are presented as mean values (n = 3) ± standard deviation.

The results are interesting as they are somewhat analogous to those achieved in the accompanying spot and wash analysis. The results from both showed that high molecular weight grades of PVA were able to improve or at least not decrease the retention of azoxystrobin over a control. The treatment of medium molecular weight PVAM in particular showed excellent retention after 30 minutes of rain in both of these methods. Although this result is slightly anomalous when considering the rainfastness observed in chapter 4, it showed that the results

between microscopy analysis and LC-MS/MS show good agreement when the same batch of plants was examined. However, discrepancies between previous measurements of rainfastness highlight some drawbacks to the repeatability of the methods – likely due to the nature of using real leaf surfaces. Results from the LC-MS/MS and microscopy analysis of PVA treatments presented in the previous 2 figures are combined and compared in Figure 5.12. This figure highlights how closely the two methods match.

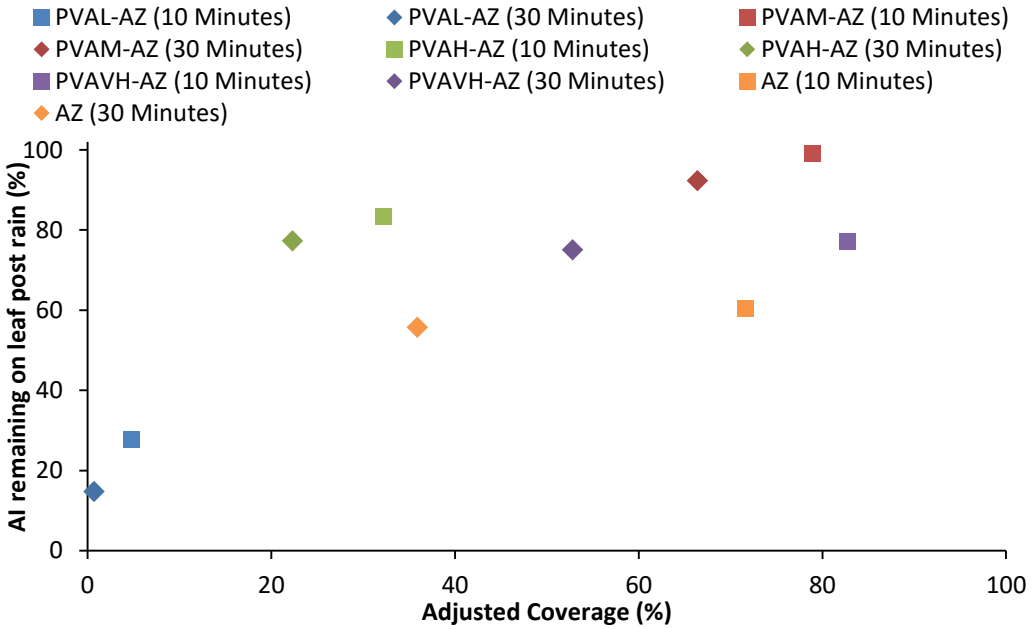

Figure 5.12 Results from Figure 5.10 for the percentage of active ingredient left on leaf surfaces plotted against the adjusted coverage values measured using microscopy from Figure 5.11.

Differences between the quantitative azoxystrobin retention determined by LC-MS/MS and the coverage values provided by microscopy analysis occur most often between results for treatments of PVA samples or Bond. These samples form annular deposits (Figures 4.2 and 5.4), so when a large amount azoxystrobin concentrates in this particular area, the coverage values determined by microscopy can underestimate the actual retention of azoxystrobin as determined by LC-

MS/MS. The LC-MS/MS values for retention of azoxystrobin formulated with chitosan correlate exceptionally well with the coverage determined by microscopy. The fact that chitosan generally forms deposits with small or no annulus at all (Figure 4.18) is further evidence for this theory.

5.5 Conclusions

In this chapter the methods established in chapter 4 to probe rainfastness of polymers alone were built upon. Firstly, the methods were adapted to examine the rainfastening effect of polymers on a model agrochemical compound. It was highlighted that the methods can be employed in this way, by simply preparing a formulation containing a fluorescent species. Results of this chapter show that certain polymers, in particular chitosan, can be used to enhance retention of azoxystrobin. Those polymers that improved the retention of an agrochemical were those that retained alone in chapter 4. The work provided further proof that length of rain events is largely irrelevant to the rainfastness of formulations.

Mass spectrometry ('spot and wash') work undertaken in this chapter aimed to validate the microscopy methods as tools for determining rainfastness. When the potential differences between batches of leaves were eliminated, the two methods showed excellent correlation. Those polymers shown to improve azoxystrobin retention via microscopy methods (Figure 5.11) also improved retention in the LC-MS/MS method (Figure 5.10).

Some discrepancies were highlighted, such as the fact that coverage values can underestimate the effectiveness of certain treatments at improving retention. For example, Bond did not improve coverage of azoxystrobin via microscopy methods but it showed enhanced retention via LC-MS/MS quantitative analysis.

However, while the microscopy analysis cannot be considered a quantitative measurement of rainfastness, it proved to still be an excellent way to quickly estimate rainfastness of formulations. With the methods that have now been established, any number of agrochemicals could be examined on a variety of leaf species quickly.

5.6 References

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Chapter 6 Concluding remarks and future work

6.1 Conclusions

This work aimed to understand rainfastness of water-soluble polymers to further the intelligent design of agrochemical formulations. This thesis examined the rainfastness of several water-soluble polymers on the *Vicia faba* leaf surface – both alone and when formulated with a model agrochemical. Novel fluorescent microscopy and image analysis methods were developed to study the actual process of wash-off of the dry deposits on the leaf surface. These methods were validated by quantitative analysis of retention of a model agrochemical when formulated with polymers. Physical characteristics were correlated with rainfastness performance in order to understand why water-soluble polymers may or may not be rainfast. The main findings from each chapter are outlined.

Chapter 2 examined the current state of the art regarding pesticide use and their formulation. It highlighted the growing trend for the development of formulation technology as a way to improve the safety and efficacy of pesticides. The excellent potential of polymers in agrochemical formulation development was highlighted in depth. Rainfastness was highlighted as a potentially untapped route to the improvement of efficacy and two mechanisms behind rainfastness of adjuvants were outlined. Firstly, super-spreading adjuvants improve the uptake and absorption of active ingredients so that they are safely inside the leaf.[1], [2] The second form of rainfast adjuvants form hard to remove deposits and films, but little is known about the types of water-soluble polymers that can be used to aid rainfastness or why.[3], [4] The focus of this thesis was to elucidate factors underlying rainfastness of water-soluble polymers.

In chapter 3 selected water-soluble polymers, including chitosan and poly(vinyl alcohol) (PVA), were characterised in order to understand the results from rainfastness measurements in the proceeding chapters. It was shown that the most water-resistant polymers were those of high molecular weight and high degree of crystallinity and it was hypothesised that these would prove the most rainfast samples. The polymers were fluorescently labelled and the procedures for the labelling were explained.

Chapter 4 presented the novel methods for measuring rainfastness of fluorescent species using image analysis. It was shown that high molecular weight PVA (50 – 92 kDa) with a high degree of crystallinity and chitosan over a moderate molecular weight (62 kDa) were rainfast polymers. Chitosan was particularly resistant, even at high intensity rain (30 mm/h). Readily water-soluble polymers such as low molecular weight PVA, carboxymethyl cellulose, dextran and a range of pectins were shown to have almost no resistance to rain wash-off. Washing methods were compared and it was shown that the lab-scale washing was equivalent to medium intensity simulated rain (10 mm/h). Therefore, unless high intensity rain is required, lab-scale experiments to measure rainfastness are adequate replacements for simulated raintower washings. As several grades of PVA were able to retain indefinitely at 10 mm/h rain, but were removed almost immediately in 30 mm/h rain, rainfastness of polymer deposits is likely dependent on the intensity of rain rather than volume or amount of rain.

Chapter 5 demonstrated that the microscopy methods established in chapter 4 could be used to measure the rainfastening effect of polymers on a fluorescently active pesticide. Those polymers which retained alone in chapter 4 were able to improve rainfastness of azoxystrobin. Chitosan, even at reduced concentrations,

was able to rainfasten azoxystrobin particularly well. Findings were validated using a quantitative ‘spot and wash’ measurement of azoxystrobin retention via mass spectrometry. Despite some discrepancies between rainfastness measured with both methods on different ‘batches’ of plants, when the same batch was used the results correlated very well. Thus the impact of the leaf surface, which can be variable with age and conditions, should always be taken into consideration when comparing results such as these.[5]

6.2 Future Work

There are several options for continuing to build on the work presented in the preceding chapters. Work in future could continue to probe the fundamental underlying factors of polymer rainfastness. Work to investigate the drying of droplets into deposits could prove fruitful in understanding at what point polymers become rainfast and why. Investigations could focus on both the drying time and the conditions which the droplets were dried. Conditions such as temperature and humidity could ultimately affect the crystallinity and resistance to wash-off of deposits – as they do with drying polymer films.[6] This would require a large controlled environment such as a glasshouse with which to keep plants under controlled conditions. This further work would look at separating the influence of crystallinity and molecular weight. This would be achieved by measuring the rainfastness of a PVA sample above the molecular weight threshold of 35-50 kDa described in chapter 4 and by carefully controlling crystallinity of the dried deposits.

The methods could be applied to a whole range of plant surfaces. The literature review highlighted that leaf surfaces have a variety of morphologies and wax

chemistries. Differences occur between species but also between plants of different growth stages. Studies could be used to understand rainfastness of agrochemical formulations on agriculturally relevant crop species at various growth stages. The methods could be used to screen a wide range of polymer-agrochemical formulations – with the stipulation that the agrochemical is fluorescently active.

Finally, future work could investigate the efficacy of polymer-azoxystrobin formulations used in this work via field trials. For PVA-azoxystrobin formulations it was suggested that PVA and azoxystrobin aggregate together in the annulus. Potentially the agrochemical could become entrapped in the annulus and lose efficacy, even if rainfast. The effectiveness of PVA-azoxystrobin formulations to protect crops would highlight if this is a reality.

6.3 References

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