

Design and Development of a Novel Plant
Cuticle Model using 3D Printing

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Abstract

The agrochemical penetration rate through the cuticle is dictated by wax composition. Cuticle wax composition varies between plant species, resulting in the same agrochemical displaying variable penetration rates as a function of the plant the agrochemical is applied to. Subsequently, methods to maximise cuticle penetration have been identified including surfactant use and the interactions of cuticle waxes, surfactants and agrochemicals has been intensively studied. However, elucidating the contribution of specific waxes and specific mixes of waxes upon these interactions is challenging due to the compositional complexity of cuticle waxes. As such, predictive modelling of these interactions is challenging and models predicting penetration as a function of the cuticle wax and agrochemical physiochemical properties are not possible. Subsequently, agrochemical permeation must be determined experimentally.

This thesis aimed to produce a model of the cuticle waxes comprised of cuticle wax analogs using a 3D printing technique, hot-melt inkjet printing, enabling various cuticle interactions to be investigated and the contribution of specific waxes and wax mixtures to be eluded. Three waxes were selected: dotriacontane, 1-docosanol and stearic acid based on ToF-SIMS analysis of the wax composition of *Solanum Lycopersicum* epicuticle and intracuticle surfaces. DSC, FT-IR, XRD and a rheometer were used to measure crystallinity, thermal properties, viscosity, eutectic properties, and printability. All tested waxes were printable at 90 °C and presented 50% crystallinity or greater like reconstituted cuticle waxes.

The wax analogs were used as material to 3D print wax films, approximately 50 - 200 µm in thickness, and material characterisation was conducted to analyse the wax powder and printed film. FT-IR showed printing did not impact the film composition; however, FT-IR and XRD showed crystallinity significantly decreased following printing. Contact angle measurements of water and two alcohol ethoxylate surfactant solutions containing 20 and 7 ethoxylate units Synperonic A20 and A7 respectively, were conducted to enable testing of the wax composition's effect on wetting. Both wax composition and topography significantly changed contact angle, however it is predicted this was driven predominantly by topography. This is because the contact angle on a surface formed in confocal contract with a flat substrate presented no significant change as a function of composition.

Imaging of the films showed surface wax crystalloids mimicking the morphology of cuticle crystalloids. The crystalloids' structure and distribution were controlled via changing the drops per inch (DPI), substrate temperature and film thickness. Raman Spectroscopy images

showed crystalloids predominantly formed at the interface of print tracks, where wax phase separation occurred. It was theorised crystalloids formed because of localised cooling of heterogeneously distributed waxes, that freeze at different temperatures, leading to phase separation and the local purification causing short range ordering and crystalloid formation.

Azoxystrobin penetration through printed films was performed using a Franz cell and azoxystrobin solubilised in water, Synperonic A20 and A7. Aqueous Azoxystrobin permeated 1-docosanol and a 1-docosanol stearic acid binary wax. Interestingly, permeation did not correlate with % crystallinity or sorption rate. However, it matches literature showing cuticles with large alcohol wax compositions permitted rapid permeation. Similarly, the addition of stearic acid to an alcohol wax film enhanced permeation, suggesting wax mixing modifies agrochemicals permeation. This highlights the correlation between wax composition and permeation rate, and that specific waxes and wax mixing induce rapid penetration. However, no azoxystrobin permeated through any other film when solubilised in water, nor when solubilised in A7 or A20. It is theorised excessive portioning into the A7 and A20 micelles prevented permeation through any of the tested films.

Finally, *Z. tritici*, a fungal spore, attachment to printed wax films was tested to assess the relationship between wax composition and topography on fungal attachment. No correlation was found between surface morphology and attachment. However, a correlation between attachment and composition was observed as attachment was significantly increased on films comprised largely of stearic acid. Similar trends were not observed for 1-docosanol or dotriacontane. This matched literature showing *Z. Tritici* preferentially attaches to cuticles containing large quantities of carboxylic acids.

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1. Chapter 1 – Literature review

1.1. Introduction and cuticle function

The aerial surface of almost all plant surfaces consists of a multi-layered membrane called the cuticle¹. The cuticle structure is projected from the external periclinal and anticlinal cell wall of epidermal cells², forming a complex multilayer structure (Figure 1-1).

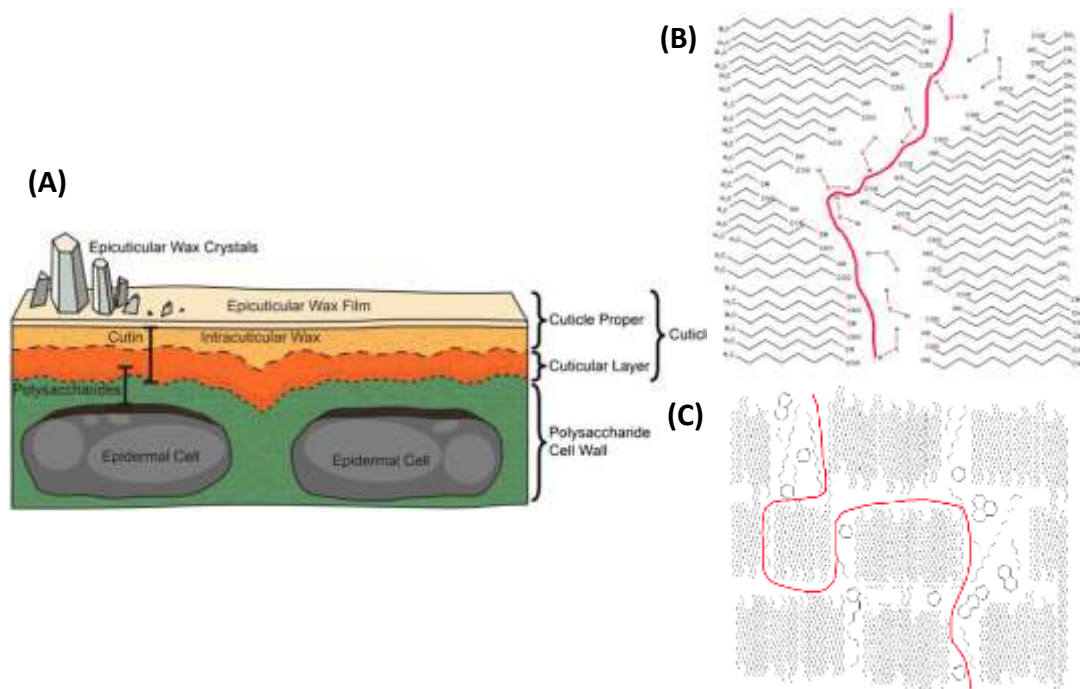


Figure 1-1 (A) Representation of the cuticle structure and underlying cells. Reproduced from Yeets and Rose, 2013³, (B) a representation of a proposed pathway for polar compounds comprised of polar chain ends and water that is used to move hydrophilic molecules through the cuticle, where the red line shows the pathway traversed and (C) a representation of the proposed pathway through the cuticle for lipophilic molecules where the red line shows the tortuous pathway lipophilic molecules traverse.

The cuticle is comprised of a range of materials with two predominant components. The first principal component, cutin and/or cutan, comprises the bulk of the cuticle⁶ and is a polyester consisting of a family of monomers, mainly C_{16} and C_{18} fatty acids^{5,8}. The second component, wax, impregnates itself upon and into the cutin polyester, forming distinct wax regions within the bulk cuticle⁹. Wax comprises long-chain aliphatics presenting different functionalities such as alkanes, fatty acids and esters, and cyclic waxes such as pentacyclic triterpenoids¹. In addition to wax and cutin, further compounds such as polysaccharides and phenolic compounds can be identified¹⁰.

Regions where wax impregnates itself into the cutin and/or cutan polyester, and is a small component of the overall bulk intracuticle, vs the regions where wax comprises the majority of the bulk material, epicuticle, define the two major unique layers of the cuticle. These layers are identified by the presence, or lack thereof, of cutin respectively³. These layers are typically not distinct layers and form diffuse interfaces, unlike what is shown in Figure 1-1. These regions visually are typically indistinguishable, but histology^{1,2,5} and electron microscopy³ techniques can derive the location of cutin through staining or visualisation of electron density.

The cuticle is involved in attenuating UV irradiation¹¹, limiting the attachment of microorganisms¹², limiting pathogenic insect interactions¹³ while enabling symbiotic interactions¹⁴ and regulating the ingress of endogenous chemicals and egress of water through transpiration¹⁵. Regulating permeation of exogenous and endogenous materials, particularly water, through the process of transpiration is often recognised as the cuticle's primary function¹⁶. The plethora of functions fulfilled by the cuticle explains why cuticles are found on and around almost all plant surfaces, as tissues lacking a cuticle would present no barrier to transpiration into the environment and the other functions the cuticle provides.

The capability of the cuticle is derived from its complex multi-layered and multi-material nature¹⁷ caused by the heterogeneous distribution of different cuticle components throughout these different regions and layers of the cuticle. This results in the cuticle presenting various surfaces, structures, and chemicals that give different functions that protect the plant. These functions result in exogenous materials interacting with the cuticle differently depending on the physicochemical properties of the exogenous materials and the location within the cuticle, Figure 1-1B and C. It is crucial to understand both the structure and composition of the cuticle to understand how the cuticle enables such depth of interaction.

1.2. Cuticle structure

The cuticle was originally discovered in 1672¹⁸ and defined as an external aspect of the leaf and later redefined as a distinct, homogenous covering above the epidermal cells of the leaf. It is, however, now understood that the cuticle comprises an external multilayer structure presenting heterogeneous structures and composition¹⁰.

The multilayer nature of the cuticle can be understood differently depending on the analytical technique, i.e. electron imaging¹⁹ or immunochemical²⁰ studies used to map or image this bilayer structure. A prevailing, classical cuticle model describes three lipidic layers

projected from the periclinal and anticlinal epidermal cell walls²¹. The uppermost surface, the plant-environment interface, is called the epicuticle layer. This structure comprises very-long-chain aliphatic compounds (VLCA) or cyclic chemicals such as pentacyclic triterpenoids or sterols, often called cuticle waxes, forming a thin layer²².

The VLCA compounds can display considerable chemical heterogeneity concerning chain lengths and functionality. Waxes range in chain length from C₂₀ to C₄₀, and present n-alkanols, alkanolic acids, n-alkanes and alkyl esters functionalities within the bulk cuticle wax²³. SEM images of epicuticle surfaces reveal wax crystallites protruding from the cuticle surface. The term wax crystal to describe these surface structures is widely used within the literature. Epicuticle crystal structures are highly morphologically variable, with reports of structures ranging from thin films to thick multi-layered wax structures forming crusts on the cuticle surface. The different morphological forms of wax crystal have been characterised²⁴, however, only a small proportion of these structures' crystalline order has been characterised, so the term wax 'crystalloid' will be used throughout this thesis²⁴ to indicate these structures might not be crystalline.

The exact number and morphologies of different wax crystalloids vary by author and how they characterise these structures. The definitions used in this thesis are based on the work of Barthlott, W and Neinhuis, C²⁴, who classified 23 crystalloids based on chemical and morphological features: Films, smooth layers, crusts and fissured layers, which comprise waxes that cover the cuticle surface forming layered structures instead of projecting out from the cuticle, Figure 1-2. Other structures are Granules, platelets, plates, rods, tubules, threads and rosettes, which comprise structures that project upwards from the ubiquitous wax film. Additionally, syntopism crystalloids describe structures where two or more morphologies are simultaneously present. Finally, many morphology classifications can be further separated into subcategories such as coiled rodlets²⁴, Figure 1-2.

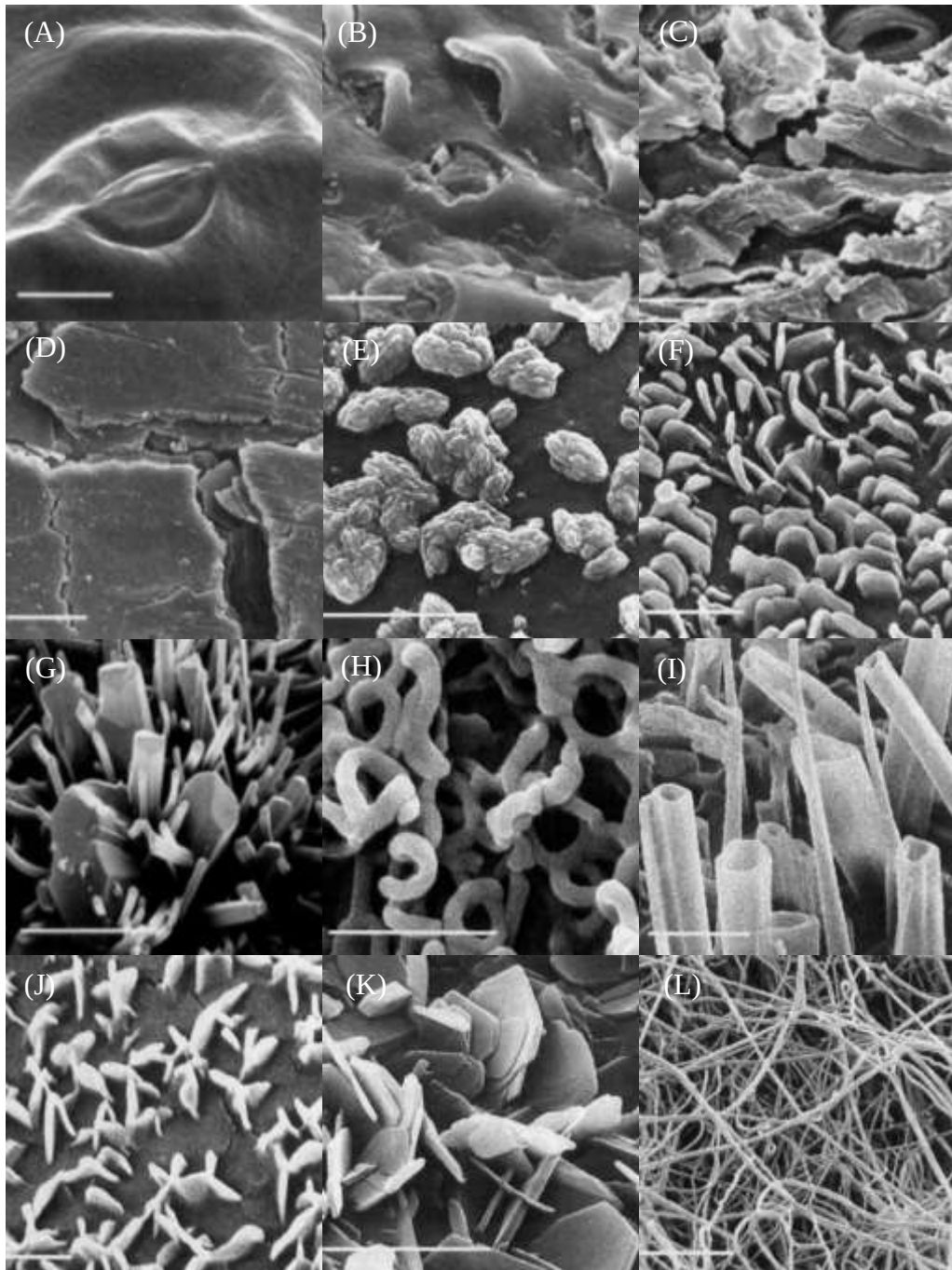


Figure 1-2 SEM images of (A) *Hydrocotyle bonariensis* Films, scale = 10 μm , (B) *Termilinalia cf. seyrigii* smooth layers, scale = 10 μm , (C) *Cynanchum Sarostmma* crust, scale = 20 μm and (D) *Frerea indica* fissured layer, scale = 20 μm , (E) *Aegiceras corniculatum* granules, scale = 5 μm , (F) *Habropetalum dawei* entire platelets, scale = 1 μm , (G) *Ascmiythia densiflora* polyangular rodlets, scale = 10 μm μm (H) *Buxus sempervirens* L. coiled rodlets, scale = 1 μm , (I) *Amorphophallus maximus* tubules, scale = 1 μm , (J) *Calliandra haematoma* rosettes, scale = 2 μm , (K) *Lecythis chartacea* plates, scale = 5 μm and (L) *Drosera burmanni* threads, scale = 2 μm . Image adapted from *Classification and terminology of plant epicuticular waxes*²⁴

These crystallite structures, which vary with species, plant age, and location²⁴, modify surface properties, including wettability²⁵; this later property can lead to more significant agrochemical runoff and reduced agrochemical penetration²⁷. Epicuticle crystalloid morphology is determined by wax composition which is known to change as a function of

plant age, species and location²⁴. This is more clearly shown when specific waxes phase separate from the bulk epicuticle waxes and form crystalloids dominated by a singular wax. Examples include nonacosan-10-ol and beta-diketone, preferentially forming tubule structures^{28,29} which are not frequently observed when similar waxes are absent in the cuticle. Platelet structures, described as flat crystals attached to the plant surface, are typically dominated by triterpenoids³⁰ or primary alcohols³¹. Subsequently, platelet structures are rarely seen when waxes lack these functionalities. Interestingly, the crystalloid structure is often preserved following recrystallisation, suggesting self-assembly drives crystalloid growth^{32,33}. However, the substrate used does impact the morphology of the crystalloid, particularly regarding the substrate polarity³⁴.

However, there is growing evidence that waxes comprising a small proportion of the overall wax composition can determine crystalloid growth and morphology²⁸. This was theorised following imaging of nonacosan-10-ol waxes that failed to form tubule structures depending on the bulk wax composition³⁵. In addition, it has been observed that some minor waxes resulted in a high prevalence of longitudinally ridged rodlets on the cuticle surface³⁵.

The presence of epicuticle crystalloids has rendered it unclear if the epicuticle wax is crystalline or amorphous given the presence of morphologically tubal structures and smooth films, which are commonly associated with crystalline and amorphous structures, respectively, meaning many have defined the epicuticle layer as amorphous. Despite this, crystallographic evaluation of epicuticle wax and bulk wax shows predominantly crystalline features; however, some regions of amorphous appearance have been observed³⁶. The quantity of these amorphous regions depends on the wax composition^{37–39}.

The two layers below the epicuticle are the intracuticle and cuticular layers and form a diffuse interface with the epicuticle layer where components, particularly wax, from all layers can be found⁴⁰. The intracuticle layer is comprised primarily of cutin or cutan^{6,45}, forming a large matrix. A range of waxes and phenols impregnate upon and within the cutin, forming the intracuticle waxes⁹. The cuticular layer presents composition and structure similar to the intracuticle layer^{6,45}. However, polysaccharide fibrils project from the pectin lamella of underlying cell walls anchoring the cuticle to the epidermal cells^{42,43}. Subsequently, the cuticle is not a discrete structure but a specialised lipidic modification and extension of the cell wall. Regardless, almost all cuticles present intracuticle and cuticular layers comprising most of the cuticle mass and thickness. The cutin monomers are linked via ester bonds and comprise approximately 40%-60% of the mass of the cuticle^{6,45}, depending upon the species.

Exceptions exist; for example, *Triticum aestivum* presents an epicuticle layer comprising the majority of cuticle thickness⁴⁴.

Many papers investigating cuticle functionality group different layers to simplify the terms used. Frequently, epicuticle and intracuticle layers are called the cuticle proper, as the significant wax presenting regions of the cuticle free of pectin⁴⁵. Additionally, the intracuticle and cuticular layers can be considered simultaneously. This is sometimes done despite the cuticular layer often presenting a lower wax concentration, particularly early in organ ontogenetic, when the cuticular layer is almost entirely comprised of pectin and devoid of cutin or wax⁴⁰. It should be noted there is no defined endpoint at which the cutin polyester will not be able to penetrate further. Rather, it is found at the interface of the epicuticle and intracuticle layers there is a gradual reduction in the mass of the cutin/cutan polyester as measurements move upwards into the epicuticle layer⁴⁶. Figure 1-3C shows this via the colour gradient between the intracuticle and epicuticle layer and shows an exaggerated representation of the wax structure in the epicuticle, Figure 1-3A, and intracuticle, Figure 1-3B. As shown, and as will be discussed in Section 1.3.1 and 1.3.2, the wax component of the epicuticle presents longer chain lengths, relative to waxes in the intracuticle, is less polar and is arranged in structured crystalline blocks that form a barrier to external substances. Intracuticle waxes in comparison are impregnated as crystalline structures within the cutin polyester. As will be discussed in Section 1.5, the cutin of the intracuticle layer provides a pathway through which hydrophilic compounds can permeate the lipophilic cuticle. Additionally, the irregular arrangement of cutin and wax structures in the intracuticle layer presents an irregular structure that results in the formation of a tortuous barrier that lipophilic substances must navigate to permeate the cuticle.

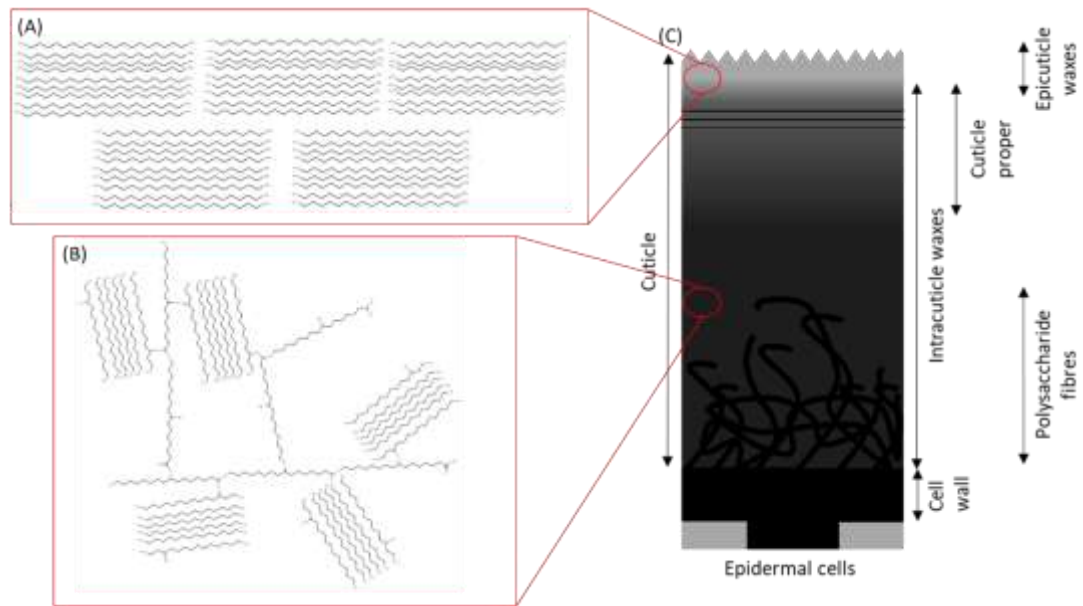


Figure 1-3 (A) Shows a representation of the chemical structure of crystalline wax structures in the epicuticle layer, (B) shows a representation of crystalline waxes impregnated onto a cutin polymer chain forming a tortuous network and (C) shows a representation of the cuticle. The darker regions show the intracuticle layer whereas the light grey region shows the epicuticle layer. The gradient highlights the interface between the epicuticle and intracuticle layer is a diffuse interface with no defined border. The red circles highlight that the structures found in (A) are present in the epicuticle and the structures shown by (B) are present in the intracuticle.

Using Transmission electron microscopes, a series of lamellate structures of epicuticle and intracuticle waxes mixed with no interference from cell wall structures, cellulose or polysaccharide fibres can be observed⁴⁵. These structures are proposed to be a consequence of local mixing of components of the interfacing layers of the cuticle. Subsequently, the cuticle should be considered a composite membrane with an asymmetric and layered structure⁴⁷.

For this thesis, the focus regards the wax composition, and thus the cuticle will be considered a bilayer structure based on the composition of the cuticle waxes in each region. Subsequently, the epicuticle is considered a unique layer, and the intracuticle and cuticular layers, presenting a similar composition of wax, will be considered together as the intracuticle layer.

Beyond observing internal structures and layers, it is also possible to observe the variance of the overall cuticle features, such as wax coverage and cuticle thickness. Cuticle thickness depends on the plant species and organ sampled. Cuticle thickness varies between 0.1 μm - 10 μm in leaves⁴⁹ and 2 μm - 25 μm in fruit⁵⁰, with some species displaying much thinner or larger cuticles such as *Arabidopsis* with a 22 nm thick cuticle⁷ or *Ariocarpus fissuratus* with a 225 μm thick cuticle⁵¹.

This variability was studied to elude why different cuticles display different properties and physical features. Variability primarily appears based on response to evolutionary pressures and the plant's environment. Cuticles within arid environments are rich in long-chain waxes with an increased proportion of cyclic wax component, as found in *Ziziphus nummularia*, compared to temperate plant cuticles⁵². Such behaviour is also seen as a function of the location on the leaf itself. Surfaces facing away from the plant, the adaxial surface expresses a unique wax composition relative to abaxial surfaces facing the plant⁵³. It is thus clear that cuticles present specific waxes and polymeric characteristics to adapt to unequal challenges⁵³. Similar changes can be induced by rapidly changing environments. For example sudden drought causes a significant increase in wax coverage⁵⁴.

Considering macroscale features, a wide array of cuticle structures can be formed where the cuticle conforms to the underlying anticlinal and periclinal cell walls (Figure 1-4A). Morphologically, this causes undulations on the cuticle surface (Figure 1-4B), influencing the nature of surface-environmental interactions, particularly wetting⁵⁵.

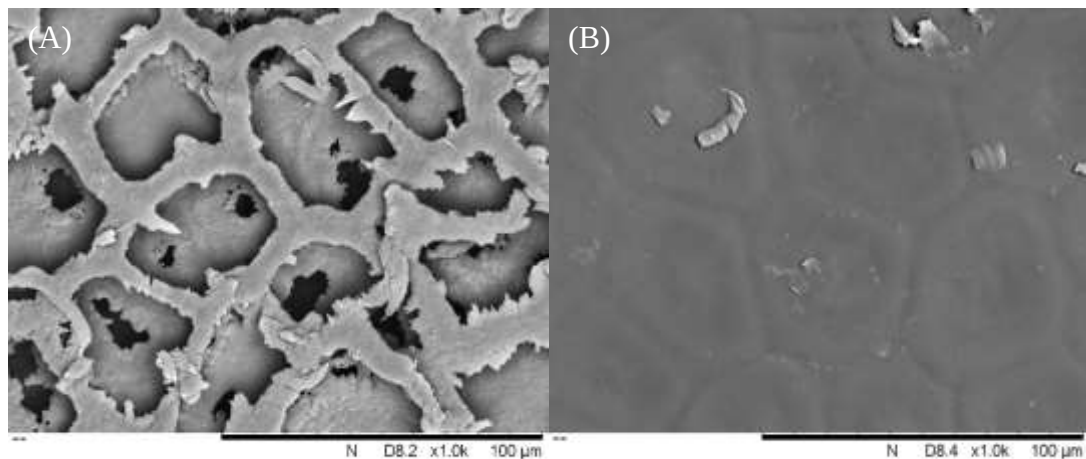


Figure 1-4 SEM images of *Solanum Lycopersicum* (A) intracuticle and (B) epicuticle surfaces.

An alternative macroscale surface feature are hair-like trichomes forming projections from the cuticle surface. Trichomes are often found on flowering plant surfaces, increasing the surface area of the plant and increasing the hydrophobicity⁵⁶ of the surface while not being directly attached to the plant's vascular system.

1.3. Cuticle composition

Unfortunately, the structural and chemical complexity of the cuticle has made investigations of the localisation and function of different components challenging. Cuticle morphology and chemical composition were investigated for many years using histological techniques

involving staining and microtoming⁵⁷. However, these techniques cannot readily identify microscale structures such as cuticle pores or provide detailed composition breakdowns of the cuticle, limiting their use⁶⁵.

In recent decades, continual analytical advancements, particularly spectroscopy, have enabled thorough investigations of spatial cuticle composition. Subsequently, the overall composition within each cuticle layer has been broadly characterised. The most significant discovery was that the cuticle is almost entirely comprised of wax, cutin and pectin¹, distributed throughout the cuticle (see Section 1.2). Additionally, modern spectroscopy techniques have enabled the identification of the unique chemical composition of each wax and cutin monomer that make up these components simultaneously, thus enabling a more thorough evaluation of the bulk composition⁶⁰.

Subsequently, it has been identified that the cutin monomers and the cuticle waxes consist of a wide range of structures within even a single cuticle and vary widely between species, age of the plant⁶⁰ and environmental growing conditions^{46,61}. Many cuticles present upwards of 10 cutin monomers and more than 50 unique waxes²³. This wide range of variability has been observed not just interspecies, dictating response to different environmental needs as an example, but also intraspecies. This can be observed within cuticles extracted from different locations on the same plant, from different varieties of the same species, based on the age of the plant and environmental growing conditions^{46,61}.

It is essential to understand which environmental factors can change the wax composition and quantify these changes, i.e. an increased wax amount following and during drought or higher light intensities⁶². This composition variability is thought to be the source of interspecies functional variability as this composition adapts to gradual and rapid changes in the environment to ensure survival⁶².

Unfortunately, because the cuticle can display a wide variety of compositions that affect cuticle functionality and the specific mix of compounds changes cuticle function⁶³, understanding the role of each component is challenging. Subsequently, structure-function relationships have not yet been derived despite the importance of this relationship on plant behaviour or the prediction of induced composition modifications. However, this does not preclude the ability to isolate these components from the cuticle for study and modelling of these components in isolation. While this cannot be used to predict the behaviour of the entire cuticle, or replicate functions and behaviours induced by interactions between different components, they can enable understanding of the structure-function relationship

of individual components. Such models can subsequently be built upon to gain an understanding of the structure-function relationship of the cuticle. As such, much research has sought to understand the function and behaviour of the cuticle components, Section 1.3.1 and 1.3.2, which has been used to develop models of the cuticle, Section 1.4. Understanding this, this thesis seeks to further current understanding of the cuticle through investigating the effect of composition, and component interactions on a variety of processes including agrochemical permeation, sorption, and fungal attachment.

1.3.1. Cutin and cutan

To understand the composition-function relationship, details of critical components must be understood. Considering the bulk polyester comprising most of the cuticles mass^{4,6,41}, immunological assays have shown that the polyester is almost entirely comprised of two families of monomers: cutin and cutan¹⁰. The polyester matrix produced is responsible for much of the cuticle's mechanical properties, particularly viscoelasticity and elasticity⁶⁴. In addition, cutin and cutan help regulate organogenesis⁶⁵, restricting growth by applying a force onto the growing organ as part of epidermal-growth-control theory⁶⁶. Additionally, it helps prevent pathogenic infection through presenting a physical barrier pathogens cannot easily cross; however, some pathogens can degrade the cutin and cuticle, limiting the utility of this function⁶⁷. Finally, the cutin/cutan polyester is the primary site of wax impregnation⁹, upon and within, potentially forming a channel through which hydrophilic compounds can penetrate the highly lipophilic cuticle barrier⁶⁸.

1.3.1.1. Cutin

In almost all cuticles, cutin monomers dominate with a small amount of cutan⁶⁹. Subsequently, cutin's chemical structure is relatively well documented as a lipidic polyester composed principally of hydroxylated and epoxy-hydroxylated C₁₆, and C₁₈ esterified fatty acids with a terminal hydroxyl group in many monomers a hydroxyl group or an epoxide at the mid-chain⁷⁰, Figure 1-5.

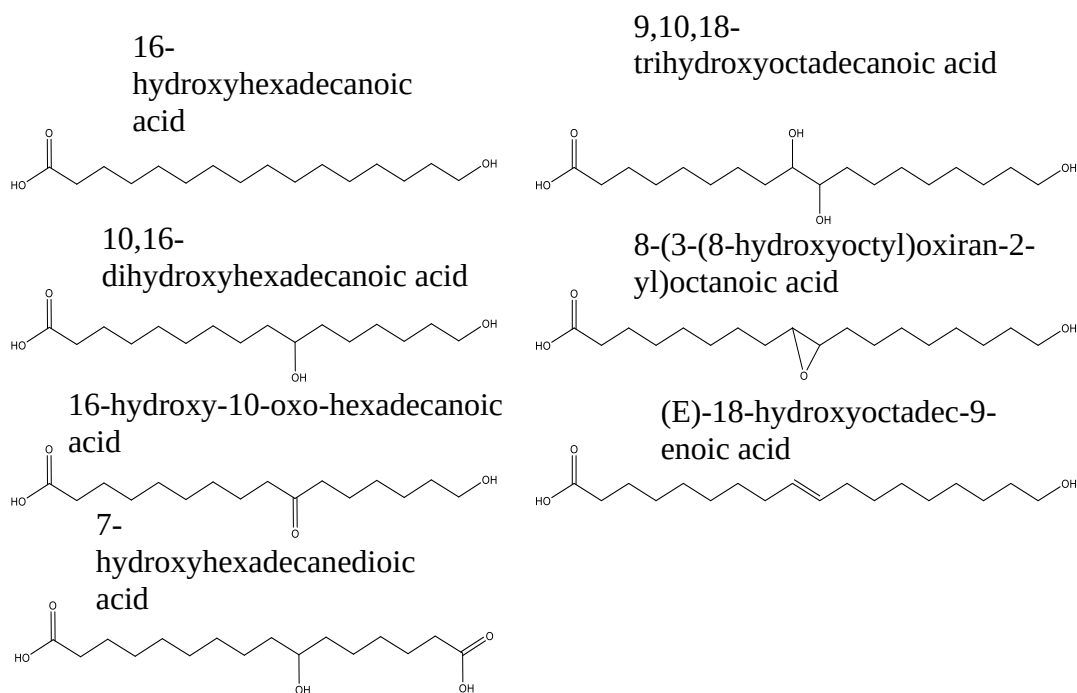


Figure 1-5 Examples of typical cutin monomers. The first column shows C_{16} cutin monomers, and the second column shows C_{18} cutin monomers.

9,16- and 10,16-dihydroxyhexadecanoic acids are the most common C_{16} cutin monomer, whereas 9,10,18-trihydroxyoctadecanoic and 9,10-epoxy,18-hydroxyoctadecanoic acids are the most characteristic monomers of the C_{18} cutin, with 10,16-dihydroxyhexadecanoic acid being the most common tomato fruit cuticle cutin monomer^{71,72}.

1.3.1.2. Cutan

Cutan is poorly understood as an acid- and base-hydrolysis resistant hydrocarbon polymer⁷³ found following wax extraction and cutin hydrolysis⁷⁴.

In stark contrast to cutin, the chemical composition of cutan is currently unknown and its chemical and functional role is poorly understood⁷⁴. It is proposed that cutin aids the formation of ester bonds at mid-chain hydroxyl and epoxide groups along with glycerol¹⁰. Several monomeric structures have been proposed, including a polysaccharide polymerised through polyethylene units to an aromatic structure with a high degree of carboxylic and hydroxyl functionalities forming ester bonds⁷⁵.

Some recent work questions the connection between cutin and cutan, suggesting cutan is related to waxes and is a product of wax biosynthesis⁷⁶. If this is correct, it would help guide further investigations into the poorly understood role of cutan vs the far better understood cutin monomer/polymer.

1.3.2. Wax

Considering wax composition, cuticle wax is highly variable as a function of plant species⁷⁷, age⁷⁸ and location on the plant⁵³. As already described, cuticle waxes are defined broadly as the C₂₀ to C₄₀ aliphatic compounds found throughout the cuticle, often presenting a single functional group forming n-alkanols, alkanolic acids, n-alkanes and alkyl esters¹. In addition, many species show further compositional changes induced by environmental factors and changes⁵⁸. Figure 1-6 shows examples of possible cuticle waxes demonstrating the variability of waxes found in the cuticle.

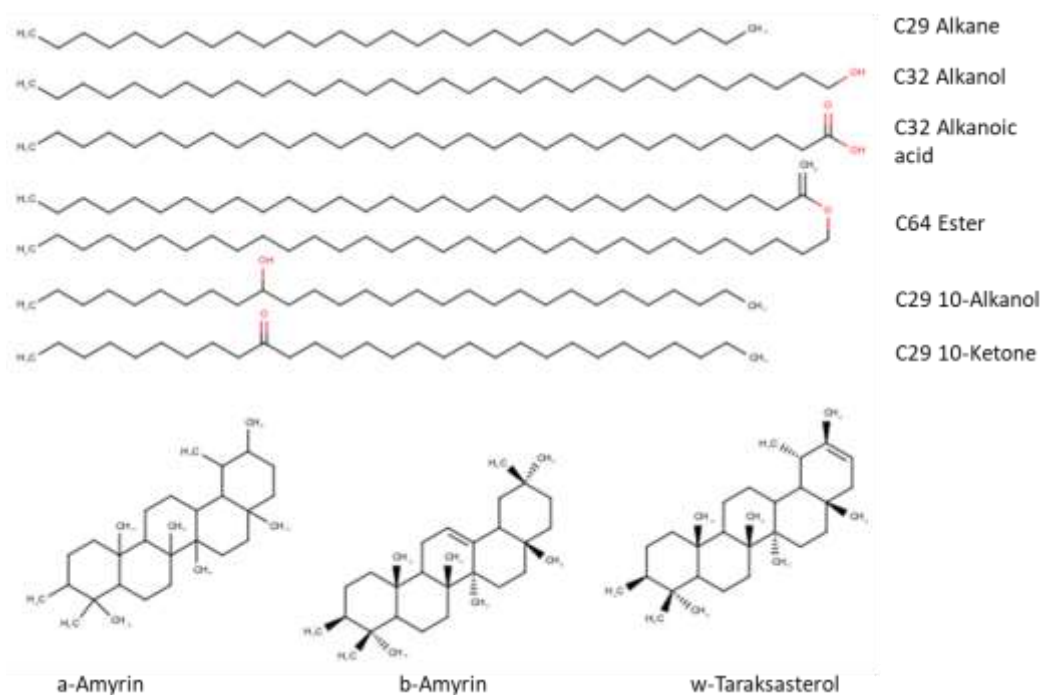


Figure 1-6 Examples of typical cuticle wax molecules showing a range of aliphatic waxes and three triterpenoid waxes.

This variable wax composition has been relatively well investigated using organic solvents, typically chloroform⁸⁰, to extract wax. Subsequently, extracted material is analysed, often using mass spectrometry techniques, particularly gas chromatography mass-spectrometry, to identify composition⁸⁰.

From these studies, trends regarding chain length, wax compositions, the specific mixing of different waxes and a range of ubiquitous waxes in almost all plant waxes. This is critical as wax composition⁸¹, the mixing of waxes^{38,81} and distribution of chain lengths modifies⁸² the crystalline structure of cuticle waxes and thus the properties of the cuticle waxes⁸³. A trend observed in almost all cuticles is wax chain length control in different species. Cuticle waxes present as a homologous chain length series. Where a homologous series is present, waxes

with specific required functionalities dominate with either odd⁸⁴ or even⁸⁵ chain lengths. This is thought to be associated with the biosynthesis of waxes⁸⁴. Distribution of chain length is determined by functionality with fatty acids, aldehydes and primary alcohols dominated by even chain length homologues and alkanes; secondary alcohols and ketones are dominant in odd chain length homologues¹.

Concerning wax functionality, the bulk of waxes are characterised by long-chain fully saturated hydrocarbon structures that may carry a single primary oxygen-containing functionality¹. Subsequently, waxes frequently comprise a homologous series of hydrocarbons with chain lengths between C₂₀ and C₄₀. Ubiquitous waxes are found in almost all plant species: n-alkanes, n-alcohols, n-aldehydes and fatty acids¹. These waxes often comprise upwards of 50 chemical entities per cuticle¹, with the most abundant chain lengths being between C₂₉ and C₃₁^{78,86}. Further analysis also reveals the ubiquitous presence of esters composed of fatty acids and primary alcohols of chain lengths between C₁₆–C₃₄ and C₂₀–C₃₆, generating esters with typical chain lengths between C₃₆–C₇₀¹. A single functionality of wax either dominates cuticles, i.e. *H. Vulgare* leaves, which presents a wax composition comprised of 89% primary alcohols⁸⁷ or display a composition with a wide variety of functionality, i.e. *Zea mays*⁸⁸.

While not ubiquitous, other waxes have been identified, including secondary alcohols. Depending on the species, secondary alcohols may present a broad chain length distribution⁸⁹ or a singular chain length^{90,91} and defined secondary hydroxyl- group location such as nonacosan-10-ol⁹⁰. Alkanediols and ketols often accompany secondary alcohols with a matching or similar chain length as the associated secondary alcohols⁹¹. It is suggested that alkanediols and ketols are produced following the biosynthesis of secondary alcohols as products of further reactions⁹². A further subcategory of waxes found in specific taxa are triterpenoid waxes. Triterpenoids are more widespread than secondary alcohols, and a small subselection of cuticles present high concentrations of triterpenoid waxes⁷⁸, while many cuticles present limited or no triterpenoid waxes. Of the over 200 triterpenoid carbon skeletons identified, only a small number are present in plant waxes, the majority being pentacyclic triterpenoids⁸².

The limited variety of secondary alcohols and identified triterpenoid carbon skeletons are associated with the limited nature of wax biosynthesis. This is highlighted by the hydroxyl function in the 3 β position of almost all identified triterpenoid waxes associated with the immediate product of the initial step in triterpenoid biosynthesis⁹³.

Solvent extraction and evaluation of cuticle wax have enabled the extensive study of isolated cuticle waxes, which can be conducted quickly, within 5-20 seconds¹. Subsequently, this has helped develop an understanding of wax composition as a function of species and highlighted the relationship of the wax composition with the environmental and physical role. Unfortunately, solvent extraction destroys the material structure of the cuticle wax layers and is non-specific, limiting the capability for spatial investigation⁸⁶. Subsequently, solvent extraction cannot determine if two regions present different wax compositions despite authors understanding the possibility of unique epicuticle and intracuticle waxes, with both regions presenting different waxes with specific roles⁹⁴

Subsequently, some groups have aimed to extract wax selectively, often using mechanical processes followed by exhaustive solvent wax extraction⁷⁸ enabling spatial wax composition evaluation⁹⁵, sometimes using selective cryo-extraction methods⁷⁸. It has been proven that the epicuticle and intracuticle present unique compositions of waxes⁷⁸.

The epicuticle surface of the cuticle is almost entirely comprised of long-chain aliphatic waxes⁹⁶⁻⁹⁸, with a small amount of polysaccharides and phenols⁵⁸. Exceptions including *Ziziphus nummularia* exist, which present many triterpenoid waxes in the epicuticle layer; however, such examples are likely an environmental adaptation to arid environments⁵². The intracuticle wax is typically impregnated⁹ into cutin and presents shorter chain lengths^{96,98} and a larger number of polar and cyclic waxes such as secondary alcohols, triterpenes and flavonoids⁹⁹ compared with epicuticle waxes⁹⁶. It is proposed that the polarity of these waxes prevents permeation through the lipophilic wax matrix, trapping intracuticle polar waxes close to the site of biosynthesis^{95,96}. Interestingly, fatty acids accumulate within the epicuticle layer despite their polarity⁹⁸.

It is thought that the epicuticle and intracuticle waxes fulfil different roles. Epicuticle waxes regulate environmental interactions, including surface wetting²⁶ and UV radiation protection¹⁰⁰. In contrast, intracuticle waxes dictate exogenous compound penetration^{101,102} and limit water loss by transpiration^{48,103}. Subsequently, intracuticle wax variability as a function of species, location and age determine agrochemical penetration with cyclic waxes allowing greater agrochemical penetration⁸¹. This occurs as changes in intracuticle wax composition lead to variability in the organisation of intracuticle crystalline domains with greater cyclic wax quantity resulting in higher penetration rates. This results from cyclic waxes tending to form amorphous regions between crystallite structures²³, explaining why

epicuticle waxes are predominantly entirely crystalline with their limited quantity of cyclic waxes, whereas the intracuticle waxes present greater amorphous regions^{37–39}.

Such studies have been critical in enabling locating barriers within the cuticle. For example, triterpenoid waxes minimise transpiration via the cuticle¹⁰⁴. Identifying triterpenoid waxes localised in the intracuticle layer⁹⁶ suggests the transpiration barrier is located in the intracuticle layer, a theory further supported by later studies⁸⁰.

Unfortunately, evaluation of the wax spatial distribution is focused currently on important commercial plants. Subsequently, while it is likely that such composition variability is a shared feature across most cuticles^{1,78}, this cannot yet be confirmed with many researchers still using bulk extraction methods to evaluate wax composition. Subsequently, understanding the implications of spatially distributed waxes is difficult.

As indicated, much of the functionality of the wax component is derived from the formation of an amorphous or crystalline structure within the cuticle. The process by which waxes crystalize is a three-step process: Super-saturation and/or super-cooling, nucleation, and crystal growth. Initially the temperature of the wax must fall below the isothermal crystallization temperature or the concentration of wax in solution must exceed the saturation concentration to induce the formation of a stable nuclei. If these conditions are not met only metastable nuclei will form wherein the kinetic energy of the wax molecules exceeds the energy of interaction between molecules of wax. Subsequently, Brownian motion will break the nuclei apart and stable crystals will not form. Once appropriate conditions to form a stable nuclei are met, wax molecules around the nuclei and crystal will become incorporated into the lattice in a defined and stable arrangement. This can occur around a nuclei of the wax molecules itself, so the crystal formed is comprised entirely of the wax, or it can occur due to wax contacting an impurity or structure such as a glass vial wall which then become the nuclei of crystallisation. Irrespective of the origin of the nuclei, wax molecules will continue to arrange into a periodic crystalline lattice provided the conditions to initiate crystallisation are maintained¹⁰⁵. Often crystallisation occurs simultaneously in multiple regions of the wax provided the appropriate conditions are met. As such, a solidified wax will comprise of multiple crystals, of different sizes, rather than being a single crystal.

When considering wax composition and the role of waxes rather than evaluating different species, attempting to find trends from bulk data derived for species with different environmental concerns and requirements, mutant plants can be grown. Mutant plants

present a modified composition compared with commercial and wild type plants⁸² allowing selective removal of waxes and comparisons within a single species. Mutant plants thus enable investigation of specific waxes, including impact on the penetration of agrochemicals⁸², thorough evaluation of cuticle properties in the wild type cuticle where the wax is present and the mutant where it is removed. Similar studies have conclusively shown that decreasing wax chain length increases agrochemical permeation. Unfortunately, wax composition modification often modifies cuticle ultrastructure resulting in many mutant cuticles presenting voids and loss of reticulate structure visible with SEM¹⁰⁶. Such mutant cuticles in almost all cases show extensive degradation of the cuticle structure, Figure 1-7

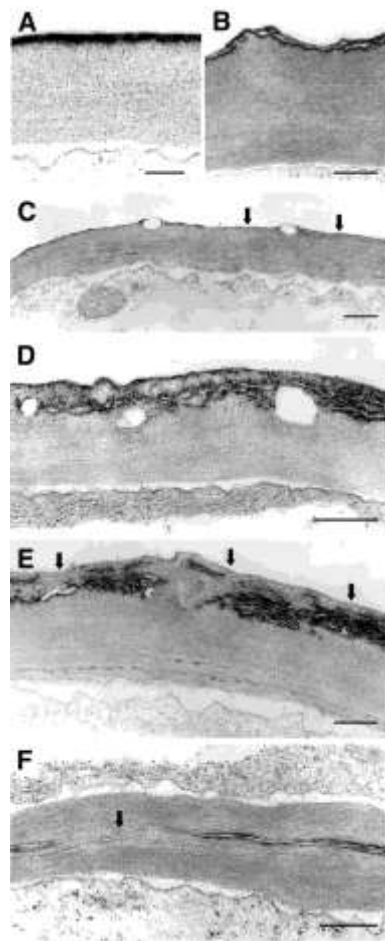


Figure 1-7 Transmission electron microscope (TEM) images of mutant cuticles ultrastructure. (A) wild-type cuticle. (B) mutant cell wall showing thin, misshaped boundaries. (C) arrows show large scale disruptions and absences within the mutant cuticle. (D) voids formed within the cuticle layer. (E) irregular layers are bulging from the cell wall where the cuticle might be expected and (F) a lack of cuticle membrane at the fusion between two cells—taken from Kurdyukov et al., 2006¹⁰⁷.

Subsequently, despite considerable research, no clear and comprehensive structure-function relationship has been developed regarding wax composition against the functional role of wax, including agrochemical penetration.

1.4. Models of the native plant cuticle

The plant cuticle is a complex structure presenting a host of properties. Given the cuticles' broad functionality, attempts have been made to model plant cuticle features via biomimicry to develop various material applications such as packaging^{108,109}, display material¹¹⁰ and reduction of vaporisation¹¹¹. Often this involves designing films and membranes replicating features of the cuticle through the incorporation of similar surface crystalloids¹¹¹ or bilayer polymer structures¹⁰⁹. Beyond biomimicry, the development of models might help elude features and properties of the plant cuticle itself, enabling, for example, the development of better agrochemicals.

A key area of investigation is hydrophilic and hydrophobic permeation through the cuticle and the effect of additives on transport. With similar, complex barrier systems, such as skin, *in vivo* testing is difficult with scope for incorrect analysis if the barrier is poorly understood or the experiment poorly designed. Such challenges are present when evaluating permeation through the leaf¹¹². For this reason, there has been some attention regarding *in vitro*^{23,113,114} and *in silico*¹¹⁵ modelling of the cuticle. Subsequently, several leaf model systems have been developed. These will be summarised briefly, with an evaluation of native leaves, considering the advantages and disadvantages of each.

1.4.1. Native and intact cuticle

The native leaf represents a leaf attached to the plant, whereas an intact cuticle is a leaf or plant surface removed and used quickly after. Native leaves represent an ideal model of the native plant as all main factors are retained, such as epidermal cells, metabolic processes, and cell signalling in response to xenobiotic compounds penetrating the cuticle, are still intact¹¹². Intact cuticle models capture the bulk of relevant barrier properties and wax characteristics as a native plant leaf¹¹². As a result, native leaves are thought to be the ideal substrate for a range of tests, penetration testing to assess the capabilities of novel agrochemical formulations²³. Unfortunately, due to the complexity of these structures and the impact of the underlying leaf structures controlling factors such as the concentration gradient and surface humidity, it is challenging to collect accurate data that can be correctly attributed to specific aspects of the plant cuticle. Subsequently, determination of the steady-state of permeation is challenging to identify, and thus determination of accurate kinetic information is difficult²³. Additionally, they require reliable washing protocols to ensure only the penetrating substances are measured¹¹⁶.

For these reasons, native leaves are infrequently seen in the literature for measuring agrochemical permeation across the cuticle, and instead, more abstract, simplified models of the native leaf are used.

1.4.2. Isolated cuticle

Isolated cuticles are a cuticle structure extracted from the leaf of interest. Isolation of the cuticle is achieved by exposing the leaf to an enzyme bath of pectinase and cellulase for approximately one week¹¹⁷ resulting in the underlying epidermal cell and pectin fibrils being metabolised, leaving the insoluble and unmetabolised cutin and cutan polymer impregnated with waxes¹¹⁷. Subsequently, because isolated cuticles present the cuticle structure without the underlying epidermal cells or pectin fibrils, analysis can evaluate only the wax and cutin polyester without the complexity introduced by underlying biological structures.

For this reason, isolated cuticles are regularly utilised for agrochemical research. Isolated cuticles can be modified to selectively remove the epicuticle wax using gum Arabic¹¹⁸ or the entire wax component using organic solvents⁸⁰. Subsequently, isolated cuticles are considered the 'gold standard' for modelling specific plant species cuticle behaviour, allowing investigation of processes that occur primarily within or upon the cuticle with limited interaction from basal bulk leaf components. This includes surfactant interaction¹¹⁹, assessing wax crystalline¹²⁰ properties and critically agrochemical penetration¹¹⁴.

Unfortunately, some cuticles are not suitable for isolation, and an attempt to isolate the cuticle will produce a membrane unsuitable for further use. Reasons include the cuticle layer being too thin. For example, the *A. thaliana* leaf cuticle is only 15 nm - 29 nm thick⁷, meaning handling post-extraction can cause damage¹²¹. Alternatively, cuticles that present many stomata or trichomes present holes where these structures were located¹²². This and similar physical issues limit isolated cuticles' utility when studying agrochemical penetration as the membrane will be potentially leaky, enabling the penetration of compounds via different routes. This is predicted to affect 65% - 90%¹²³ or 70%¹²² or all isolated cuticles. This suggests isolated cuticles are not an accurate cuticle model for penetration modelling as most papers reporting on this topic do not report cuticle validation within their methodology. This uncertainty is supported by data confirming extraction impacted *Citrus aurantium* cuticles' barrier properties to water¹²⁴. Interestingly due to the complex nature of water diffusion through the cuticle, occurring via both hydrophilic and lipophilic pathways, Section 1.5.2, it is difficult to assess which cuticle structure was disrupted or the mechanisms by which this

disruption occurred¹²⁴. For these reasons, alternative models have been sought that can be consistently produced from all species of plants.

1.4.3. Reconstituted wax film

Reconstituted wax films are often used when assessing only the interaction of waxes with the test system or in situations wherein the cuticle cannot be consistently isolated without the risk of structurally compromising the cuticle²³. Reconstructed waxes are typically produced by extracting the waxes of interest using a solvent such as chloroform and allowing the solvent to evaporate following drop or spin casting, leaving a film of wax¹²⁵.

Regarding lipophilic agrochemical penetration, reconstituted wax films do not replicate the original cuticle wax structure or arrangement and have been noted to have a modified internal structure, as observed using NMR¹²⁶. However, the diffusion coefficients and desorption of agrochemical within these reconstituted wax films presents similar trends as an isolated cuticle or whole leaf¹¹⁴. Permeance is typically one order of magnitude slower through a reconstituted wax film¹¹⁴ compared to intact cuticle; however, trends regarding environmental and surfactant modification¹²⁷ of permeance are consistent. A study of an isolated cuticle where permeation changed as a function of the surfactant used demonstrated these same trends when permeation was tested on reconstituted waxes derived from the same species¹¹⁴. These results were observed regardless of whether the wax was reconstituted from a solvent or a melt or if the wax was reconstituted alone¹¹⁴ or within a cutin biopolymer¹²⁸ matrix. Furthermore, sorption and desorption rates and surfactant interactions were retained when tested against native leaves^{113,114}. This highlights the importance of wax composition on agrochemical penetration and its associated impact on the physical ordering of the wax component. It also highlights that the cutin is typically not involved when assessing lipophilic agrochemicals interactions with the cuticle. This is consistent with experimentation showing that following the reintroduction of reconstituted waxes into a wax extracted cuticle that whilst the absolute permeance was modified, permeation trends relating to size selectivity and lipophilicity remained unchanged¹²⁸.

Unfortunately, because the extraction method involves the bulk extraction of both epicuticle and intracuticle waxes, the physical spatial distribution of cuticle waxes is not preserved. Subsequently, polar pores required for polar agrochemical penetration, Section 1.5.2, are affected, and the rate of polar pore sorption, penetration and desorption is significantly different to the isolated cuticle compared with reconstituted waxes limiting their viability as models of hydrophilic agrochemical penetration¹²⁹.

Subsequently, while some aspects of cuticle wax structure are modified by extraction and reconstitution, causing reduced permeance¹¹⁴, the film retained the same general properties and behaviours^{128,129}.

Direct comparisons of the reconstituted wax and wax within an isolated cuticle cannot be performed using XRD due to the absorption of X-Rays by the cutin. However, waxes' physical properties and behaviours are generally retained following reconstitution, meaning many tests and evaluation of cuticle wax behaviour is conducted on reconstituted wax films, including XRD evaluation¹²⁰. This allows wax behaviour investigations without interference by cutin or other cuticle components. Thus, evaluation of the crystalline nature of cuticle waxes is often conducted on reconstituted cuticle waxes^{120,126}. Reconstituted waxes are often used due to the ease of production compared with cuticle extraction.

Unfortunately, solvent reconstituted wax films lack macroscale epicuticle surface features such as crystalline structures that were initially present and therefore present substantially modified surface morphology³⁴. When the wax is reconstituted from a melt, it has been observed that the surface morphology can be modified through control of the melt and cooling processes¹³⁰.

1.4.4. Model wax film/cuticle

It is well understood that the cuticle composition is the primary factor controlling agrochemical penetration and must be considered when developing agrochemicals. The current most commonly used model, isolated cuticles, presents multiple major issues limiting its utility^{121,122,124}. Hence there is interest in developing model films that better reflect cuticle wax composition or replicating behaviours of native cuticles ranging from surfactant interactions¹³¹ to composition^{132,133} while avoiding isolated cuticles associated issues.

Recent physical modelling of the cuticle has mainly used thin wax films with similar chemical functionality and chain length as cuticle waxes to model the complex wax composition¹³¹⁻¹³⁴. Only two papers have offered alternative approaches^{135,136}. One evaluated surfactants interaction using tristerine¹³⁶, and the other assessed the directionality of transpiration using a cutin inspired via an artificial graded nanocomposite membrane¹³⁵.

Five papers were identified utilising waxes predominantly. Carreto et al.¹³² used a binary system of 1-tetradecanol and 1-octadecanol to model cuticle wax-wax interactions. Coward, JL¹³³ generated FT-IR spectra of nonacosan-1-ol wax films to understand and compare against the FT-IR of plant waxes. Fagerstrom et al. published two papers, one characterising a dotriacontane/ 1-docosanol system modelling native cuticles¹³¹ and a second evaluating the

plasticisation and penetration through isolated cuticles and the plasticisation of a dotriacontane/ 1-docosanol model cuticle¹³⁴. Overall, these papers conclude that complex wax systems can be modelled using a small number of waxes that partially model native cuticles, particularly surfactant interactions¹³⁴, while maintaining complex wax-wax interactions¹³². Subsequently, while model waxes as a tool to predict penetration has not yet been attempted, provided the correct waxes and production method are utilised, such a film might be possible.

However, model cuticles are relatively new and present issues, such as poorly defined topography and variable thickness, i.e. nonacosan-1-ol films due to the production process¹³³. There is scope for producing and developing model cuticles for agrochemical penetration testing, with suitable topography for penetration testing and wax crystallisation modelling native cuticles.

1.5. Cuticle penetration

1.5.1. Penetration of Lipophilic compounds through the cuticle

It is clear that cuticles are highly complex structures involved in a wide variety of environmental-leaf interactions, determined by the composition and structure of the cuticle¹³⁷. The cuticle is also where agrochemicals are often applied, called follicular application.

Follicular application involves applying an agrochemical formulation aerosol directly onto the plant's surface. Following contact between the agrochemical formulation and the cuticle, several factors affect the formulation's efficacy, principally the retention at the surface, penetration into the cuticle and transport to the site of action⁹. Here, cuticle composition determines epicuticular waxes and epicuticle crystalloid morphology which impacts wettability and the interaction volume between the agrochemical spray and the surface^{26,28}. Further, wax composition, partially the intracuticle wax composition, impacts the penetration rates of agrochemicals¹³⁸.

Follicular application has many advantages that have made it the most common application methodology, including ease of application, relatively low environmental harm potential, the ability to mix agrochemicals to be applied simultaneously time and the quick and uniform response¹³⁷.

Following follicular application, penetration of lipophilic and hydrophilic compounds has been observed. However, following follicular application, the relationship between lipophilicity, wax composition and permeation rates is poorly understood. It is predicted that due to the cuticle's heterogeneous and lipophilic nature, the penetration pathways of hydrophilic and lipophilic agrochemicals will differ¹³⁷.

The most accepted pathway by which lipophilic compounds penetrate the cuticle is via a sorption-diffusion mechanism through which exogenous compounds sorp onto the surface of the cuticle, following follicular application, and subsequently diffuse through the cuticle^{102,121} after which they desorb from the cuticle as the penetrating agrochemical enters the underlying epicuticle cells.

As part of this pathway, two regions of interest exist that relate to the heterogeneous nature of the cuticle structure and composition. A 'limiting skin', a region of cutin encrusted with waxes proposed to be located within the intracuticle layer¹⁰¹, limiting diffusion¹⁰¹ and a 'sorption compartment' located within the intracuticle layer where sorption and diffusion is rapid¹³⁹. Penetration by this sorption-diffusion mechanism is proposed to be a passive process described by Fick's law wherein molecules move down their chemical concentration gradient. If steady-state conditions are applied, permeation is a linear process wherein the total concentration of agrochemical that permeates the cuticle will increase with time²³. Subsequently, several models have arisen to characterise this pathway, such as Equation 1-1 and Equation 1-2:

Equation 1-1

$$J = PAC_d$$

Where,

Equation 1-2

$$P = \frac{KD}{\Delta x}$$

Where J is the flow rate ($\text{mol m}^{-2} \text{s}^{-1}$), P is permeance (m s^{-1}), A is the exposed area, K is the membrane water partition coefficient, D is the diffusion coefficient, and Δx is the thickness of the membrane. This can be used to understand permeance as a function of film thickness and partition coefficients.

Alternatively, if this data is not available, permeance can be determined via Equation 1-3a and Equation 1-4^{81,114}:

Equation 1-3

$$P = \frac{F}{\Delta c}$$

and

Equation 1-4

$$F = \frac{\Delta M}{\Delta t A}$$

Where F is flux ($\text{mol s}^{-1} \text{m}^{-2}$), Δc the change in concentration within the receiver cell, ΔM is the mass of active that permeated, Δt is the time taken, and A is the area through which permeation occurs.

Additionally, several studies have tested singular aspects of the permeation process, such as sorption tests wherein cuticles are exposed to a solution containing the active of interest, which is allowed to sorb into the cuticle and the change in concentration within the sorbing medium is measured to evaluate the quantity sorbed into the material¹¹⁴. Alternatively, tests evaluating desorption and diffusion wherein cuticles or reconstituted waxes saturated with radiolabelled actives are exposed to a sink solution, and the movement of the active out of the wax or cuticle is measured^{114,140}. These studies showed that the diffusion coefficient varies significantly due to the cuticle tested and wax composition. It is understood that many artificial waxes present a crystalline arrangement wherein amorphous material surrounds crystalline domains forming a torturous pathway. Based on the above finding and nuclear magnetic resonance and X-ray diffraction studies of the cuticle waxes^{120,126,141}, it was identified that agrochemicals permeating via the lipophilic pathway must pass through a similar amorphous pathway¹²⁰. This would explain the diffusion coefficients variance as a function of composition due to tortuosity, thought to be controlled by crystallinity and crystalline structures¹⁴², spatial arrangement¹²⁶ and the capability of the active to sorb¹⁴³.

In similar work, it was identified that negative logarithms of the diffusion coefficients plotted against the molar volume of the tested agrochemical (MV , mol cm^{-3}) generates a linear relationship, Equation 1-5, highlighting the impact of size selectivities on agrochemical sorption and subsequent penetration:

Equation 1-5

$$D = D_0 e^{-\beta MV}$$

Where D_0 is the diffusion coefficient of a molecule with a hypothetical zero MV , as determined by the Y-intercept, and β is the size selectivity of the cuticle or wax, highlighting

the dependence of D on MV. It was determined β varied between 0.016 to 0.037 and 0.017 to 0.044 in reconstituted waxes and isolated cuticles, respectively^{144,145}. It was suggested due to this study that the amorphous cuticle regions forming the tortuous lipophilic pathway were identical regardless of the cuticle tested as the size selectivities identified were insignificantly different. If this is the case, only tortuosity and rate of sorption and desorption determine the permeation rate of the active¹⁴⁵.

Understanding this, cuticle tortuosity is derived from interactions between crystalline waxes and the amorphous regions between wax crystals¹²⁰. This is supported by studies showing the crystalline domains formed from excluded regions through which permeation cannot occur^{94,126}. Subsequently, aliphatic compounds incorporated into these crystalline domains during reconstitution of the wax have significantly lower mobility than aliphatic compounds allowed to permeate through the amorphous regions^{94,126}. These amorphous regions must thus form pores and channels that transverse throughout the cuticle waxes.

The nature of these amorphous regions is controlled by wax composition; however, an evaluation of wax composition's impact on penetration has not been conducted. It is currently understood that long-chain aliphatic compounds rather than cyclic waxes form the permeation barrier⁸¹, likely as long-chain aliphatic waxes form crystalline units that generate the tortuous barrier, as seen in many similar nanocomposite materials. Further, it has been suggested that chain length modifies the formation of crystalline domains and thus, the tortuosity of the wax barrier³⁶ and reducing chain length increases permeation of agrochemicals^{81,82}.

Considering the tortuous network lipophilic compounds permeate through (schematically shown in Figure 1-8), these networks form where cuticles waxes are organised into four distinct zones³⁷ based on crystal organisation and the chemical structure of the wax³⁷⁻³⁹. The bulk of waxes self assemble as a highly ordered, regularly arranged series of aliphatic chains, zone A, with an orthorhombic crystal lattice at low temperatures or a hexagonal phase below its melting point³⁹. These regions often align themselves as flakes protruding towards the cuticle surface and are excluded regions where diffusion cannot occur¹⁴⁶. Because wax making up zone A show variable chain length, some flakes will present regions mostly comprised of chain ends. These are often located between flakes, forming a solid amorphous region, zone B¹⁴⁷, presenting motional freedom allowing agrochemical diffusion.^{36,38} The final region, zone D, forms from waxes that cannot be incorporated into the orthorhombic crystal lattice due to low melting points or steric hindrances. When zone D waxes are present

sufficiently, they cluster in zone B forming larger amorphous channels. Through these amorphous regions, zone B and D, diffusion can occur^{51,148}. As a result of this arrangement lipophilic compounds travel through a tortuous pathway, resulting in the pathlength of diffusion being far greater than the thickness of the cuticle, as shown by the red line in Figure 1-8. This results in a decreased rate of permeation¹⁵.

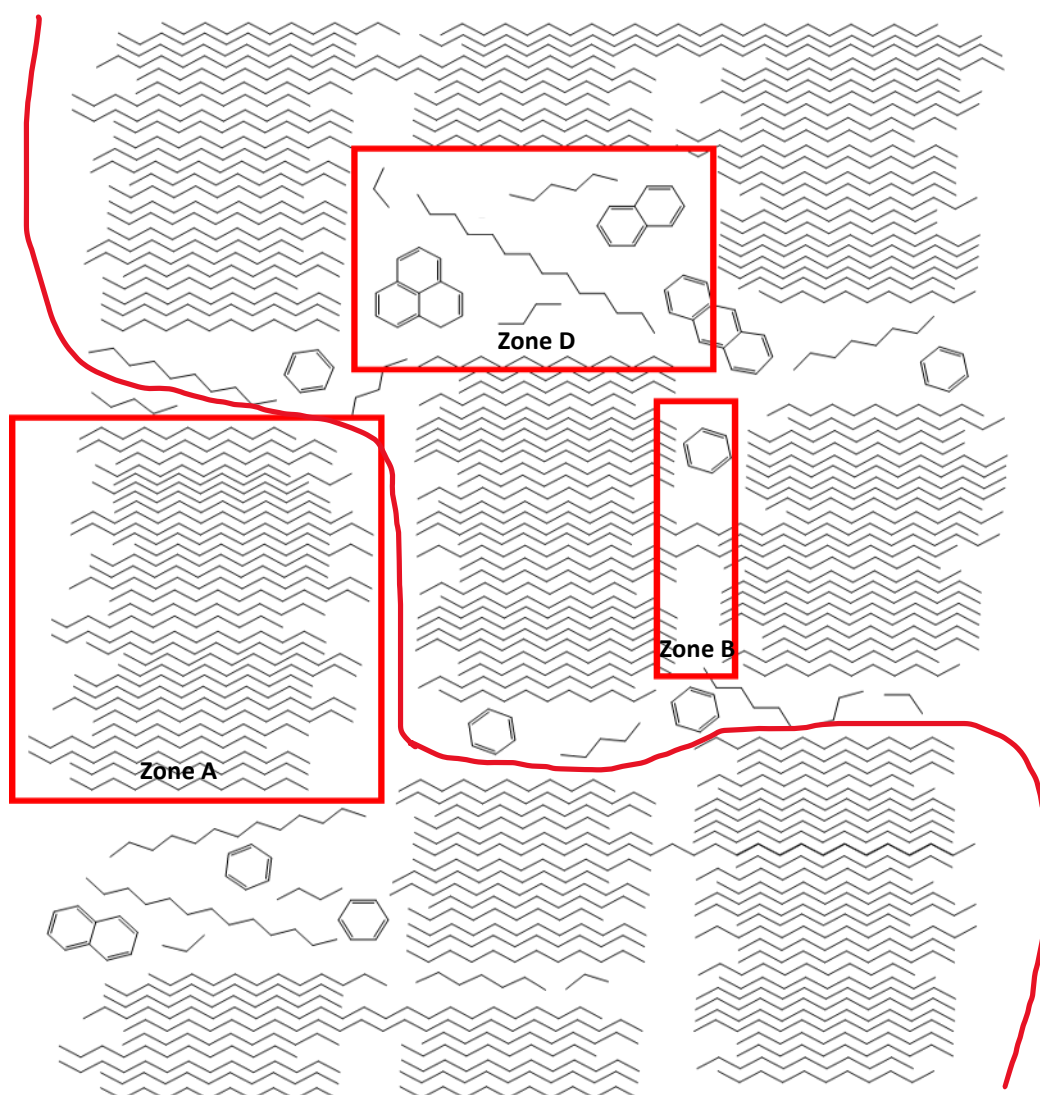


Figure 1-8 The structure and distribution of aliphatic and cyclic waxes within the cuticle, forming a tortuous amorphous network that lipophilic compounds must permeate. Red boxes highlight examples of each distinct region of the crystalline cuticle arrangement. The red line shows a possible pathway for a lipophilic agrochemical through this tortuous network.

Due to the ordering of zone A, crystalline flakes will form regularly spaced “bricks”, forcing diffusing compounds through a tortuous pathway, extending the diffusion pathway, partly explaining why diffusion rate is not dictated by cuticle thickness¹⁰². This theory is supported by the ability of surfactants to increase cuticular uptake of foliar-applied lipophilic agrochemicals^{149,150}. Current evidence suggests surfactants disrupt interactions between crystalline domains in the intracuticle waxes resulting in fluidisation of the amorphous wax as shown by irreversible fluidisation of waxes following surfactants application to wax in a quartz crystal microbalance^{102,134}. It is thus suggested the increase in penetration rates is driven by an increase in pore size, as the surfactant infiltrates the pores and solubilises the waxes present to increase the size of the pore enabling greater lipophilic penetration^{102,134}. Additionally, it is thought surfactants act to solubilise waxes, particularly surface waxes, resulting in the formation of new pores¹⁵¹. Subsequently, it is suggested that these crystalline - amorphous wax networks are involved in agrochemical penetration. It was noted that these surfactants did not interact with the crystalline domains of the cuticle wax¹⁵¹. A representation of the impact surfactant exposure induces on the cuticle wax structure can be seen in Figure 1-9 which shows an increase in pore size, generation of pores in the upper surface and infiltration of alcohol ethoxylate surfactants into the pores between crystalline wax flakes. These processes all decrease tortuosity, as shown by the red line a lipophilic molecule could travel.

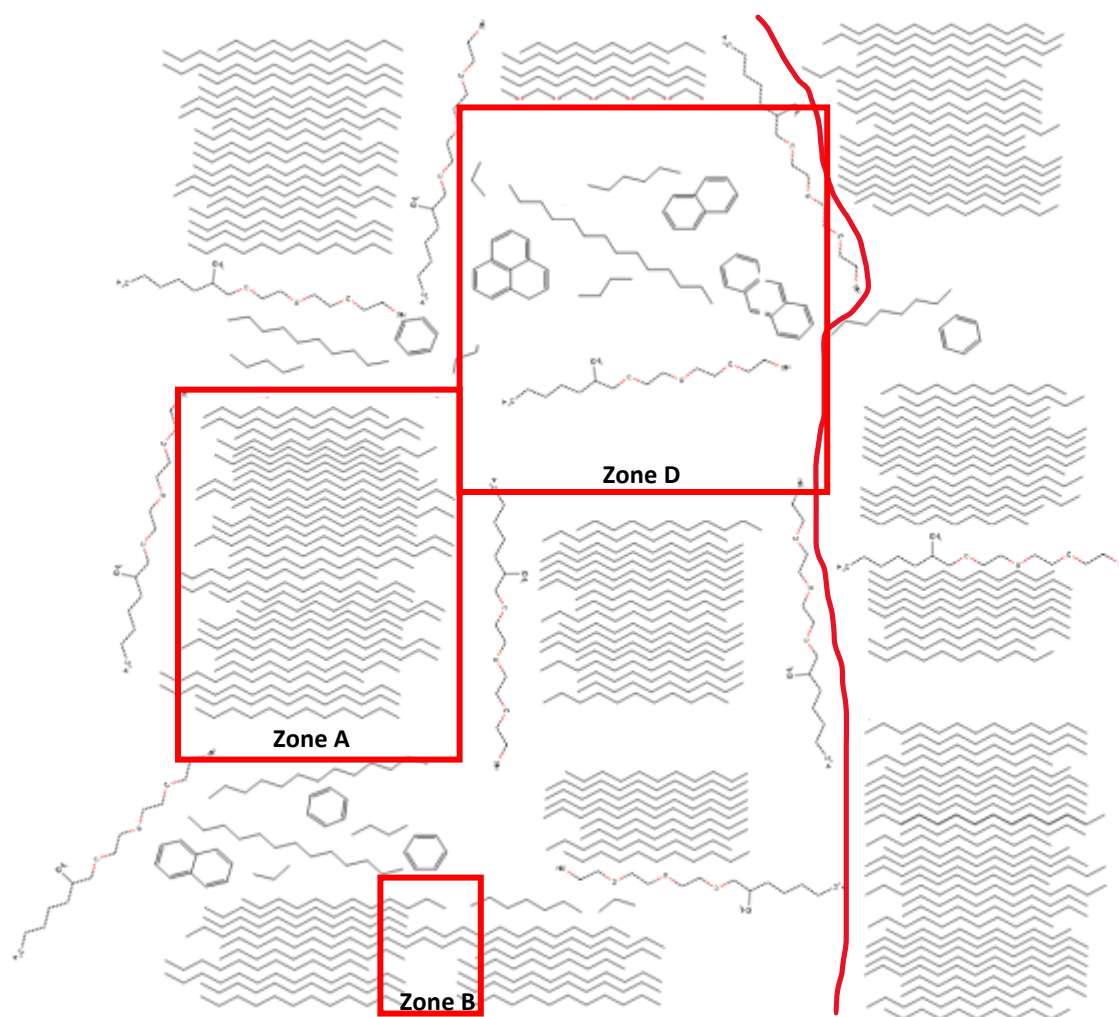


Figure 1-9 The structure and distribution of aliphatic and cyclic waxes within the cuticle, as seen in Figure 1-8, following the application of surfactant. Red boxes highlight examples of each distinct region of the crystalline cuticle arrangement. The red line shows a possible pathway for a lipophilic agrochemical through this tortuous network. Alcohol ethoxylate with 8 carbons and 3 ethoxylate groups are shown in the pores.

To highlight the importance of the cuticle waxes barrier properties regarding regulating the penetration of lipophilic compounds, measurements of agrochemical penetration through isolated cuticles wherein wax has been extracted show penetration rates many magnitudes greater than through untreated cuticles¹⁵². It is clear that the cuticle wax not only forms a complex internal structure but presents the primary barrier to lipophilic agrochemical penetration.

1.5.2. Penetration of hydrophilic compounds through the cuticle

Lipophilic agrochemicals comprise the majority of small molecules utilised as pesticides, herbicides and insecticides. Subsequently, this permeation pathway is relatively well

understood, Section 1.5.1. However, nutrients, growth factors and ions found in fertiliser are typically hydrophilic. Unfortunately, the hydrophilic permeation pathway through the cuticle is less understood than the lipophilic pathway, and there is no conclusive view on the mechanism or location of the hydrophilic pathway¹³⁷.

Initially, it was predicted that hydrophilic and ionic compounds penetrated the cuticle via the lipophilic pathway^{102,153} at a slow rate due to their poor solubility within waxes¹⁵⁴. This was based on observations showing water was affected by the same factors that affect lipophilic penetration: temperature⁶⁸, surfactant concentration¹⁵⁵ and wax extraction⁴⁸. Water was thus assumed to penetrate through the lipophilic pathways despite its poor solubility in the wax. However, this theory was challenged after observing hydrophilic compounds penetrating the cuticle significantly faster than predicted from their solubility and particle size¹²⁹. Furthermore, surfactant use¹⁵⁶ and increased temperature¹²⁹ that typically increase lipophilic penetration had an often insignificant effect on hydrophilic compound penetration. Similarly, humidity significantly impacted hydrophilic but not lipophilic compound permeation¹⁵⁶ and cuticle wax extraction, which significantly increases lipophilic penetration but frequently has an insignificant impact on hydrophilic penetration¹⁵⁷. It was subsequently suggested that hydrophilic compounds penetrate via a novel pathway not reliant on free volumes and interactions with the diffusion medium called aqueous polar pores^{122,153}.

Whilst polar pores have been experimentally verified¹⁵³, their location, structure and size is an area of active research⁹. Several locations have been postulated: through the cuticle, via cracks within the cuticle or stomata and trichomes¹⁵⁸. Regardless of location, the most prevalent theory is that hydrophilic moieties found within waxes, the cutin polyester and polysaccharide fibrils accumulate, enabling water absorption and the formation of a hydrophilic channel¹⁵⁹. If this accumulation extends across the entire depth of the cuticle channels, hydrophilic compounds would then permeate via these hydrated pathways, that are currently believed can only form in the presence of water¹⁶⁰. This theory understands that cuticles extend from epidermal cell walls⁴² via pectin fibrils, structures that are typically rich in polar and amphiphilic relative to the surrounding wax. The pectin fibrils anchor the cuticle to the cells using a hydrated pectin matrix and proteins, and thus can be used as a channel through which hydrophilic compounds permeate the cuticle¹⁶¹. A representation of the proposed polar pore can be seen in Figure 1-10. Permeation is a thermodynamic process and occurs more rapidly when the solute is more soluble. If a hydrophilic solute can permeate through these hydrated molarities, where solubility is higher than the lipophilic wax, permeation should become more rapid⁴⁶. Supporting this theory, it is known that absorbed

water molecules cluster within the cuticle¹⁶² and electrical conductivity studies show polar ions readily penetrate the lipophilic cuticle¹⁶³. The rates of penetration shown in these tests could only be achieved if the molecule is diffusing through the cuticle at a rapid rate, which would not be possible in the lipophilic wax matrix. Further, factors that increase hydrophilic permeation also increase cuticle hydration and the formation of hydrophilic channels¹⁶⁴. For example, increased humidity swells the cuticle as water permeates the membrane, expanding the polar pores and modifying the proposed tortuous dynamic polar continuum¹⁶⁵. As discussed in Section 1.5.1 factors such as cuticle hydration do not significantly change the rate of permeation of lipophilic compounds. This further suggests the two types of molecules use alternative pathways to penetrate the cuticle.

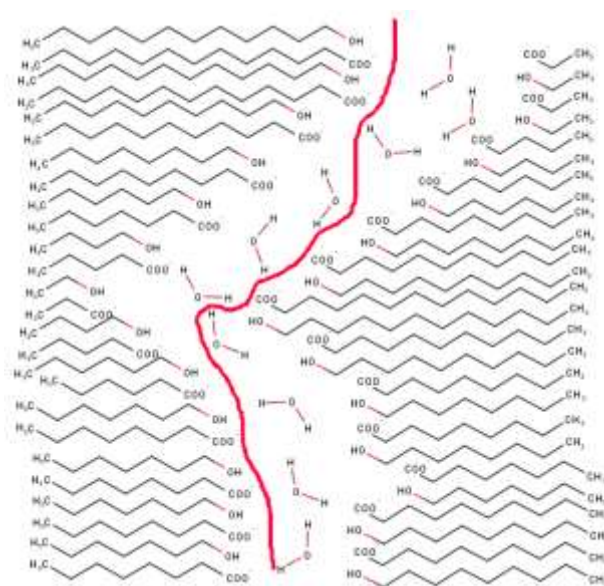


Figure 1-10 A representation of the proposed structure of the cuticle polar pore. As shown the pore is a location where polar and amphiphilic heads accumulate along with water to form a hydrated channel that hydrophilic compounds are capable of permeating down. The red line shows a possible pathway a hydrophilic molecule could travel.

As yet, polar pores have not been imaged conventionally^{9,47}, and the cuticle as a dense membrane precludes the formation of physical pores that interact and compete with the lipophilic wax matrix. Considering the location and size of the proposed polar pores, the most conclusive literature involves identifying first-order penetration of ions and hydrophilic compounds. Such first-order penetration only occurs if the molecular volume of the ion is smaller than a species-specific size exclusion limit ranging from 2.4 nm in *Populus × Canadensis* cuticles¹⁶⁷ to 0.3 nm in *Hedera helix* leaf cuticle¹²⁹. Considering first-order penetration only occurs with ions smaller than these values, it is proposed that polar pores are around 2.4 nm wide in *Populus × Canadensis* cuticles¹⁶⁷ and 0.3 nm wide in *Hedera helix* leaf cuticle¹²⁹. If hydrophilic and lipophilic compounds penetrate the cuticle via the same

pathway, hydrophilic and lipophilic solute size selectivity should be equal. This, however, is not observed, further suggesting that aqueous solubility determines penetration pathway^{129,167,168}. However, small uncharged hydrophilic compounds such as water and urea can permeate via both pathways due to their small size and high mobility¹⁶⁹.

An alternative theory is that polar and hydrophilic compounds penetrate the cuticle via the stomata, which regulate water loss. This is based on observations of greater polar agrochemical penetration through cuticles presenting stroma structures¹⁷⁰, primarily when environmental conditions favour stroma opening¹⁸⁷, and imaging radiolabelled agrochemicals as they penetrate the cuticle shows polar agrochemicals clustered around the stomata¹⁶⁷. Permeation via the stomata would enable rapid permeation due to the proximity of the underlying cells compared with permeation through the whole cuticle.

While it is understood that stomata induce some follicular penetration, the exact location is unknown, with most research focused on whether it occurs through the stroma or through the peristomal stomata cuticle¹⁷¹, which was thought to be highly permeable. This is because the density of stomata and trichomes has been shown to have a variable effect¹⁸⁴ on hydrophilic permeation, whereas changes to the cuticle structure and composition can significantly decrease permeation¹⁷⁵. This, however, might vary as a function of species with the accumulation of hydrophilic compounds observed in trichomes of soybean but not in tomato leaves¹⁷⁵. A 1972 paper by Schönherr and Bukovac¹⁷⁶ halted this debate by showing that water is incapable of inducing stomatal opening, and thus applied aqueous solution would not enter the stomata or permeate the cuticle. However, this does not consider the impact of surfactants that can reduce the surface tension of the applied droplet of agrochemical formulation allowing movement of the droplets into the stomata, nor the lack of uniformity of a native leaf that can open the stomata¹⁷³. Once the agrochemical formulation infiltrates the stomata the pathlength from the bottom of the stomata to the underlying epidermal cells is significantly shorter than from the surface of the cuticle, enhancing the speed of penetration¹⁷³. Currently, the prevailing theory regarding stomata in polar and hydrophilic compounds penetration is that stomata form the site where specific compounds¹⁶⁷ permeate and only in situations when the stomata wettability is increased, i.e. in the presence of bacteria¹⁷⁷. This occurs through the generation of trans-stomatal water clusters through which diffusion occurs between the stomata and underlying epidermal cells^{167,177}.

Regardless of the polar pore location, it is known that wax composition changes can induce changes in the polar pathway, as demonstrated by several mutant plant studies. Here mutations that modify cuticle wax composition were induced, enabling the study of wax composition changes on water penetration^{87,89,110}. A reduction in average chain length⁸² and increased cyclic wax content increase the movement of water across the cuticle¹⁰³. In addition, the extraction of epicuticle waxes only increases penetration by a small amount, whereas extraction of the entire wax layer significantly increases penetration suggesting, like in the lipophilic pathway, the intracuticle waxes controls penetration⁸⁰. Similar studies have highlighted the importance of cuticle composition and the spatial distribution of different components, as it has been shown that VLCAs, not cyclic waxes, control the transpiration barrier⁸¹.

1.6. Goals of the thesis

This thesis aims to develop 3D printed wax films with similar wax compositions and physical properties as native cuticle waxes. Such a model film could evaluate the impact of wax composition on the physical and chemical properties of the wax film and its interaction with external materials such as agrochemical solutions.

To achieve this, a range of waxes will be assessed for physical suitability for printing and thin films of waxes printed in a controlled manner using hot melt inject 3D printing. Commercial waxes will be used that have similarities to broad intracuticle and epicuticle waxes such as chain lengths, functional groups and wax mixing. To vary the films produced, the composition printed will be modified. Additionally, changing printing parameters will enable modification of the surface topography. 3D printing will be used to assess the capability of hot melt printing to print wax thin films with variable composition as a function of location. Possibilities include interspacing discrete wax droplets of varied compositions within the same wax layer and printing layers with different compositions. Producing similar films using casting methodologies would be challenging, necessitating 3D printing methods.

It is hoped that a printed film will model the broad cuticle wax behaviour, particularly agrochemical penetration and fungal attachment. These wax films could then be used to enable more detailed investigations of these topics, including the role and impact of chemical functionality on wax properties and the role of topographical features on environmental-wax interactions in a controlled manner. Tests to be conducted on printed film include investigation of surface morphology, agrochemical penetration, and the fungal attachment to wax films. While the waxes printed are not intended to be identical to native cuticle waxes,

the impact of mixing waxes, chain length and specific functional groups on agrochemical penetration and fungal attachment will be eluded. These results might enable future work to relate the properties of printed film to native cuticles. If successful, this would aid in developing predictive models of cuticle wax function and the development of a structure-function relationship relating wax composition and properties such as permeation that have been challenging to elude using prior models of the cuticle.

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2. Methods

2.1. Inkjet printing

2.1.1. Actuation

Inkjet printing, or as defined in ASTM standard 52900, material jetting, is an additive manufacturing technique defined by individual droplets being deposited onto a substrate in a predetermined pattern. Often inks will be ejected as a liquid column which will ideally coalesce into a single droplet during flight¹.

Currently, two significant forms of inkjet printing exist, continuous inkjet printing (CIJ) and drop on demand (DoD)², with several novel methodologies in development^{2,3}. In principle, both techniques vary concerning ink dispensing. CIJ involves using a continuous column of ink broken into droplets using a piezoelectric transducer that applies a magnetic field onto a charged ink column².

The piezoelectric transducer's waveform can be modified to change droplet properties via modifying the ink column break up⁴. Unfortunately, inks printed with CIJ must either be possible to charge or contain a conductive material⁵ so that deposition can be controlled by passing droplets through a set of charged electromagnet deflectors that deflect droplets to their deposition location². Due to the nature of droplet deposition, complex patterns can be produced using this technique. Subsequently, CIJ is used extensively within the textile and printing industry⁶. As a tool for research, the use of charged inks is not always possible, and thus an alternative droplet deposition methodology must be sought.

DoD involves the formation of droplets through the ejection of ink from a reservoir². This is either achieved using a thermal actuator that rapidly vaporises a small volume of liquid. The generated vapour bubble upon collapse triggers a shockwave causing droplet ejection⁷. Alternatively, piezoelectric actuators can be used wherein a piezo element will contract or retract based on the applied voltage⁸. Users can modify and optimise droplet formation velocity and angle by modifying applied voltage and how long the dwell time is for each Section of the piezoelectric contraction or relaxation^{2,8}.

Unlike Continuous inkjet printing (CIJ), DoD does not use a continuous stream of ink deflected to the target location. Instead, the printhead moves, so droplets are ejected above the required printing location². Subsequently, charged droplets are not required, and DoD deposition is more accurate². Additionally, the printhead can be moved upwards once a layer is printed, enabling greater ease when printing 3D structures².

For either system to work, the deposited droplets must solidify before an additional layer is dispensed, enabling deposition of a new layer of ink upon the prior printed layer. This is often achieved through cooling the liquid sample^{9,10}, solvent evaporation^{2,5} or induction of polymerisation via either on-line or post-processing UV irradiation and photopolymerization¹¹.

2.1.2. Droplet formation

For inks to print successfully, the inks must display appropriate physical properties. These properties relate to the droplet overcoming surface tension barriers at the printhead nozzle^{12,13} and ensuring the ink column coalesces into discrete droplets following the collapse of ejected ink columns under viscous forces⁴. An ideal ink following ejection coalesces into a single droplet with no modification of print angle due to the ink column collapse. This is predominantly controlled by viscosity and surface tension⁴.

Viscosity affects droplet ejection¹⁴, droplet coalescence¹⁵ and ink-substrate spreading¹⁶. High viscosity inks often cannot be ejected due to poor flow within printhead cartridges and nozzle blockage¹⁷. Additionally, excessively low or high viscosity, i.e. outside of 1 cP – 20 cP, results in the formation of satellite droplets¹⁵. This results from the ink column failing to coalesce into a single droplet. Instead, ink deposits as a column of ink or coalesces into two or more droplets^{15,18}, as shown in Figure 2-1, resulting in aberrant droplet deposition.

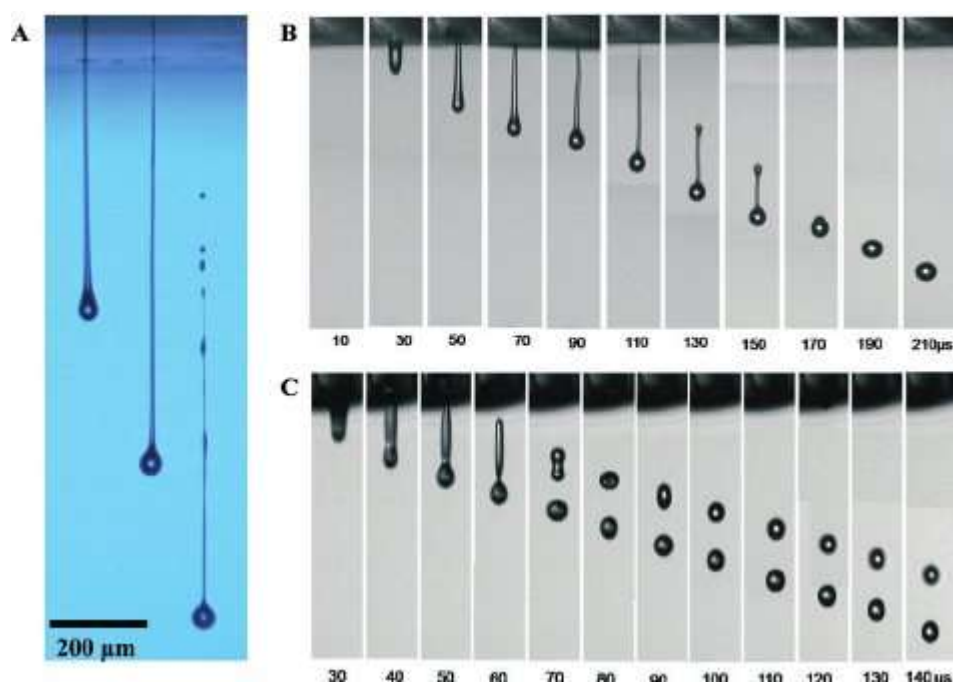


Figure 2-1 Images of different droplet coalescence following inkjet printing. A shows tailing and satellite droplet formation, B shows proper droplet formation after 170 μ s, and C shows satellite droplet formation after 70 μ s¹⁹.

High ink surface tension results in the inability of the ejection mechanism to overcome the energy barrier posed by the ink at the nozzle²⁰. Subsequently, droplets will fail to eject from the printhead. Additionally, high surface tension inks readily form satellite droplets in flight⁴.

Most commercial DoD inks should have a Z number, the reciprocal of the Ohnesorge number which relates viscous, inertial and surface tension forces, between 1 and 10²¹. Below 1, viscous forces dominate, and increasingly large pressure pulses must be applied to generate droplets with low velocities²¹. Above 10, the ejected droplets will travel with high velocities and form large liquid columns leading to extensive satellite droplet formation behind the main droplet²¹. Modifying surface tension and viscosity can be induced using excipients²² or changing factors such as printhead temperature to control the Z number via controlling viscosity and surface tension.

2.1.3. Droplet substrate interactions

Following the ink and substrate contact, the ink will spread outwards, depending on the substrate, ink properties, and the initial jetting conditions^{16,23}. Four phases of spreading have been identified¹⁶: kinematic, spreading, relaxation, and wetting. The initial kinematic phase involves droplet-substrate initial interactions and the formation of a shockwave that spreads the droplet radially. This is principally affected by droplet velocity and surface roughness²³. The spreading droplet forms a thin disk called a lamella, where mass accumulates on edges due to capillary forces²⁴. The disk will continue spreading until inertial forces, pulling the spreading droplet back to a sessile droplet, overcome capillary forces driving spreading²². If the capillary forces are sufficiently large, the droplet's edges will be ejected, causing splashing and ejection of satellite droplets²⁴.

Regardless of if splashing occurs, the remaining material will relax when surface tension forces overcome capillary forces, and the droplet relaxes^{22,25}. Once the droplet impacts, energy is dissipated, and the droplet will take on a consistent shape, often forming a spherical cap structures^{9,10}.

The process of solidifying the relaxed droplet depends on the droplet's composition and the material's physicochemical properties. Many monomer droplets are UV cured, inducing polymerisation and the formation of a solid from the liquid monomer solution¹¹. Droplets containing solid material dispersed within the droplets: ceramics, metals, or nanomaterials will deposit the dispersed solid material following solvent evaporation² of the ink carrying the dispersed solid. Alternatively, a phase change can be induced, such as the solidification

of waxes following hot-melt wax inkjet printing^{9,10}. Solidifying droplets is critical to ensure subsequent layers can be accurately deposited.

As part of this PhD, inkjet printing was used to print wax films that could comprise the model cuticles. This was achieved by printing waxes using a PiXDRO LP50 (Meyer Burger, Netherlands) inkjet printer equipped with a Spectra SE-128 printhead (Fujifilm, Japan). Identifying the optimal printing parameters was critical to producing consistent wax films for each wax printed.

2.2. Thermal Differential scanning calorimetry (DSC)

Thermal DSC is a frequently used technique within polymer and lipid research fields, enabling the identification of thermally induced phase transitions, including crystal polymorphisms, melting and recrystallisation transitions²⁶. It is broadly defined by its ability to measure energy changes and the enthalpy associated with phase transitions and report the temperature at which these phase transitions occur²⁷.

Two types of DSC exist. Both measure heat flow through different designs and principles: Power compensated²⁸ and Heat flux²⁹. This thesis used a power compensated Perkin Elmer, DSC 8000.

Power compensated DSC present two identical furnaces with individual heating blocks and thermocouples wherein a sample and reference are placed in separate furnaces. Samples are placed in a sealed aluminium pan for tests below 600 °C in small quantities (>10 mg). The reference chamber will be filled with a reference material that undergoes no thermal change within the tested temperature range. Sapphire, iridium and empty aluminium pans are the most common reference materials, with aluminium being ideal as a cheap reference for low temperature (>600 °C) measurements²⁶. Subsequently, samples will be heated either at a constant temperature rate change, called dynamic measurements. or maintained at a constant temperature, called isothermal measurements^{27,30}.

Both chambers undergo the same thermal profile with a negative feedback power compensation system, ensuring that the reference and sample chamber will remain at the same temperature regardless of heat flow changes during phase transitions. At a phase transition point, samples remain at the transition temperature until the phase change is complete²⁷. Subsequently, the negative feedback system maintains a known thermal gradient by increasing or decreasing heat flow as appropriate to allow the material to rapidly complete the phase change. To measure the heat flow required to complete the phase

change, the heat flow into or out of the sample is baselined against the heat flow into the reference material at the same temperature range. This allows the DSC to identify the heat flow associated with the phase change and plot this against temperature³¹. The area under the peaks of this heat flow vs temperature curve is the total enthalpy change associated with the phase change. Typically, samples are heated and cooled twice, with the second thermograph being used to generate results^{27,32}, as the first cycle is primarily used to eliminate thermal strain associated with the manufacturing of the sample. Figure 2-2 shows a common DSC thermograph.

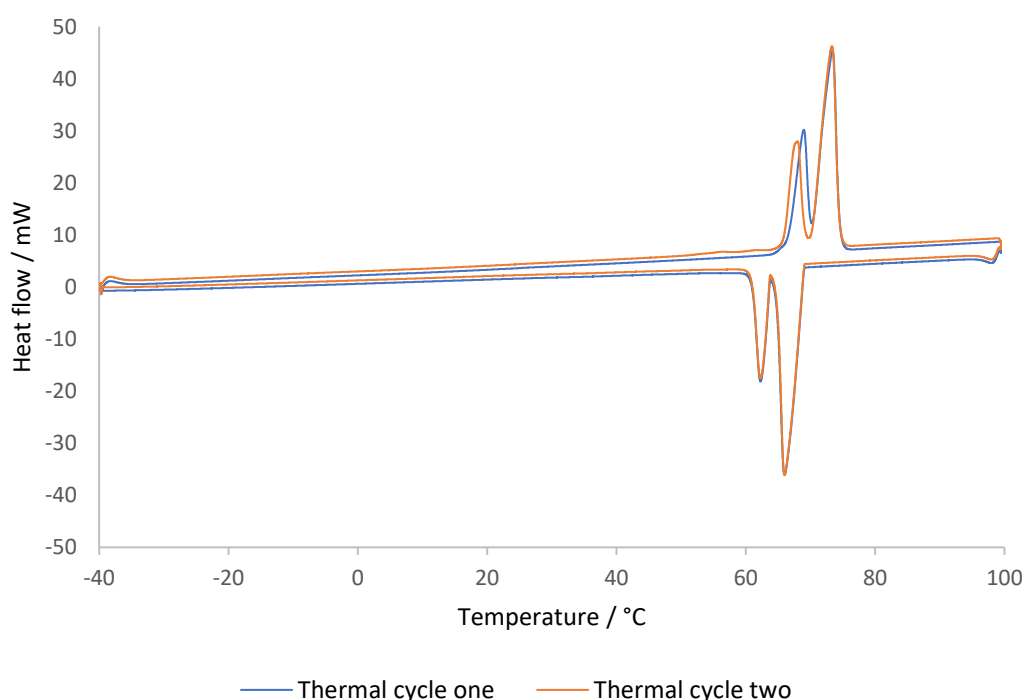


Figure 2-2 DSC thermograph of dotriacontane powder.

Mathematically the process used by power compensated DSCs follows Equation 2-1²⁸:

Equation 2-1

$$\frac{dQ}{dt} = C_p \times \frac{dT}{dt} + f(T, t)$$

Where $\frac{dQ}{dt}$ is the heat flow normalised by recorded sample mass (J/min*g), C_p is the heat capacity at the tested conditions (J/g K), $\frac{dT}{dt}$ is the rate of temperature change set by the user (K/min), and $f(T, t)$ is the heat flow induced when a phase transition occurs. Subsequently, the DSC will rapidly modify the heat flow to maintain the programmed temperature change, so the phase transition is terminated rapidly. For endothermic phase transitions, i.e., many

melting transitions, the DSC will increase heat flow while exothermic phase transitions, i.e., many crystallisation transitions, result in the DSC decreasing heat flow^{27,32}.

Understanding this, materials with different thermal properties will present unique thermographs³¹. However, when miscible materials are tested together, a unique thermograph is formed associated with a eutectic material, often with an endothermic melting peak at a lower temperature than the absolute melting points of each component material^{33,34}. DSCs are often used to identify the formation of these eutectic materials and predict the composition of a completely miscible set of materials provided information on the original materials is given by Equation 2-2, Equation 2-3 and Equation 2-4:

Equation 2-2

$$\ln x_a = - \frac{\Delta H_m^a}{R} \left(\frac{1}{T_0^a} - \frac{1}{T_m^E} \right)$$

Equation 2-3

$$\ln x_b = - \frac{\Delta H_m^b}{R} \left(\frac{1}{T_0^b} - \frac{1}{T_m^E} \right)$$

Equation 2-4

$$x_a + x_b = 1$$

Where x_a and x_b are the molar fraction of components a and b, ΔH_m^a and ΔH_m^b are the enthalpies of fusion for components a and b (J/mol) and T_0^a , T_0^b and T_m^E are the melting points of components a, b and the eutectic point, respectively (K)³⁴.

In this work, DSC was principally used to identify the melting points of the tested waxes. For a material to be ejected using the LP50 printer and Spectra SE-128 printhead printheads, the material must be liquid below 90 °C.

Additionally, it was used to identify the temperature of recrystallisation to ensure the substrate is held below this temperature, ensuring sample solidification, which would be critical to enable the deposition of additional layers onto a solid rather than a liquid surface. DSC was also used to investigate eutectic interactions between waxes when mixed. This increased the repertoire of available waxes for printing and enabled some aspects of the complex cuticle wax-wax interactions to be modelled.

2.3. Thermogravimetric analysis (TGA)

TGA is a means of measuring thermally induced degradation of a sample via on-line gravimetric analysis of a known quantity of sample as a function of time or temperature²⁷. Samples of interest are weighed into a crucible, often comprised of a material that only degrades at high temperatures, such as ceramics, and positioned upon a precision balance. Samples within the crucible will be heated either at a constant heating rate, called dynamic measurements or at a constant temperature, called isothermal measurements²⁷. Additionally, changing the environment-specific reaction or degradation process can be observed. A vacuum or nitrogen environment can be used as an inert gaseous environment. Alternatively, an oxidising (oxygen or ambient air) or reactive (hydrogen) environment can be utilised depending on the information to be determined^{27,35}. This gas flows over the sample to provide an environment in which the sample can degrade and purge any gasses evolved from the sample as it degrades that might impact further degradation.

Principally TGA is utilised to understand decomposition patterns³⁶ and phase changes³⁵ and solid-gas reactions³⁷. This is achieved by observing sample mass as the sample is exposed to different gasses or temperatures for differing times resulting in mass loss or gain depending on the chemical-physical properties of the material and any induced reactions³⁷.

The most straightforward evaluation using TGA, thermal degradation, will produce a step size graph showing the mass loss induced as each component within the sample material degrades at specific temperatures³⁷. Figure 2-3 shows an example. TGA can also identify phase changes associated with no mass losses, such as melting, glass transitions, or crystalline rearrangements³⁸. This is done by analysing the TGA thermograph alongside a DSC thermograph, or simultaneous differential thermal analysis (DTA), a process similar to DSC, is carried out within the TGA³⁸.

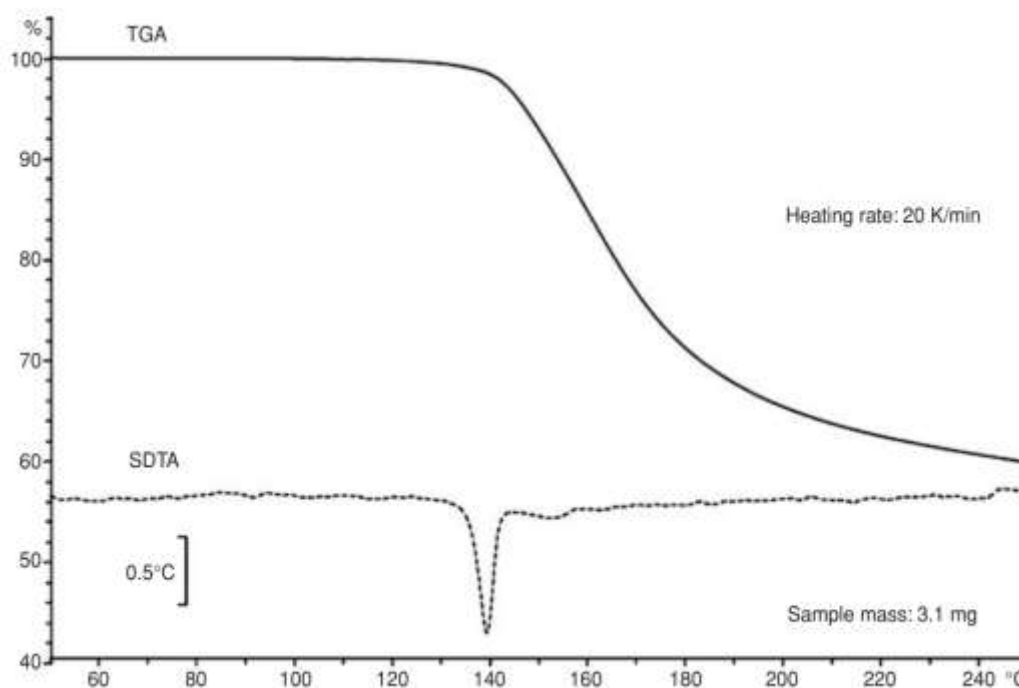


Figure 2-3 TGA, top, and DSC, bottom thermographs show acetic acid degradation in TGA and the associated phase change observed in DSC27.

TGA was used in this PhD to identify the degradation points of each of the waxes to be printed at elevated temperatures. It was critical to identify if exposure to operational temperatures would induce mass changes through thermally induced degradation. Approximately 10 mg of wax waxes were separately analysed in a Nitrogen environment from 30 to 600 °C at a heating rate of 20 °C/min. Operationally samples would only be exposed to a maximum temperature of 100 °C. Subsequently, if it was identified at 100 °C, degradations had not begun, the wax would be deemed sufficiently thermally stable for printing. TGA was also used to investigate the degradation of mixed waxes and identify if mixing had induced changes to the physicochemical properties of the waxes.

2.4. Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR- FT-IR)

ATR-FT-IR is a vibrational spectroscopy technique frequently used alongside alternative spectroscopy techniques such as Raman spectroscopy. Primarily FT-IR enables analysis and identification of the chemical functional groups within a sample by identifying IR radiation - samples interactions³⁹. When samples are exposed to specific wavelengths of IR radiation, the energy of the IR radiation will be absorbed, promoting the transition of molecules from a lower-energy state to an excited state, inducing bond vibration³⁹. The absorbance of IR radiation occurs at different wavelengths depending on the functional group present³⁹. As

such different functional groups can be discriminated, meaning each material has a unique FT-IR fingerprint, and thus, it is technically possible to distinguish between all molecules³⁹.

Many FT-IR techniques have been developed, i.e. transmission, specular reflectance, diffuse reflectance, photoacoustic infrared spectroscopy and attenuated total reflectance (ATR)^{39,40}. ATR-FT-IR can be collected with no sample preparation and can be used on both liquid and solid samples⁴¹. ATR-FT-IR passes IR radiation through a crafted ATR crystal at a predetermined angle, typically 45 °, so the IR radiation is repeatedly reflected off the crystal and sample, maximising exposure to the sample before detection to maximise absorbance, aiding sample identification⁴¹. Despite the IR radiation being confined to an ATR crystal sample, exposure to the IR radiation is achieved when IR radiation is reflected from the surface, and an evanescent wave is generated that penetrates the sample⁴². The interactions of the sample and this evanescent wave are measured as per absorbance of different wavelengths of IR radiation and plotted as either transmission or absorbance as a function of wavenumber^{41,42}. Figure 2-4 shows an example of an ATR-FT-IR spectrum.

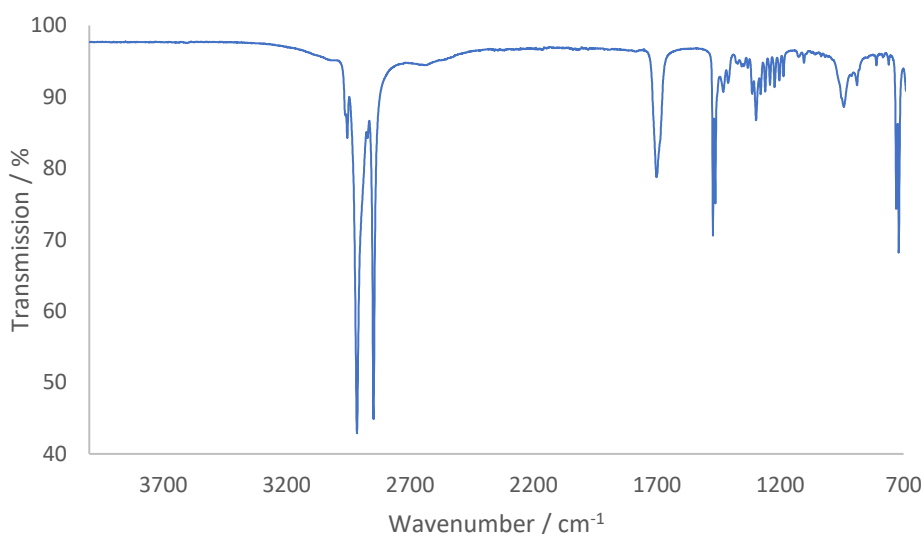


Figure 2-4 FT-IR spectra of dotriacontane mixed with stearic acid 50: 50 % w/w

ATR-FT-IR was used to evaluate samples composition and the chemical nature of single component waxes: dotriacontane, 1-docosanol and stearic acid waxes, and binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid within this PhD. Critically it enabled identification of contamination post-printing. Additionally, FT-IR was used to evaluate the crystallinity of the wax samples in both the powder and printed state.

2.5. X-ray Diffraction (XRD)

XRD is a common technique used to measure the nature and structure of crystalline or amorphous structures and identify the atomic spacing of molecules within a crystalline arrangement⁴³. Using XRD information on the crystalline structure, phase structure, lattice parameters, and crystalline grain size can be derived^{43,44}.

Crystalline materials present a regular periodic microstructure related to atomic or molecular distribution within the crystalline structure generating clear peaks in the XRD analysis⁴³. An amorphous structure is isotropic with no regular pattern or distribution, meaning, on average, the same properties are present in all directions, which display as a halo in XRD analysis of such materials⁴⁵. In contrast, crystalline structures are anisotropic and present different physical properties in all directions⁴³.

XRD probes these structural features by projecting an X-ray originating from a cathode ray tube before passing the X-ray through a monochromator and a collimator through the solid material, often as a powder. X-rays are rastered over the surface at a range of 2θ values at each point and are scattered elastically and inelastically⁴³. If the material comprises periodic crystalline structures, the material will act as a diffraction grating, and the x-rays will show constructive interference at small 2θ values for long repeated distances between crystalline structures^{43,46}. In addition, constructive interference will occur at high 2θ values for short repeating distances between individual molecules or atoms^{43,46}. The width of the peak can be used to give information on the grain size of the crystalline structure probed⁴⁴.

In this PhD, XRD was used to probe the crystalline structure of the powder and printed wax films to investigate if the printing process-induced changes to the waxes' crystalline properties. Furthermore, XRD was used to investigate the correlation between crystallinity and crystal structure and the film's physical properties. It is through the crystalline arrangement and % crystallinity of the cuticle waxes is correlated with the permeation of lipophilic agrochemicals⁴⁷. Beyond this, XRD was utilised to investigate the eutectic crystalline structures of the wax in both powder and printed form.

Similar XRD measurements of reconstituted and powdered waxes have been extensively studied^{48,49}, with a recent study cataloguing an extensive range of extracted and native cuticle waxes^{46,50}. It is observed that the XRD diffractograms of closely related crystalloids on the cuticle surface of different species can show extensive differences^{46,50}, and there is no clear correlation of crystalloids structure as a function of XRD diffractograms, unlike what was originally proposed⁴⁹.

2.6. Drop size analysis (DSA)

Contact angle, defined as the angle between the tangent observed at the gas-liquid interface at the intersection point of gas, liquid and solid through the liquid phase and the solid-liquid interface (Figure 2-5), is frequently used to evaluate the wettability of solid surfaces⁵¹. The contact angle is modelled assuming a non-reactive droplet on an ideal surfaces' contact angle will be determined by an equilibrium of three interfacial tension at the gas, liquid and solid intersection. The Young equation describes this using Equation 2-5:

Equation 2-5

$$\gamma^{sv} = \gamma^{sl} \gamma^{lv} \cos \theta$$

Where γ^{sv} is the solid-vapour interface tension, γ^{sl} is the solid-liquid interface tension, and γ^{lv} is the vapour-liquid interface tension, and θ is the contact angle.

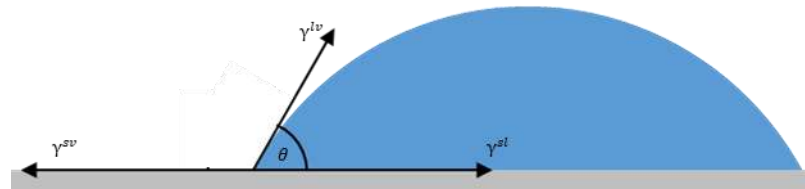


Figure 2-5 Schematic of the triad of interfacial tensions present at the edge of a droplet.

The contact angle can derive important information regarding surfaces' properties and interactions with dispensed liquids. For example, contact angles can be observed over time to investigate droplet evaporation⁵² or evaluate a surface response to the applied liquid⁵³. If the droplet induces modification of the substrate properties, it might be expected that changes in contact angle will be induced⁵³. Furthermore, the droplet's wetting mode, Wenzel or Cassey-Baxter, can be derived by observing the run-off angle to indicate the nature of droplet pinning⁵⁴.

Beyond the measurement of sessile droplets, it is possible to determine the advancing and receding contact angle by dispensing further liquid into a sessile droplet⁵³. This describes the contact angle where surface-droplet pinning is overcome, and the further addition or removal of liquid causes the surface area of the droplet-substrate interface to increase or decrease, respectively^{53,55}. On an ideal flat surface, the lack of pinning induced by surface roughness means the addition or removal of liquid would cause an immediate change in droplet-substrate interface surface area. Subsequently, these measurements allow the investigation of pinning and surface roughness. This is shown in the measurement of contact angle hysteresis, the difference between the advancing and receding contact angle that is

generally caused by surface topography where heterogeneous surface structures present barriers to the movement of the contact line between droplet and substrate^{53,56}.

A drop size analyser measures contact angle by depositing a known quantity of liquid onto a surface of interest. This is achieved using a driver motor, inducing the deposition of a droplet of known volume from a syringe equipped with a straight blunt needle of known internal diameter. The substrate is subsequently driven upwards to meet and extract the pendant droplet. Subsequently, images of the droplet are taken at a known frequency after droplet transfer to observe changes in the contact angle over time. This is achieved by drawing a baseline between droplet and substrate and fitting the droplet shape to known models of sessile droplets wither within independent software, i.e. ImageJ, or within software equipped to the DSA.

For this thesis, the wettability of a surface was defined with respect to the contact angle. Surfaces were highly hydrophilic if the contact angle was $< 40^\circ$, highly wettable if the contact angle was $40^\circ < n < 90^\circ$ and wettable if the contact angle was $90^\circ < n < 110^\circ$. If the contact angle identified was $110^\circ < n < 130^\circ$, the surface was defined as non-wettable and highly non-wettable if the contact angle was $130^\circ < n < 150^\circ$.

Static contact angles were collected from the top and bottom surface of the printed wax films using Mili-Q water and a range of surfactant solutions. Static contact angles were used to identify how the contact angle of a range of liquids changed over time and to understand how print topography and film composition affected the spreading of applied droplets.

2.7. Rheology

Rheology is broadly understood as the study of flow within a fluid. It can be defined as the non-recoverable deformation of an infinitely small aspect relative to adjacent points⁵⁷. The primary deformation to consider in this report is shear flow, the deformation of hypothetical lines within the fluid relative to one another⁵⁷. This phenomenon can widely be seen when fluid passes through a tube where fluid near the casing will have a lower velocity due to interaction with, and resulting shear on, the internal tubing surface.

Shear flow can be described using shear deformation (γ) and shear stress (σ). The shear deformation is described as the deformation of a unit rectangle of fluid into a parallelogram through force, where the total angular change measures deformation. Shear stress is the force per this unit area required to induce this deformation and generate a velocity within the fluid. Velocity depends on the distance from the flow and the presence of stationary

elements, generating a layered structure wherein different fluid layers possess different velocities, which increase as the measurement moves away from the stationary element⁵⁷. This variation in velocity is defined as shear rate, whereas resistance to this flow is the dynamic viscosity (η).

As per viscosity measurements, two forms of liquids exist, Newtonian and non-Newtonian. Newtonian liquids display a uniform viscosity as a function of shear rate at the measured shear rate values⁵⁷. It is not appropriate to describe a liquid as perfectly Newtonian, as it is predicted that all Newtonian liquids will present non-Newtonian properties at sufficiently high shear rates⁵⁷. Instead, materials should be described as presenting Newtonian properties at the tested shear rates. The viscosity of Newtonian fluids is defined by Equation 2-6:

Equation 2-6

$$\sigma = \eta \dot{\gamma}$$

Non-Newtonian fluids present a non-linear relationship between shear rate and viscosity, including shear thinning, where the viscosity decreases exponentially with increasing shear rate or shear thickening, where viscosity increases exponentially with increasing shear rate⁵⁷, Figure 2-6. This is a simplification, and many liquids present some combination of these two properties.

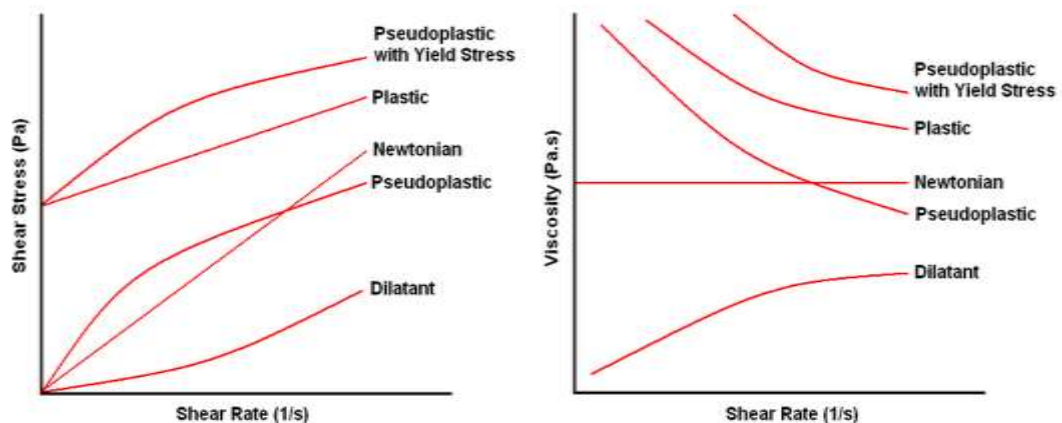


Figure 2-6 The rheological relationship between Shear rate and Shear Stress for a series of fluid classifications and the relationship between Shear rate and viscosity for a series of fluid classifications⁵⁸.

In most cases, non-Newtonian properties are observed due to a micro-structure present within the fluid before testing or develops during testing. For shear-thinning materials, an internal microstructure causes inter-particle attraction or entanglement. As the shear rate increases, these structures break apart, thus reducing viscosity⁵⁹. Similar properties are

observed within fats where fat crystal networks act as a solid due to the complex molecular packing of wax crystals until deforming stress exceeds the yield value, and viscous flow is induced⁵⁹. After this, further increases of shear rate shows no change in viscosity as the fluid will behave as a Newtonian fluid following the breakdown of fat crystal networks⁵⁹. Such materials are characterised as Bingham plastics when apparent yield stress must be overcome to induce flow, as seen with fats and waxes^{57,59}.

To collect data regarding the viscosity of a sample, a rheometer is one of the most common analysis methods. A rotational rheometer was used in preference to a shear rheometer as rotational rheometers better replicate the conditions experienced by the fluid as it passes through the nozzle⁶⁰. Rotational rheometers work by compressing liquid samples between a cup, a substrate that holds the sample, and a bob, a substrate that acts on the sample and measures the viscous response⁵⁷. Many rotational rheometers use a plate and cone/plate as the bob and cup, respectively. The cone and plate arrangement geometry is the most common as it ensures the shear rate experienced across the sample is the same as per Equation 2-7⁶¹.

Equation 2-7

$$\sigma = 3T/2\pi a^3$$

Where T is the torque, which is known to increase with distance from the centre, and a is the cone's diameter at the tested location.

Unfortunately, rotational rheometers are limited to shear rates of 10^3 s^{-1} , whereas the shear rate at the printhead nozzle is as high as 10^7 s^{-1} ⁶². Subsequently, it was critical to understand the flow properties of the tested waxes. Rheometers cannot readily exceed 10^4 s^{-1} shear rates due to residual heat build-up at higher shear rates⁵⁷. Increased temperatures decrease viscosity which would impact the measured results⁶³.

As already stated, waxes and fats are a form of non-Newtonian fluid called Bingham plastics. Many models have been produced attempting to measure the viscosity of similar non-Newtonian fluids, the selection of which depends on the material studied and shear rates utilised. The cross model, described by Equation 2-8, is the most general description of time-independent shear thinning behaviour using two constants, K' and m , in addition to η_0 and η_∞ , the viscosity at the asymptote of a very low and very high shear rate respectively⁶⁴.

Equation 2-8

$$\frac{\eta_0 - \eta}{\eta - \eta^\infty} = (K' \dot{\gamma})^m$$

Using such models, it is possible to predict the viscosity of a Bingham plastic at shear rates that would not be possible to measure using a traditional rotational rheometer and thus predict viscosity at values relevant to the nozzle of the Spectra printhead used⁵⁹.

In this PhD, rheology measurements allowed the prediction of ink printability between 70 and 90 °C. By measuring the shear rate between 10^{-1} - 10^3 s⁻¹, the fluid properties of the inks were identified, and shear viscosities at the nozzle were predicted. Using this, it was possible to identify if the ink would be printable at the tested conditions and if inks were liable to cause damage to the printhead.

2.8. Time of Flight secondary ion mass spectroscopy (ToF-SIMs)

ToF-SIMs is a highly surface-specific and sensitive analytical technique used to characterise and analyse a surface's chemistry⁶⁵. Unlike alternative techniques used to probe surface composition, it can precisely determine the surface chemical composition through identifying analyte fragments⁶⁶. Similar techniques struggle to achieve this, for example, energy-dispersive X-ray spectroscopy can only determine the elemental composition⁶⁷, whereas Raman spectroscopy struggles to differentiate similar compounds such as waxes of different chain lengths^{68,69}.

This is achieved by using a primary ion beam that bombards the surface and is rastered within an ultra-high vacuum. This bombardment results in a collision cascade and the formation of secondary ions of the surface molecules, with the generated ions either being whole molecules, molecular ions, or fragments of the original surface composition⁶⁶. These ions are subsequently attracted towards the detector. Subsequently, the different mass/charge ratios of the ion cause different acceleration of ions, with lighter ions accelerating more rapidly, resulting in separation. Most ions generated in the collision cascade are singularly charged, either negative or positive, as such ions are separated based on the ion's molecular weight, not charge. This sputtering and subsequent detection of different weight ions generates a spectrum of ions at each location the primary beam is directed to, thus generating a spatial image of ion distribution and thus chemical composition across the surface⁶⁶.

It should be noted that only approximately 1% of the material the ion beam sputters from the material surface will become charged due to the collision cascade. Most of the material sputtered remains uncharged and is vented as uncharged waste, which cannot be accelerated and thus separated based on weight⁶⁶. Due to the highly destructive nature of this test, a

maximal ion intensity is recommended wherein one primary ion is generated per 10 nm² of the sample. This is called the static limit⁶⁶.

Each pixel in the produced image of chemical composition presents a unique spectrum depending upon the chemical composition of that pixel. The resolution of individual pixels ranges between 0.2 µm - 1 µm⁶⁶ with modern Orbi-SIMs systems capable of even greater resolutions⁷⁰. Further to this, by repeatedly sputtering the exact location, either as a single point or as part of a larger image, it is possible to progressively penetrate the sample such that different depths of the surface can be imaged⁷¹. Using this, compositional changes⁷¹ or the movement of exogenous compounds through the sample can be imaged⁷². Figure 2-7 shows the general construction of a ToF-SIM system.

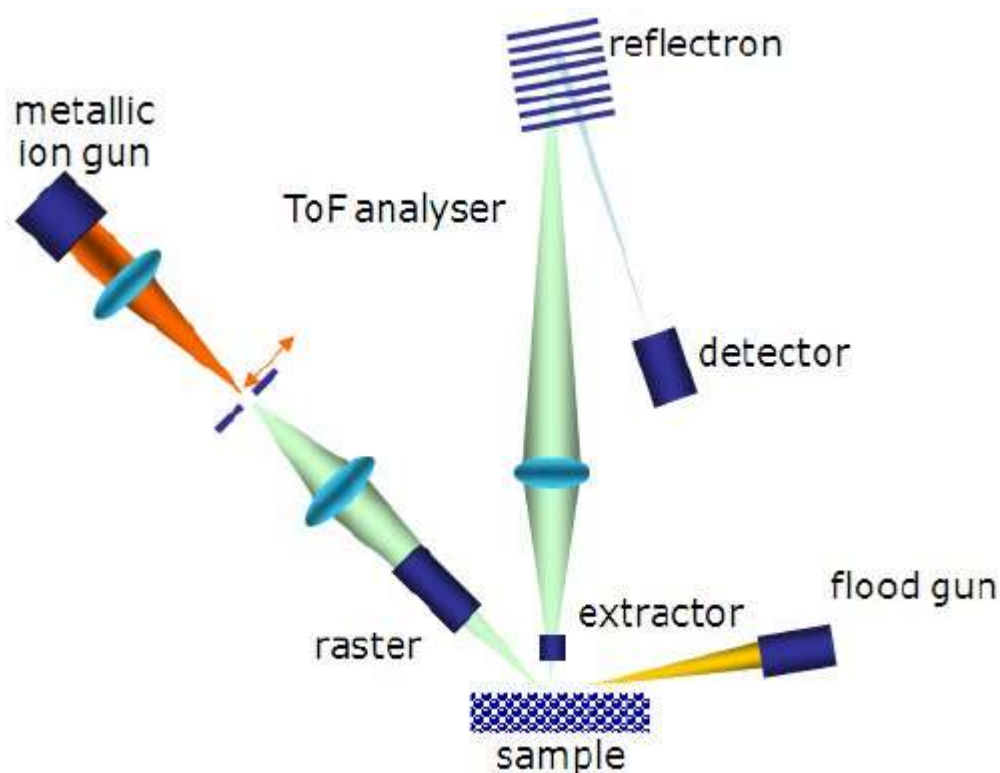


Figure 2-7 Schematic of the ToF-SIMs assembly⁶⁶.

Regarding the interactions of the primary ion beam with the surface, it is essential to understand that the surface ideally should be conductive. As the primary ions interact with the surface, these ions will increase the sample's surface potential. If the sample is not conductive, this leads to charge accumulation, decreasing or eliminating secondary ion signals for many ions that would otherwise be emitted⁷³. An electron flood gun irradiates the sample's surface with low energy electrons to minimise or eliminate this effect. These electrons will be attracted towards the now positively charged regions of the sample and

eliminate the positive charge⁶⁶. However, it is essential to keep the electron dose below 6.3×10^{18} to minimise surface degradation or electron-simulated ion emissions between pluses of primary ions irradiation⁷⁴. A technique used either in tandem or as an alternative is using a fine metal grid in close contact with the insulating material imaged⁶⁶.

Once the samples have been sputtered, it is crucial to understand that the electrical state of the region sputtered can affect the nature of the spectra produced through changing the potential of different ions to be formed and the intensity at which they are generated. This is termed the matrix effect^{66,75,76} and means different chemical environments containing the same material concentration will observe changes in the ionisation of this material resulting in either suppression or enhancement of the materials ions intensity^{75,76}. This means ion intensity is not uniformly correlated to the concentration of the original molecule in a range of different chemical environments, and thus determination of absolute surface concentration is challenging⁷⁷. As a result, ToF-SIMS is not a quantifiable system as standards of the analyte to be quantified will be challenging to produce such that the matrix effect is not introduced⁷⁶. Subsequently, standards often show the intensity of the secondaries that is not directly proportional to the concentration. An additional disadvantage of ToF-SIMS compared with alternative imaging techniques such as Raman is that imaging can only be conducted in an ultra-high vacuum environment. Subsequently, in vivo imaging of biological tissues is impossible, and the experimentation and operation are very costly, limiting wide-scale adoption of ToF-SIMS⁶⁵.

For these reasons, in this thesis, quantification of ToF-SIMS data will not be sought. Instead, ToF-SIMS will be used to guide material selection. Regarding the PhD goals and the use of ToF-SIMS principally, ToF-SIMS will be used to evaluate the chemical composition of waxes present on the epicuticle and intracuticle surfaces of the *Solanum Lycopersicum* leaf cuticle surfaces. This information will identify insight into heterogeneous wax distribution, and a range of commercial waxes will be selected for further analysis and printing.

2.9. Scanning electron microscopy (SEM)

Scanning electron microscopy is an imaging technique that utilises a beam of electrons to image surfaces and generates maps of elemental composition, topographical features and grain structures^{78,79} within a high vacuum environment. Resolutions achievable can be as high as 0.5 nm for speciality research equipment⁸⁰. Additionally, SEM variants have been developed, cryo-SEM and environmental SEM as examples. Such variants can image samples

within a low vacuum environment and a range of biological samples with minimal sample degradation that would be induced in a standard SEM^{81,82}.

SEM is often used when imaging a sample using an optical microscope is deemed unsuitable for reasons such as optical microscopes' relatively poor resolution, depth of focus or where the sample could not be readily imaged, i.e. transparent samples. Some of the key advantages of SEM leading to its widespread adoption include the capability for sub-micrometre resolution and minimal depth of field⁷⁸.

The operation of SEM is a complex topic; however, a brief overview will be given. SEM operates by generating an electron beam by removing electrons from a source, tungsten thermal filament sources being the most commonly utilised⁷⁸. Electrons are generated through a thermionic process wherein the filament is heated using a high-voltage anode to generate free electrons across the filament, which are attracted to an applied strong magnetic field⁷⁸. The filament is designed to focus free electrons to a point forming a beam as they are liberated from the filament. The generated beam is subsequently collimated using a series of electromagnetic lenses. The final focused beam is rastered over the surface of interest using a series of variable electromagnet lenses, so the beam contacts all areas of interest⁸³. The size of the raster pattern depends on the user's desired magnification, with smaller regions rastered at high magnifications⁸³.

The incident electron beam penetrates the sample to a depth of several micrometres as determined by the material density and the accelerating voltage applied onto the electron beam resulting in the generation of a teardrop-shaped volume of interaction (Figure 2-8). Subsequently, a series of radiation and electrons are produced from different locations within the teardrop volume of interaction⁸⁴. This occurs as electrons are liberated from the outer shells of atoms on or near surfaces upon contact with the electron beam due to the energy transfer event occurring upon contact. Subsequent interactions with released electrons or radiation can trigger the release of further radiation from alternative positions within the volume of interaction⁸⁴.

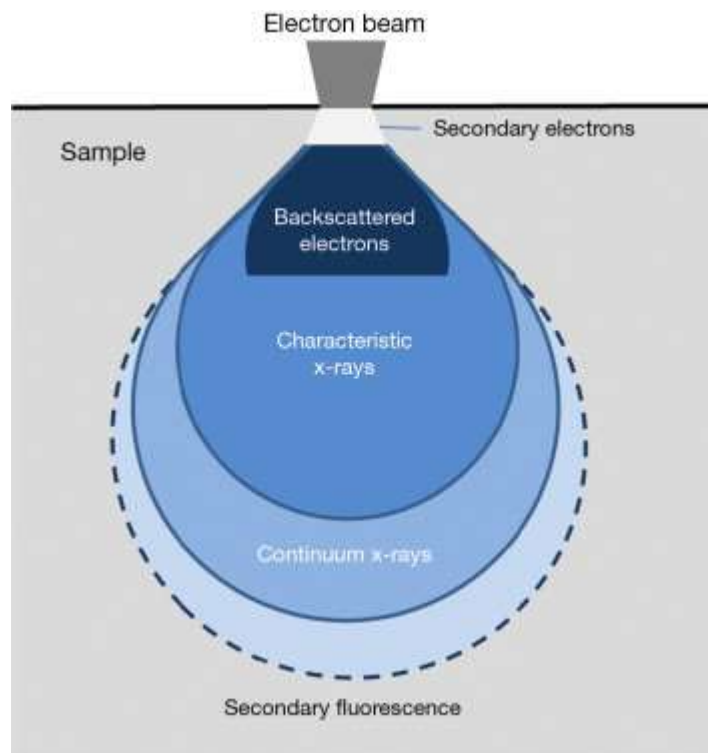


Figure 2-8 Teardrop-shaped volume of interaction between the incident electron beam and substrate of interest⁸⁴.

Generated X-rays, Auger and backscattered electrons enable specific properties of the sample to be probed: bulk chemical elemental analysis⁸⁵, surface elemental analysis⁸⁶ and grain structure⁸⁷, respectively. Secondary electrons generated from the conduction or valence bands of the substrate material are imaged using a photomultiplier system used to create a map of secondary electrons generated as a function of location. Given that steep surfaces and edges interact with the incident beam to a more considerable extent, they present a greater surface area to the beam due to the sloped texture meaning more secondary electrons will be generated from these locations, subsequently allowing topographical features to be mapped. The intensity of secondary ions detected as each area is converted into a greyscale image wherein scale is determined by electron intensity^{84,88}. Figure 2-9A shows an example of a secondary electron SEM image showing the topographical features of the impregnated and projected cuticle wax crystalloids from the cutin matrix. Figure 2-9B however shows the cutin matrix following wax extraction. The change in morphology is an indication the cuticle wax component drives the formation of sub features such as crystalline surface structures, Figure 1-2, that protrude from the cutin matrix. It also indicated following removal of these waxes that the general structure is retained suggesting the cutin/cutan matrix forms the bulk structure such as coating of the periclinal cell walls and impingement into the void spaces between anticlinal cell walls. Considering Figure 2-9 it is clear SEM is a powerful tool to observe morphology and can be used to observe

morphological changes within a single samples or to compare the same sample following treatment via a secondary method as shown in Figure 2-9 before and after wax removal via solubilisation.

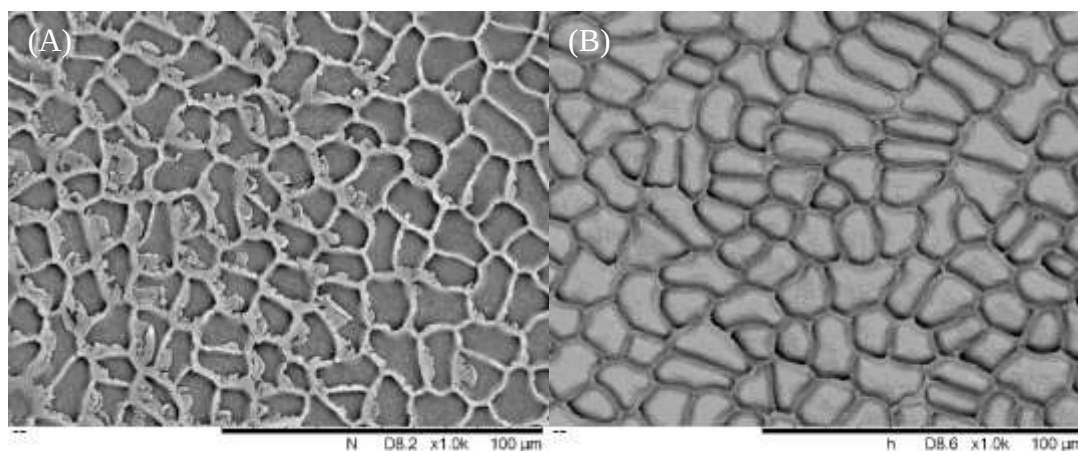


Figure 2-9 Secondary electron image of the intracuticle surface of Citrus Aurantium (A) untreated and (B) dewaxed following 60 seconds of exposure to chloroform.

Unfortunately, to image a sample, the surface must be conductive. Non-conductive surfaces rapidly show charge building upon the sample surface, obscuring the image or disrupting the electron beam. As such, samples must either be naturally conductive or coated in a conductive material, often gold or platinum or rarely carbon or iridium⁸⁹. The sample must be capable of being exposed to a vacuum of at least 10^{-4} torr while undergoing limited or no changes in morphology that might affect the generated image. Subsequently, most biological samples cannot be imaged natively using SEM due to the need to coat samples and the degradation induced by the vacuum^{90,91}.

Within this PhD, SEM was primarily used to image produced wax films as composition and printing parameters were changed to understand the effect on surface topography. Additionally, SEM was used to observe the impact surfactants induced on the surface printed films over time.

2.10. Raman Spectroscopy

Raman spectroscopy is a chemical imaging technique based on a form of inelastic light scattering called Raman scattering⁹². Typically, upon incidence of light with matter, the radiation will perturb the charge distribution resulting in a molecule in the ground state being excited to a higher virtual energy state. If the molecule relaxes from this virtual energy state back to the ground state, a photon is released equal to the energy inputted from the original interaction. This is called Rayleigh scattering and is an elastic scattering event⁹². Because the

energy released equals the initial energy inputted, information on the chemical composition of the material that induced the scattering cannot be determined⁹². Inelastic scattering occurs when the molecule in a virtual energy state relaxes to a different energy state than the ground state. The energy released will vary compared with the incidence photon, and thus, information can be derived. This is called Raman scattering⁹².

Two forms of Raman scattering exist, Stokes scattering, where the frequency of the incident photon was greater than the released scattered photon, and anti-Stokes scattering, wherein the incidence photon has a lower frequency than the released scattered photon⁹³.

It was determined that the presence of specific chemical functionalities would induce specific Raman scattering⁹². Subsequently, the wavenumber of the incidence radiation induces inelastic scattering in different bonds with the intensity of the observed scattering related to the concentration of the bond inducing scattering⁹². Subsequently, Raman scattering could be used to understand and determine the chemical functionality of different molecules based on the nature of the observed Raman scattering⁹². This is similar to the principles used to determine chemical composition using ATR-FT-IR. By rastering, a monochromatic beam of light at a surface of interest, the chemical composition is mapped by observing the locations where specific scattering events occur associated with different materials⁹³.

The most common form of Raman Spectroscopy measures vibrational Stokes bands and thus are the form of scattering typically used to analyse and map surfaces. Alternative techniques exist, including surface-enhanced, tip-enhanced, stimulation and transmission as examples⁹³.

As part of this thesis, Raman spectroscopy was used to observe the spatial chemical composition of different waxes within a binary printed wax film comprised of stearic acid and 1-docosanol. This was done to observe if phase separation was responsible for the extensive crystalloid structures observed on the surface of the wax film.

2.11. High-performance liquid chromatography (HPLC)

HPLC is one of the most common, powerful, and versatile chromatographic separation methods available and is frequently used to quantify the concentration of known compounds within a solution⁹⁴, identify the identity of unknown compounds⁹⁵ and separate materials within complex mixtures⁹⁶.

Reverse-phase HPCL operations involve the separation of materials contained within a solution of interest using an HPLC column. Material separation is achieved by injecting a small quantity of the solution of interest into a liquid flow comprised of an aqueous and organic

phase, called the mobile phase⁹⁷. The column separating materials has a 2.1 mm – 4.5 mm internal diameter and is 200 mm - 300 mm in length. The length of this internal space is packed with C₁₈ silica, the stationary phase, that interacts with solubilised material injected into the mobile phase. The degree of interaction dictates how long a material will take to pass through the column and is determined by a range of factors. These include analysis conditions such as type of column⁹⁸, column dimensions and experimental temperature⁹⁹ as well as a range of material physicochemical properties, principally the chemical composition and polarity¹⁰⁰. Typically materials with lower polarity are the first to elute as they interact with the stationary phase to a smaller extent compared with more polar molecules⁹⁷. In this way, the pH of the mobile phase is critical to control analyte ionisation and thus polarity¹⁰¹.

Following the separation of materials within the column, materials must be detected. The most common detection methods use a UV or UV-photodiode array (PDA), which directly detects material presence through observation of UV radiation absorbance¹⁰². Because the injected material is separated into discrete bands through interaction with the stationary phase, it is expected that materials can be discriminated from evaluating the UV absorbance at the timepoint they elute¹⁰². HPLC does not use a reference solution; thus, it is impossible to determine concentration using the Beer-Lambert law. Instead, UV radiation absorbed is measured at each time point and plotted against time, and either the area of the peaks generated or the peak height is measured. Peak area/height are correlated with concentration by measuring peak area/height following injection of a range of known concentrations of the injected material, allowing the production of a calibration curve of peak area/height vs concentration¹⁰³.

Increasingly more detection systems are being utilised, and detection systems are being utilised in tandem¹⁰⁴. Some of these include Electrospray ionisation detection or using a tandem system such as nuclear magnetic resonance or mass spectroscopy¹⁰⁵.

In this thesis, HPLC was utilised to measure azoxystrobin concentration within solutions of unknown concentration following Franz cell permeation and sorption of azoxystrobin into the wax film.

2.12. Franz cell penetration

Penetration, as measured using a static diffusion cell often called a Franz cell, is an *ex vivo* technique used to test the penetration of analytes through a membrane with respect to time. This technique is frequently used during the development of topical therapies¹⁰⁶ and follicular applied agrochemicals¹⁰⁷ to understand how changes to formulation and

environmental conditions affect active permeation and movement towards the site of action. Ideally, the results provided by permeation studies using *ex vivo* membranes will provide general predictions of *in vivo* results and can be used to guide more complex *in vivo* studies.

Franz cells operate by suspending a membrane of interest between two chambers, the donor and receiver chambers (Figure 2-10). The donor chamber is filled with a variable quantity of donor solution containing the analyte of interest. This solution can either be a liquid¹⁰⁷ or semi-solid¹⁰⁸, provided diffusion of the analyte into the membrane is possible. In almost all cases, the donor solutions at the start of a penetration test will contain 100% of the analyte, which will penetrate the membrane over the testing period as the analyte travels down its potential chemical gradient through the membrane of interest¹⁰⁹. Often, the quantity of donor solutions is relatively large. Thus, as the analyte diffuses into the membrane, the concentration will effectively remain the same and thus, boundary conditions between the donor and receiver solution are maintained. Subsequently, identification of penetration rates is readily possible as conditions are highly controlled^{107,109}, this is called unlimited dose testing¹¹⁰. By minimising the quantity of donor solution, it is possible to simulate live conditions more closely wherein boundary conditions change over time due to concentration changes or fluid drying, this is called finite dose testing^{110,111}. Finite dose testing is often done when testing against *in vivo* tests, such as when testing topical pharmaceuticals, whereas unlimited dose Franz cells are often used to understand broader changes such as the effect of formulation or environmental changes.

The solution found within the receiver chamber is called the receiver solution and is a sink solution used to encapsulate the analyte, so the free concentration of the analyte within the receiver solution is effectively zero¹¹¹. At set time points throughout the test period, small quantities of the receiver solution will be collected and stored to determine analyte concentration. The receiver chamber after each collection will be filled up with fresh fluid to replace the collected fluid¹¹¹.

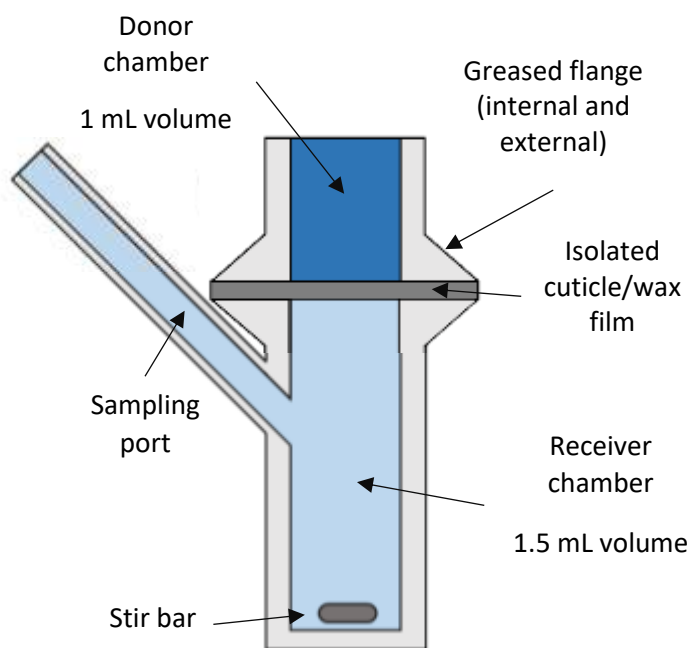


Figure 2-10 Schematic of a Franz cell.

If the membrane tested presents heterogeneous structures as a function of depth, the surface of interest must face the donor chamber, as seen when testing cuticles. Varying the surface of the cuticle exposed to the donor chamber can change permeation rate by orders of magnitude¹¹², with the epicuticle surface being termed by some as the "Limiting skin"^{112,113}.

This methodology, while relatively simple, requires the careful selection of membrane and solutions. Ideally, both should model live conditions. Subsequently, when testing agrochemical penetration, isolated cuticles are used with solutions containing no solvent that can degrade or derange the cuticle¹⁰⁷.

2.12.1. Relevant theory and modifying factors

Penetration results generated by Franz cells diffusion, often determined using chromatographic techniques such as HPLC, measure the ability of the agrochemical to penetrate through the cuticle. Agrochemicals penetrate the cuticle through different pathways depending upon the lipophilicity of the active^{113,114}. Most agrochemicals are lipophilic; thus, this work will focus on the lipophilic pathway, see Section 1.5.1. Movement through this pathway is driven by diffusion, and thus permeation occurs via Fick's first law¹¹⁵⁻¹¹⁷, Equation 2-9.

Equation 2-9

$$J_i = D_i \frac{dc_i}{dz}$$

Diffusion flux, J_i , measures how many molecules will penetrate the defined area during a set period. D_i , The diffusion coefficient is the molecular mobility of the diffusing molecule within the system tested. $\frac{dc_i}{dz}$ is the concentration gradient where c_i is the concentration and z the position and distribution of analyte. Considering this law, molecules will travel from regions of high concentration to areas with lower concentrations.

For this PhD, Franz cell penetration studies were conducted to evaluate the penetration rate of azoxystrobin, solubilised in a range of surfactant solutions through model wax cuticles produced as part of this PhD. The aim was to understand how penetration was modified by different wax compositions, print parameters, wax-wax interactions within the model film and wax-surfactant interactions. Subsequently, this data would be related to both future wax films, enabling the production of a model of any cuticle and enabling studying the agrochemical formulation-cuticle interactions that occur following application.

2.13. XTT assay

The XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)) assay directly measures cell and fungal viability and, indirectly, cytotoxicity¹¹⁸. This is achieved through measuring cellular respiration, as identified by the reduction of XTT to an active formazan derivative generated during respiration. This assay can directly measure the activity of NADPH oxidoreductase enzymes in the mitochondria enabling measurements of ATP synthesis¹¹⁹ and thus the presence of viable organisms. This assumes that viable organisms will constantly produce ATP via the NADPH oxidase enzyme, thus converting XTT into formazan derivatives that are detected and measured.

Principally this assay is used to measure cell death and viability for the high throughput screening of novel pharmaceuticals. Dead cells cannot undergo respiration, and thus the conversion of XTT would slow as the proportion of dead cells increases¹²⁰. Recent work has also used XTT and other tetrazolium-based assays to measure attachment to substrates added to the incubated cell medium, often aiming to identify attachment resistant materials¹²¹. Cells and fungal spores that fail to attach to a surface are washed away and thus cannot contribute to the conversion of XTT¹²¹.

Measurement of properties such as cell death or attachment is achieved with a colourimetric assay that directly measures the induced colour change as XTT is reduced, wherein the XTT salt is converted into an orange formazan derivative. The XTT assay shares many similarities

with other tetrazolium derivatives salt assays, such as the more well known MTT assay which differs from the XTT regarding the salt used in the assay¹²².

Concerning cell and fungal attachment studies, most have focused on identifying substrates with limited or no pathogenic attachment for healthcare applications¹²³. However, it is possible to investigate the fundamental relationship between substrate composition and topography vs attachment using such assays.

A broad methodology to test this might be to use solutions containing known concentrations of pathogens are grown and isolated before the test starts. This pathogen solution is centrifuged to form a supernatant which can be harvested and reconstituted at a known concentration. This pathogen solution will be placed within a well plate with the substrate of interest and allowed to incubate to test attachment. The cell culture is removed and washed such that all non-attaching pathogen is removed before being replaced with additional cell culture before further incubation and subsequent washing. This methodology ensures all the metabolic activity measured by the XTT assay relates to the pathogens attached to the substrate of interest after a known period with any contamination or non-attaching pathogen removed. At this point, both XTT and menadione are added to the solution in known concentrations and further incubated. The menadione (PMS) is an activation reagent that works as an intermediate electron acceptor during respiration, aiding XTT reduction to a formazan derivative that presents a yellow-brown colour capable of assessment using a spectrophotometer.

A colour change to yellow-brown indicates the presence of viable cells, whereas no colour change suggests the lack of viable cells¹²¹. The intensity of the colour is measured, allowing formazan derivative concentration determination and approximation of viable cells. When considering attachment studies, this approximation of viable cells is assumed to relate to the cells attached to the substrate of interest¹²¹. When no colour change is identified, it is thought that either the cells were removed in the prior washing stages signifying the cell did not adhere to the substrate or that the substrate is cytotoxic, resulting in cell death¹²¹.

One of the major issues with such techniques is the possibility of metabolic interference leading to false positives. To minimise false positives, secondary screens are often used to validate tests. Adhesion tests often forgo these secondary tests as they involve extensive washing protocols that likely would eliminate attached pathogens from the substrate¹²¹. Instead, it is presumed that contaminating cells and fungal spores are not present in extensive concentrations within the applied solution. Additionally, it is presumed that any

contaminant will not attach to the substrate of interest and thus will be removed during the gentle washing phase of the experiment, thus eliminating their potential to generate false positives.

In this PhD, it is proposed that the XTT assay can be used to develop an understanding of *Zymoseptoria tritici* (*Z. tritici*) attachment as a function of composition through measurements of attachment to wax surfaces modelling the epicuticle with known topography and composition.

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3. Chapter 3 - Selection and characterisation of wax-based printable inks

3.1. Introduction

The epidermis cell layer presented on the aerial surface of most plants is covered by a specialised membrane, the cuticle¹. This structure consists of two major components: cuticle waxes and a polyester called cutin². The wax components of the cuticle prevent insect-induced damage³, control water transpiration¹ and regulate the penetration of exogenous compounds⁴.

Cuticle wax can be found heterogeneously distributed throughout the cuticle². The surface presented to the environment, the epicuticle, comprises wax with no cutin biopolymer component². The underlying cuticle structure, the intracuticle layer, predominantly comprises cutin with wax impregnated throughout², forming the bulk of the cuticle mass. Polysaccharide fibres, projected from the epidermal basement cells, penetrate the intracuticle layer anchoring the cuticle to the cell layer⁵, Figure 3-1.

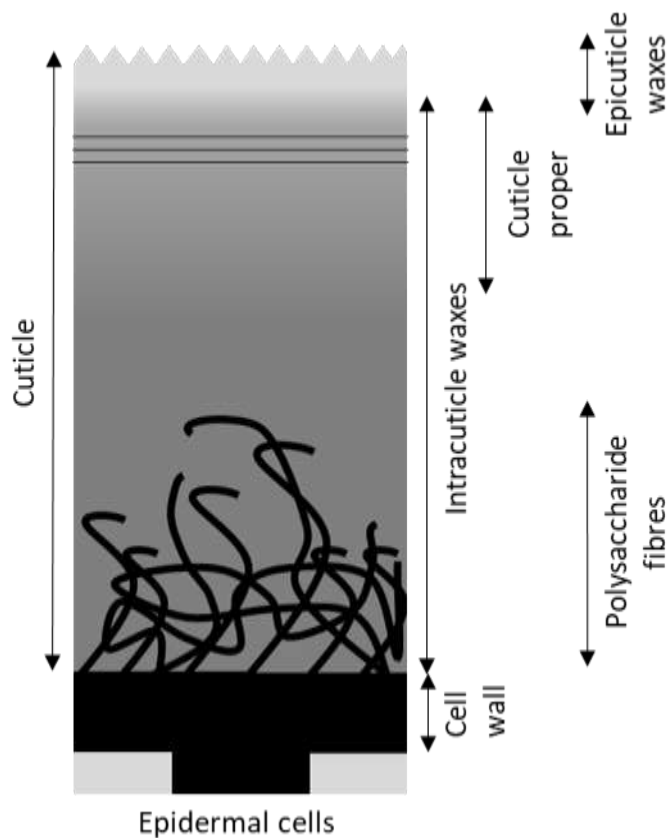


Figure 3-1 - Schematic representation of a cuticle.

Exogenous penetration through the cuticle by agrochemicals involves a three-step process: sorption-diffusion-desorption⁶. Agrochemicals sorb into the epicuticle wax, diffuse through the tortuous amorphous wax component and cutin matrix while being excluded from the bulk crystalline domains of cuticle wax, and then are desorbed from the intracuticle surface⁶. All three stages of penetration are thought to be affected by the cuticle wax composition resulting in agrochemical penetration rates varying between different cuticles⁷. Factors that modify wax composition include plant species⁸, the plant surface sampled, environmental conditions^{9,10} and the age of the plant. All these factors, therefore, are thought to modify agrochemical penetration rates¹⁰.

This difference in agrochemical permeation can be understood using two limiting factors for penetration: the cuticle wax-aqueous phase partition coefficient, $K_{w/w}$ representing the ability for the agrochemical to sorb into the cuticle, and the diffusion coefficient, D ($\text{m}^2 \text{s}^{-1}$) representing the rate of movement through the cuticle. Both factors are affected by wax composition¹¹. Unfortunately, details of the relationship between $K_{w/w}$, D , wax composition and cuticle morphology are poorly understood.

When developing novel agrochemicals, penetration testing of the active is often conducted *ex vivo* using enzymatically extracted isolated cuticle membranes¹². Such films have been used effectively with insignificant differences in agrochemical permeance observed through live leaves or isolated cuticles. Hence, isolated cuticles are considered a suitable whole leaf model regarding agrochemical and surfactant interactions.

However, the extraction process to produce isolated cuticles cannot be used on all plant or plant surface species. Furthermore, it requires the plant to grow to the development stage to be tested and often, only a small disk of the cuticle can be effectively isolated. Additionally, due to cutin extraction, isolated cuticle membranes are often too fragile to be used successfully^{13,14}. As such, high throughput analysis of agrochemical penetration through such plants is challenging due to a lack of a suitable model. This dramatically increases the cost and time required to develop novel agrochemicals and limits the utility of isolated cuticles.

Consequently, there is an incentive to develop a model to investigate penetration rates in a high throughput manner. Investigating the structure-function relationship of wax relative to penetration might enable the development of such an artificial model.

Prior work has shown that investigating the structure-function relationship of wax can generate useful information, including the location of the transpiration barrier^{15,16} and some broad trends between composition and penetration. Unfortunately, these studies are often

performed on cuticles genetically modified to induce slight controlled modification in the wax composition. This often induces additional undesired changes, i.e. morphologically modifying the cuticle¹⁷, undermining their use as an accurate model system. The impact of such modification on penetration is poorly understood, making analysis challenging¹¹. Additionally, modification of the wax composition affects the vast number of wax-wax interactions within the cuticle. These changes to composition result in poorly controlled changes to these interactions; the impact of this cannot be readily predicted.

Modifying the cuticle composition is expected to change the arrangement of the cuticle wax crystalline structure and the distribution of amorphous waxes within the cuticle. Given that penetration of lipophilic agrochemicals only occurs through the amorphous regions of the cuticle, even small composition changes impact permeation⁶. This is likely to be driven by changes to tortuosity¹⁸ induced by the change in crystalline structure and interactions.

This highlights the complexity of the cuticle system and the poorly understood impact of the wax composition. If the impact of changing composition and wax-wax interactions on the nature of permeation was understood, it might be possible to model wax matrixes found within cuticles that cannot be isolated. For this purpose, prior investigations have used binary wax films. This allows evaluation of the physical state of a wax barrier when two or more waxes can interact^{11,19} or the waxes response to exogenous compounds such as surfactants¹⁹. These films often form a eutectic phase, similarly to waxes within the native cuticle, and allow investigation of compositional and wax-wax interaction changes in a controlled manner. However, it is recognised that such films are more simplistic than the native cuticles. Additionally, no study of agrochemical penetration through such films has been conducted.

This chapter aims to identify and analyse waxes for use in a printable cuticle model. The selected waxes should, where possible, model the broad chemical heterogeneity of *Solanum Lycopersicum* fruit cuticle waxes concerning functionality and chain length. Such waxes would then be used with the intent of replicating the broad heterogeneous distribution of wax. Furthermore, the selected waxes will be investigated to elucidate if their physical properties will enable inkjet printing using a Spectra S-class printhead.

To achieve this, ToF-SIMS surface analysis was used to evaluate the composition of the epicuticle and intracuticle waxes of the *Solanum Lycopersicum* fruit cuticle. This data was then verified against bulk data in the literature. Three commercially available waxes that presented similar chemical functionality as the heterogeneously distributed waxes comprising the bulk of the *Solanum Lycopersicum* fruit cuticle wax mass were identified:

dotriacontane, 1-docosanol and stearic acid. A range of single component waxes: dotriacontane, 1-docosanol and stearic acid and binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid was generated, capturing a broad overview of possible wax-wax interactions found within the cuticle. The viscosity and melting point of the waxes were tested to assess printing viability. Because it is understood that the physical state and wax composition modifies the K_w/w and D of agrochemicals within the cuticle, DSC phase diagrams of the binary waxes were produced to better understand each binary wax's state. XRD and FT-IR were also used to assess the crystalline and compositional nature of the produced waxes. These same techniques were also used to evaluate if any chemical interactions had occurred between mixed waxes. The waxes were then drop cast to evaluate the morphology of the waxes and the impact of substrate temperature.

This is the first time this physical data has been presented regarding these waxes and associated binary mixtures. Prior work has exhaustively studied the composition of many species as a bulk wax composition; however, few reports detail the different wax compositions of the epicuticle and the intracuticle waxes²⁰.

3.2. Methods

3.2.1. Materials

Non wax materials used were *Solanum Lycopersicum* isolated cuticles, kindly donated by Syngenta (Jealott's Hill, UK), were taken from the adaxial surface of the fruit. Extraction was achieved by submersing fruit surfaces in 1% w/v cellulase and 1% w/v pectinase in a 0.02 M citrate buffer with 1 M sodium and left aside for three weeks before samples were removed and dried on clean glass slides²¹. Extraction is deemed successful if the membrane is translucent.

Wax materials used were 1-docosanol (98%), dotriacontane (97%) and stearic acid (98.5%) in powder form purchased from Sigma-Aldrich (Dorset, UK) and used without further purification. The chemical structure of these waxes can be found in Figure 3-2.

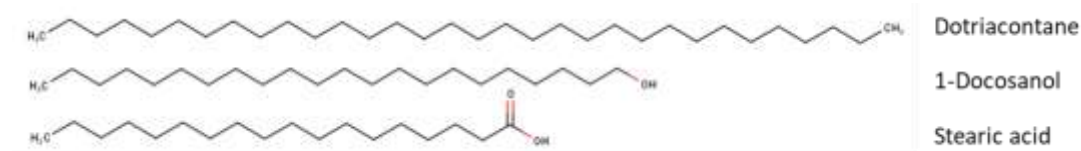


Figure 3-2 Chemical composition of the three waxes used

3.2.2. Preparation of binary waxes

Binary wax powders were prepared by mixing two dry waxes from dotriacontane, 1-docosanol or stearic acid, in a vial at ratios of 05: 95, 10: 90, 15: 85, 20: 80, 25: 75, 30: 70, 35: 65, 40: 60, 45: 55, 50: 50, 55: 45, 60: 40, 65: 35, 70: 30, 75: 25, 80: 20, 85: 15, 90: 10, 95: 05 % w/w. Binary wax powders were heated to 90 °C for 30 minutes while stirred at 200 rpm. The hot plate was then cooled to 40 °C to allow the waxes to solidify before being placed in a dark cupboard and cooled to room temperature overnight. The solidified waxes were grounded to a fine powder in a clean mortar and pestle without further sieving. Similarly, single component wax powders were heated, cooled, and ground as above to ensure each wax's thermal history was similar.

3.2.3. Drop cast wax films

Ground powder wax was heated to 90 °C in a glass vial until a molten liquid was produced. Approximately 100 mg of molten wax was placed onto PET placed on a hotplate held at 90 °C and allowed to spread until it reached spreading stopped on the heated PET substrate. The PET substrate and wax was moved physically onto a second hotplate held at 25 °C, 40 °C or 60 °C and allowed to cool for one hour. Using two hotplates, one held at 90 °C and a second at 25 °C, 40 °C or 60 °C and then physically moving the molten wax samples between them

meant the change in thermal environment could be induced almost immediately. Lowering the temperature on the original hotplate held at 90 °C to 25 °C, 40 °C or 60 °C would have taken a longer period of meaning cooling rate could not be modified. Subsequently, it would not be possible to investigate the effect of cooling rate on morphology.

Drop cast films were prepared to assess the effect of substrate temperature, and thus the cooling rate, and the impact of samples preparation on the morphology of the produced films.

3.2.4. Time of flight secondary ion mass spectroscopy (ToF-SIMs)

ToF-SIMS analysis was carried out on *Solanum Lycopersicum* fruit's epicuticle and intracuticle surface. Data was acquired with a ToF-SIMS IV instrument (ION-TOF, GmbH, Germany) equipped with a Bi_3^+ cluster primary ion gun with a single-stage reflectron mass analyser. Each ion was calibrated to an energy of 25 keV and pulsed with a current of approximately 1 pA. Surface charge compensation was applied using a flood of low energy, 20 eV, electrons using an electron flood gun. The primary ion beam was focused such that a lateral resolution of 2 μm was acquired when the beam contacted the surface with a primary ion dose of under $1 \times 10^{12} \text{ cm}^2$ to prevent deterioration of the static conditions. Spectra were acquired from a 500 μm x 500 μm area consisting of 256 x 256 pixels. Each of the presented areas was scanned five times to maximise the signal to noise ratio in negative and positive polarity.

Due to the complexity of spectra generated, a Multivariate analysis (MVA) method was used to process the data via principal component analysis (PCA)²². Datasets were exported from the surfacelab software to a general raw data format and imported into MATLAB 2018a, where it was loaded into simMVA to undergo MVA. Data was processed through peak alignment, normalisation by total ion count and a Poisson scaling²³.

ToF-SIMS and analysis using SimsMVA were performed to identify the general spectral differences between the epicuticle and intracuticle surface of the *Solanum Lycopersicum* fruit cuticle. Specifically, to investigate the heterogeneous wax distribution across the cuticle layers of *Solanum Lycopersicum* fruit cuticles and drive the selection of waxes for further analysis and printing.

3.2.5. Differential scanning calorimetry (DSC)

DSC was conducted using a DSC 8000 (Perkin Elmer, Waltham, USA) using a scanning rate of 1 °C /min, unless otherwise specified, between 50 °C to 75 °C. Samples were heated and cooled through this cycle twice. Only data from the second heating and cooling curve was

analysed to eliminate any effect of thermal stresses imparted onto the material during production.

Approximately 3 mg - 5 mg of material was weighed into an aluminium pan and hermetically sealed using an aluminium lid. An empty reference pan and lid were analysed simultaneously to identify the baseline. Gas flow was controlled at a flow rate of 20 mL/min of N₂. The DSC was calibrated using iridium ($T_m = 156 \pm 5^\circ \text{C}$, $\Delta H_f = 28.71 \pm 5 \text{ J/g}$) as per the DSC manual. The results were analysed using the Pyris software (Perkin Elmer, Waltham, USA), enabling identification of each wax's melting, solidus and liquidus point.

DSC was primarily used to identify the melting point of each tested wax to ensure it would be below the maximum operational temperature (90 °C) of the Spectra S-Class printhead. Further, it was used to investigate if the mixed waxes formed a eutectic structure by producing phase diagrams for each binary wax produced.

3.2.6. Fourier-transform infrared spectroscopy (FT-IR)

FT-IR was performed on a Frontier FT-IR equipped with an ATR crystal (Perkin Elmer, Waltham, USA). Samples were placed on the ATR crystal as a powder and compressed to maximise the contact area. Results were obtained from an average of ten scans between 4000 cm⁻¹ - 600 cm⁻¹.

FT-IR was used to identify diagnostic absorption peaks for each wax, peaks unique to each wax that could be used to identify the specific waxes tested and to confirm the waxes composition before printing or analysis. In addition, it was used to search for novel peaks or peak shifting that might result from chemical interactions between waxes, thus complementing the DSC evaluation of the eutectic nature of the wax. By evaluating the CH₂ rocking doublet, the crystallinity of each wax could be semi-quantified, and the impact of composition on crystallinity determined.

3.2.7. X-Ray Diffraction (XRD)

XRD patterns were collected using a Bruker D8 Advance with DaVinci X-ray Diffractometer (Bruker, USA). Approximately 1 g of single component and 75: 25, 50: 50 and 25: 75 % w/w binary wax was sieved using a 100 µm filter to minimise powder size polydispersity. Diffraction patterns were collected using a Bragg-Brentano geometry and a Lynxeye 1D detector. The scan rate used was 0.6 step/sec with 0.02 ° increment, and the scan range was from 2.1 to 50 °, using Cu Kα radiation ($\lambda = 1.5406 \text{ \AA}$) at 45 kV and 40 mA.

This technique was used to qualitatively compare the crystalline properties of wax powders by identifying potential amorphous halos and evaluating interplanar spacing. Such results would be used alongside FT-IR results to investigate crystallinity.

Beyond this, XRD was performed to investigate the formation of long and short distance order within the crystalline structure of the wax. Additionally, it was used further to investigate the potential eutectic nature of the wax.

3.2.8. Rheology

Using a KinexusPro rotational rheometer (Malvern, UK), Rheological testing was performed using a plate and cone geometry, PU40:PL65. Approximately 1 g of material was melted before the plate and cone were brought 0.2 mm apart and the excess material removed. Shear rate was increased from 1 s^{-1} - 1000 s^{-1} on each sample at temperatures between 70 - 90 °C with a testing interval of 5 °C. Each material was tested three times, and after each repeat, the sample was removed and replaced with a fresh sample.

Rheology was used to identify the dynamic viscosity of the tested inks and identify suitability for printing. The ideal dynamic viscosity for use in this work was between 8 cP and 20 cP as the recommended viscosity for use with a spectra S-class printhead.

3.2.9. Scanning electron microscope (SEM)

Scanning Electron Microscope imaging of the drop cast waxes was conducted by placing samples on conductive carbon tape. Before imaging, the samples were gold sputter-coated for 90 seconds at approximately 25 mA using an Agar sputter coater (Essex, UK). Imaging was conducted using a Hitachi TM3030 tabletop SEM (Tokyo, Japan). Samples were imaged at 15 keV between 50 – 2000x magnifications.

SEM was used to image the morphology of waxes solidified at different rates and thus to study the effect of cooling on morphology. Additionally, it was used to investigate whether different wax compositions affect surface morphology and the formation of surface crystallites related to the crystallites found on cuticle surfaces, Section 1.2.

3.3. Results and discussion

3.3.1. ToF-SIMS evaluation of isolated cuticles

Within the epicuticle and intracuticle layers, the wax composition is considered mainly homogeneous. Exceptions include triterpenoid wax accumulating above anticlinal cell walls²⁴ and pectin penetrating the intracuticle layer⁵. However, when comparing the wax composition between the epicuticle and intracuticle waxes, broad heterogeneity is reported allowing different waxes to control different processes²⁵. Despite this, most cuticles have not been analysed for wax composition in the epicuticle and intracuticle. Instead, only bulk composition has been measured. Even though *Solanum Lycopersicum* fruit is economically important, the wax composition has not been spatially analysed. For this reason, the epicuticle and intracuticle surface of the *Solanum Lycopersicum* fruit were imaged and analysed using ToF-SIMS and information regarding the differences in wax composition inferred.

Prior literature reporting bulk wax composition data for *Solanum Lycopersicum* fruit shows an abundance of n-alkanes and triterpenoids with smaller quantities of aldehydes, alkenes, alkanols and a range of alkanoic acids^{26,27}. Similar results are observed throughout the "Solanum" genus²⁸.

Based on bulk wax composition data, a series of waxes were identified that comprise most of the cuticle wax mass. Each wax's likely molecular ion observed in ToF-SIMS was determined based on literature fragmentation of similar chemical entities²⁹ (Table 3-1). These predictions are drawn from the observation that in the positive ToF-SIMS spectrum, alkanes form either the molecular ion (M)⁺ or lose two hydrogens (M-2H)⁺, alkan-1-ol form either the molecular ion (M)⁺ or lose two hydrogens (M-2H)⁺ and fatty acids lose either a hydroxyl group (M-OH)⁺ or gain a single hydrogen (M+H)⁺. In the negative spectrum, fatty acids lose hydrogen (M-H)⁻ whereas alkan-1-ols will form either an ion with one less hydrogen (M-H)⁻ or three fewer hydrogens (M-3H)⁻. Fragmentation of alkanes in the negative spectra gave no diagnostic molecular ion. Predicted fragmentation was specified as unknown when no appropriate reference spectra could be identified.

Table 3-1 Predicted fragmentation of known *Solanum Lycopersicum* waxes by ToF-SIMS.

Chemical Functionality	Wax	(+ Predicted fragmentation and ion mass		(-) Predicted fragmentation and ion mass	
Alkanoic acids	C ₃₀ H ₆₀ O ₂	C ₃₀ H ₅₉ O (436 u)	C ₃₀ H ₆₁ O ₂ (453 u)	C ₃₀ H ₅₉ O ₂ (451 u)	
	C ₃₂ H ₆₄ O ₂	C ₃₂ H ₆₃ O (463 u)	C ₃₂ H ₆₅ O ₂ (481 u)	C ₃₂ H ₆₃ O ₂ (479 u)	
Alkanols	C ₂₄ H ₅₀ O	C ₂₄ H ₅₀ O (354 u)	C ₂₄ H ₄₈ O (352 u)	C ₂₄ H ₄₉ O (353 u)	C ₂₄ H ₄₇ O (351 u)
	C ₂₅ H ₅₂ O	C ₂₅ H ₅₂ O (368 u)	C ₂₅ H ₅₀ O (366 u)	C ₂₅ H ₅₁ O (367 u)	C ₂₅ H ₄₉ O (365 u)
	C ₂₆ H ₅₄ O	C ₂₆ H ₅₄ O (382 u)	C ₂₆ H ₅₂ O (380 u)	C ₂₆ H ₅₃ O (381 u)	C ₂₆ H ₅₁ O (379 u)
	C ₂₇ H ₅₆ O	C ₂₇ H ₅₆ O (396 u)	C ₂₇ H ₅₄ O (394 u)	C ₂₇ H ₅₅ O (395 u)	C ₂₇ H ₅₃ O (393 u)
	C ₂₈ H ₅₈ O	C ₂₈ H ₅₈ O (410 u)	C ₂₈ H ₅₆ O (408 u)	C ₂₈ H ₅₇ O (409 u)	C ₂₈ H ₅₅ O (407 u)
	C ₃₂ H ₆₆ O	C ₃₂ H ₆₆ O (466 u)	C ₃₂ H ₆₄ O (464 u)	C ₃₂ H ₆₅ O (465 u)	C ₃₂ H ₆₃ O (463 u)
	C ₃₄ H ₇₀ O	C ₃₄ H ₇₀ O (494 u)	C ₃₄ H ₆₈ O (492 u)	C ₃₄ H ₆₉ O (493 u)	C ₃₄ H ₆₇ O (491 u)
Alkenols	C ₂₂ H ₄₄ O	Unknown		Unknown	
	C ₂₄ H ₄₈ O	Unknown		Unknown	
	C ₂₆ H ₅₂ O	Unknown		Unknown	
Alkane	C ₂₈ H ₅₈	C ₂₈ H ₅₈ (394 u)		Unknown	
	C ₂₉ H ₆₀	C ₂₉ H ₆₀ (408 u)		Unknown	
	C ₃₀ H ₆₂	C ₃₀ H ₆₂ (422 u)		Unknown	
	C ₃₁ H ₆₄	C ₃₁ H ₆₄ (436 u)		Unknown	
	C ₃₂ H ₆₆	C ₃₂ H ₆₆ (450 u)		Unknown	
	C ₃₃ H ₆₈	C ₃₃ H ₆₈ (464 u)		Unknown	
Alkene	C ₃₃ H ₆₆	Unknown		Unknown	
Iso-alkane	C ₃₁ H ₆₄	Unknown		Unknown	
	C ₃₂ H ₆₆	Unknown		Unknown	

Many of the produced peaks will likely overlap due to the compositional complexity and chemical similarity of cuticle waxes within the cuticle. Thus, the identification of diagnostic ions for each wax is critical. As Table 3-1 shows, the molecular ions often retain the original molecules' chemical functional groups, COO and OH, as examples, following fragmentation. As such, the risk of the ion peak of the molecular ion overlapping another ion is reduced as the peak is more diagnostic of the original molecule than a fragment lacking this chemical functionality. Such ions can be used as diagnostic signals to identify the wax in the complex spectra produced by imaging a cuticle. Reference spectra were not used, as purchasing or extracting specific cuticle waxes was deemed too expensive or impractical.

Figure 3-3 and Figure 3-4 show positive and negative ToF-SIMS spectra of *Solanum Lycopersicum* epicuticle and intracuticle surfaces normalised by total ion count. Epicuticle and intracuticle waxes show apparent spectral differences, likely due to chemical composition differences and related matrix effects³⁰. The matrix effect describes changes in how chemical fragmentation occurs in ToF-SIMS and the intensity of produced ions as a function of the chemical environment. Critical differences in the positive spectra between epicuticle and intracuticle surfaces are seen in Figure 3-3, including highlighted peaks: 23, 43 and 63 u. Comparing negative spectra (Figure 3-4), we see the two surfaces are similar with the expectation of the 24, 44 and 48 u highlighted peaks.

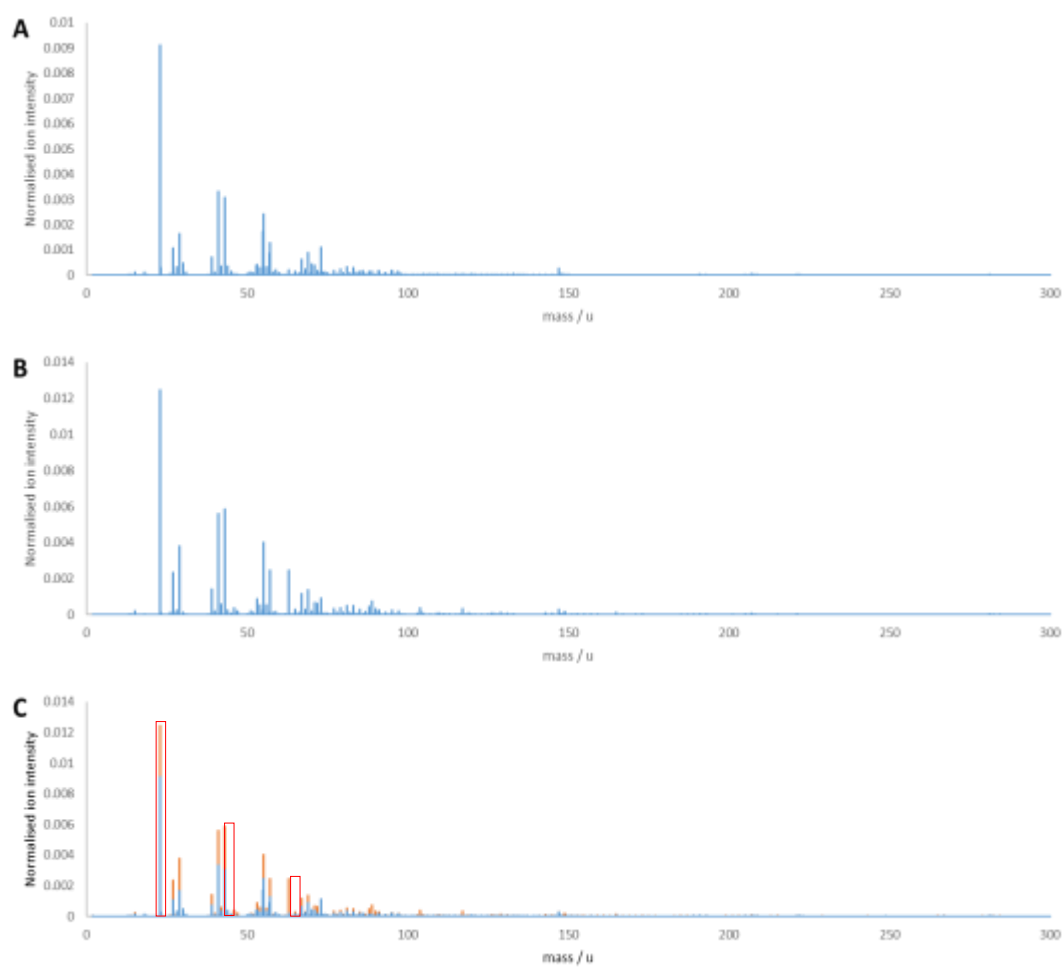


Figure 3-3 - Typical positive ion ToF-SIMS spectra recorded from the epicuticle (A) and intracuticle (B) surface of the *Solanum Lycopersicum* fruit cuticle. (C) shows the overlay of these two spectra, orange bars representing the intracuticle and blue bars representing the epicuticle surfaces. The 23, 43 and 63 u peaks are highlighted in red boxes

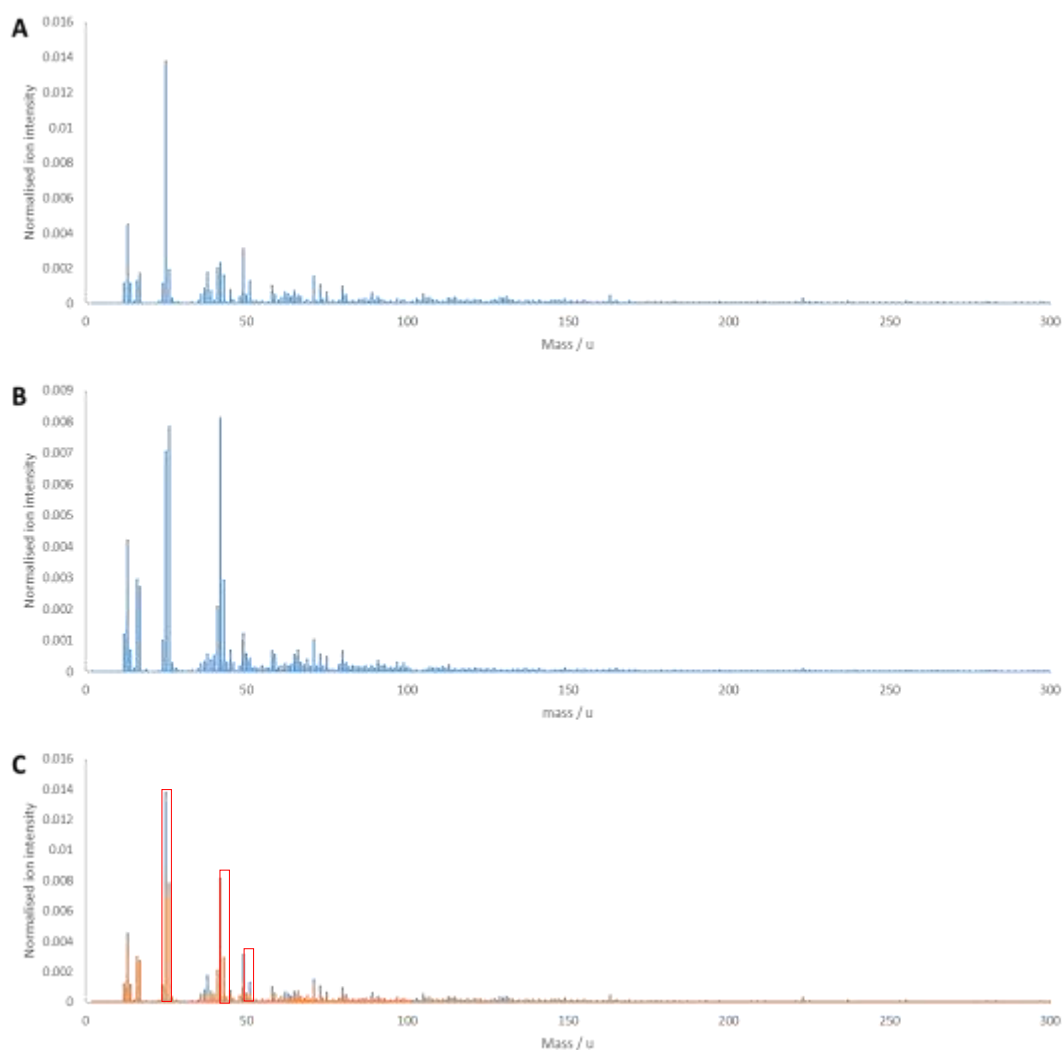


Figure 3-4 - Typical negative ion ToF-SIMS spectra recorded from the epicuticle (A) and intracuticle (B) surface of the *Solanum Lycopersicum* fruit cuticle. (C) shows the overlay of these two spectra, orange bars representing the intracuticle and blue bars representing the epicuticle surfaces. The 24, 42 and 48 u peak highlighted in a red box

While apparent differences can be observed at low ion mass values, the predicted fragmentation suggests that key fragment ions should present at higher ion masses (Table 3-1). Figure 3-5 shows ToF-SIMS epicuticle and intracuticle spectra overlay in the positive and negative spectra at the relevant mass range, 240 u – 500 u. It becomes clear that multiple peaks differentiate the two surfaces, representing fragmentation ions of waxes that are either specific to a single surface or present in higher quantities on one cuticle surface. Considering only peaks identified in Table 3-1 in the positive spectra peaks 368 u ($C_{25}H_{52}O$), 382 u ($C_{26}H_{54}O$), 396 u ($C_{27}H_{56}O$), 410 u ($C_{28}H_{58}O$) and 450 u ($C_{32}H_{66}$) are more intense upon the epicuticle surface. Considering the negative spectra peaks 353 u ($C_{24}H_{49}O$), 367 u ($C_{25}H_{51}O$), 381 u ($C_{26}H_{53}O$), and 395 u ($C_{27}H_{55}O$) present a greater intensity on the epicuticle surface, whereas peaks 451 u ($C_{30}H_{59}O_2$) and 479 u ($C_{32}H_{63}O_2$) present greater intensity on the intracuticle surface.

Whether this increased ion intensity is induced by the matrix effect or a more significant amount of fragmented chemical on specific surfaces is unknown. In all cases, the ions identified in Table 3-1 were homogeneously distributed across the surfaces. Prior literature showed a heterogeneous lateral distribution of cyclic waxes above anticlinal cell walls^{31,32}. This suggests that similar heterogeneity is either not present in the fruit of *Solanum Lycopersicum* or not observed with the identified waxes.

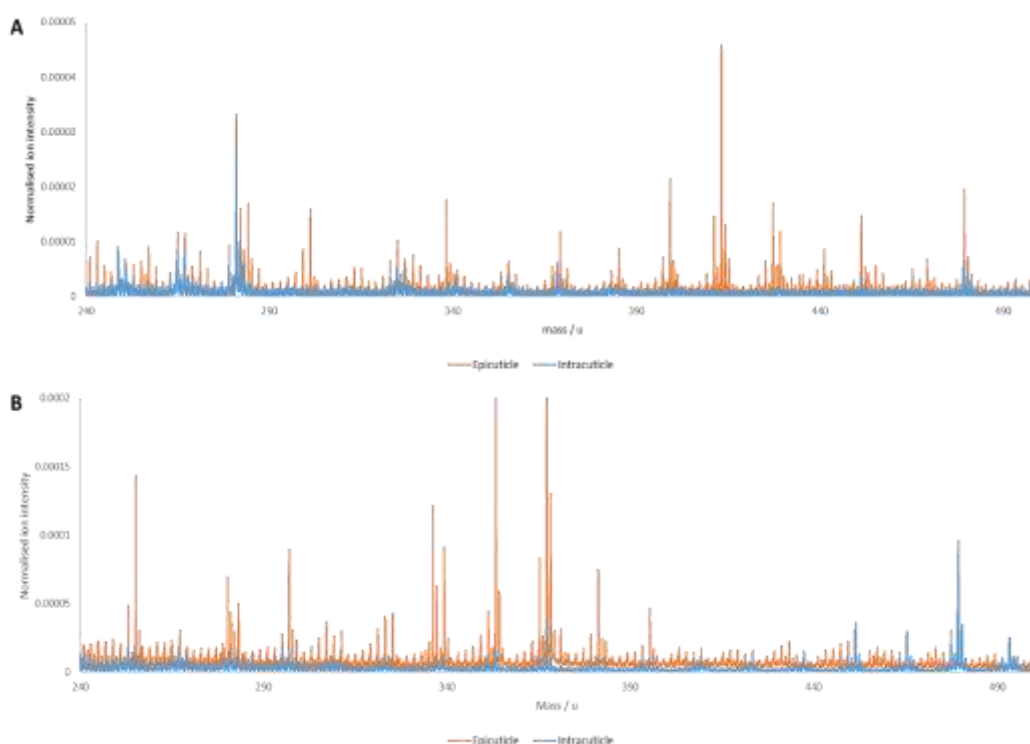


Figure 3-5 - Positive ion (A) and negative ion (B) spectra of the epicuticle and intracuticle surface of *Solanum Lycopersicum* fruit cuticle

While simple evaluation of relative ion intensity can provide information regarding surface composition, the lack of applicable reference spectra and the potential for matrix effects to modify the fragmentation of the waxes¹³¹ make quantitative analysis difficult. Hence MVA was used to identify differences between surfaces. Images of the epicuticle and intracuticle surface were collected from between three and five samples. To briefly explain MVA analysis, a surface is given either a positive or negative principle component (PC) score, which corresponds with a positive or negative value respectively on the Y-axis of Figure 3-6 (A) and Figure 3-7 (A). The Section of either a positive or negative score is determined by the distribution of ions in each sample. Two groups of ions are identified that are more dominant in each group of samples, and each set is given either an arbitrary positive or negative score. The group of ions given a positive score will subsequently present as positive peaks in the Y-axis of Figure 3-6 (B) and Figure 3-7 (B), vice versa for the group of ions given a negative PC

score. The ions on the positive and negative Y-axis of Figure 3-6 (B) and Figure 3-7 (B) are more dominant in surfaces given positive and negative PC scores, respectively.

Figure 3-6 and Figure 3-7 show some inter-surface variation between the epicuticle and intracuticle, respectively. Similar behaviour has been observed based on the location the cuticle was extracted from, as new and old plant growth will present a degree of wax composition heterogeneity³³.

However, as the data in Figure 3-8 and Figure 3-9 show, when the epicuticle and intracuticle surface are compared, these intra-surface variations are minor compared with inter-surface variations. Such variation drives the epicuticle and intracuticle PC scores to opposite ends of the PC range and couples the epicuticle samples together and intracuticle samples together.

Evaluating the MVA analysis for ions found in Table 3-1, it is possible to identify any preferential distributions of these ions relative to one another. In the positive spectra (Figure 3-8), none of the observed peaks in the loading graph (Figure 3-8 B) were ions found in Table 3-1. Thus, while it is clear that the surfaces vary chemically, these differences could not be identified as belonging to the cuticle wax and thus were not considered further.

In the negative spectra (Figure 3-9), the epicuticle surfaces were given positive PC values and the intracuticle surface negative PC values. Subsequently, 353 u, 381 u, 395 u and 493 u, representing $C_{24}H_{49}O$, $C_{26}H_{53}O$, $C_{27}H_{55}O$ and $C_{34}H_{65}O$, respectively, are associated with positive loadings and thus the epicuticle surface. 409 u, 451 u and 479 u, representing $C_{28}H_{57}O$, $C_{30}H_{59}O_2$ and $C_{32}H_{63}O_2$, respectively, are associated with negative loadings and the intracuticle surface.

Based on the observed differences in the negative spectra, it was possible to identify the distribution of the waxes of interest, Table 3-1. Four of the seven predicted molecular alcohol ions were present in the epicuticle surface $C_{24}H_{50}O$, $C_{26}H_{54}O$, $C_{27}H_{56}O$ and $C_{34}H_{70}O$. Interestingly, waxes containing carboxylic acids were confined to the intracuticle surface. Additionally, the $C_{28}H_{57}O$ alcohol was found to preferential distribute within the intracuticle layer, showing that alcohols are not confined to the epicuticle layer. The alkanes and $C_{25}H_{52}O$ and $C_{32}H_{66}O$ alcohol did not demonstrate preferential distribution and thus are distributed homogeneously throughout the cuticle. These results are summarised in Table 3-2.

These results match literature which suggests the composition of epicuticle and intracuticle shows a trend towards similar distributions of wax^{34,35}. Subsequently, it is proposed that ToF-SIMS is an avenue for the determination of spatial wax distribution in the epicuticle vs

intracuticle. Because these ToF-SIMS results broadly match the traditional Gas chromatography measured wax distribution literature that reports broad alcohol and carboxylic acid wax distribution within the cuticle. The assumption is that the observed distribution results from spatial distribution and not a matrix effect.

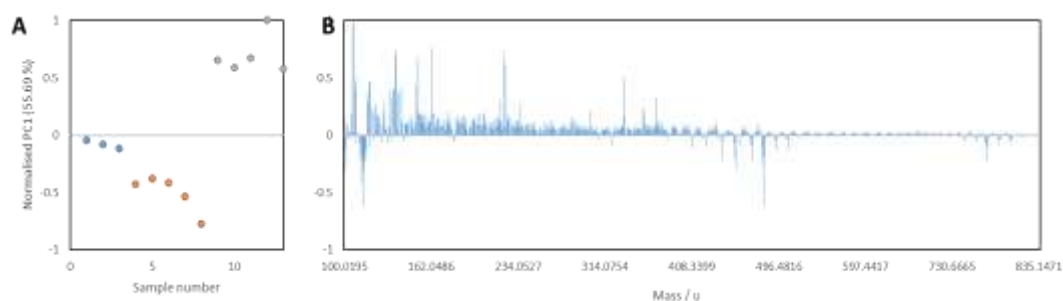


Figure 3-6 - (A) MVA scores and (B) loadings derived from negative ions collected by ToF-SIMS from three epicuticle surfaces. Different coloured dots represent repeats from the same surface.

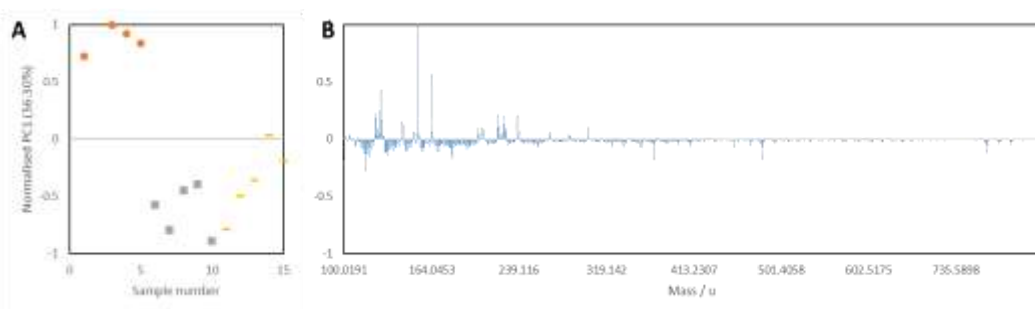


Figure 3-7 - MVA scores and (B) loadings derived from negative ions collected by ToF-SIMS from three intracuticle surfaces. Different coloured dots represent repeats from the same surface.

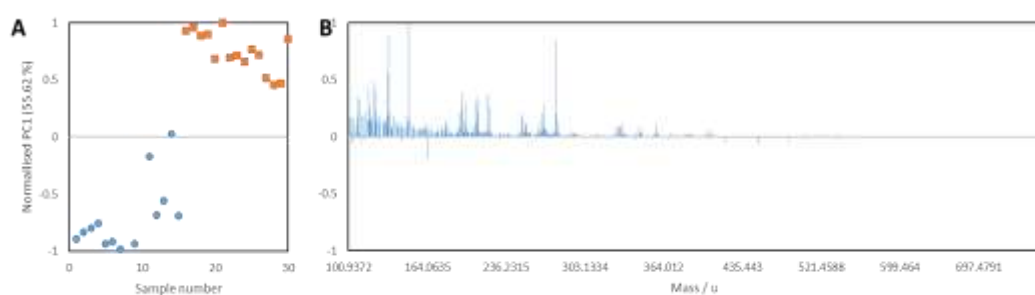


Figure 3-8 - (A) MVA scores and (B) loadings derived from positive ions collected by ToF-SIMS from three epicuticle surfaces, blue circles, and three intracuticle surfaces, orange squares.

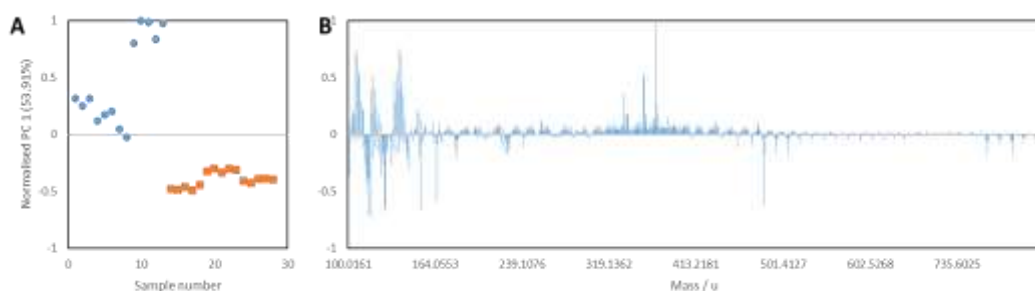


Figure 3-9 - MVA scores and (B) loadings derived from negative ions collected by ToF-SIMS from three epicuticle surfaces, blue circles, and three intracuticle surfaces, orange squares.

Table 3-2 Identified ions associated with specific surfaces.

	Identified ion / u						
	354/352	382/380	396/394	409/407	451	479	494/494
Predicted structure	C ₂₄ H ₅₀ O	C ₂₆ H ₅₄ O	C ₂₇ H ₅₆ O	C ₂₈ H ₅₆ O	C ₃₀ H ₅₉ O ₂	C ₃₂ H ₆₃ O ₂	C ₃₄ H ₇₀ O
Chemical functionality	Alcohol	Alcohol	Alcohol	Alcohol	Carboxylic acid	Carboxylic acid	Alcohol
Associated surface	Epicuticle	Epicuticle	Epicuticle	Intracuticle	Intracuticle	Intracuticle	Epicuticle

Three waxes were selected to act as analogues of the cuticle waxes for characterisation and printing based upon the results shown in Table 3-2. Stearic acid, 1-docosanol and dotriacontane, see Figure 3-2, were selected based on their chain length and cost. They were also selected based on their capability to model the waxes characterised using ToF-SIMS, which can potentially be spatially distributed using 3D printing to generate wax films modelling the imaged heterogeneity. As shown by comparing Figure 1-6 and Figure 3-2 the chemical functionality is the same in the selected waxes compared with waxes that might be found in a plant cuticle.

3.3.2. Characterisation of selected waxes

At room temperature, the three waxes selected are solid and typically powders and unsuitable for inkjet 3D printing directly. To print these waxes, they must be melted or solubilised within an inkjet printer. Here we use the solvent-free melting approach. To facilitate printing, the printed droplets of molten wax must solidify rapidly upon contact with the substrate, thereby allowing further deposition and arrangement into a predictable solid form. Furthermore, the molten wax must display physical properties matching the specification of the printhead used: molten below 100 °C and ideal viscosity between 8 mPa.s – 20 mPa.s. Materials with lower viscosity, between 1 mPa s⁻¹ and 20 mPa s⁻¹, can be printed

with increased satellite droplet formation; however, a material with a viscosity above 20 mPa.s may damage the printhead³⁶.

DSC was used to investigate these physical properties to determine the melting point of the selected waxes, while rotational rheology was used to measure the dynamic viscosity. The droplet surface tension measurement was not conducted as a safe method of maintaining a constant temperature within molten droplets within the drop size analyser was not found.

3.3.2.1. DSC Analysis

In this DSC investigation, the most critical parameter was the peak observable at the highest temperature, indicating the temperature at which complete melting (T_M) occurs instead of a crystalline phase change. Further, it is essential to identify the temperature of initial recrystallisation (TR), and thus solidification occurs. Figure 3-10 show the heating and cooling thermograms of the three single component waxes: dotriacontane, 1-docosanol and stearic acid tested. All melting points were identified within 1 °C above the melting point stated in the literature and below the maximum operating temperature of the printhead, 90 °C³⁷.

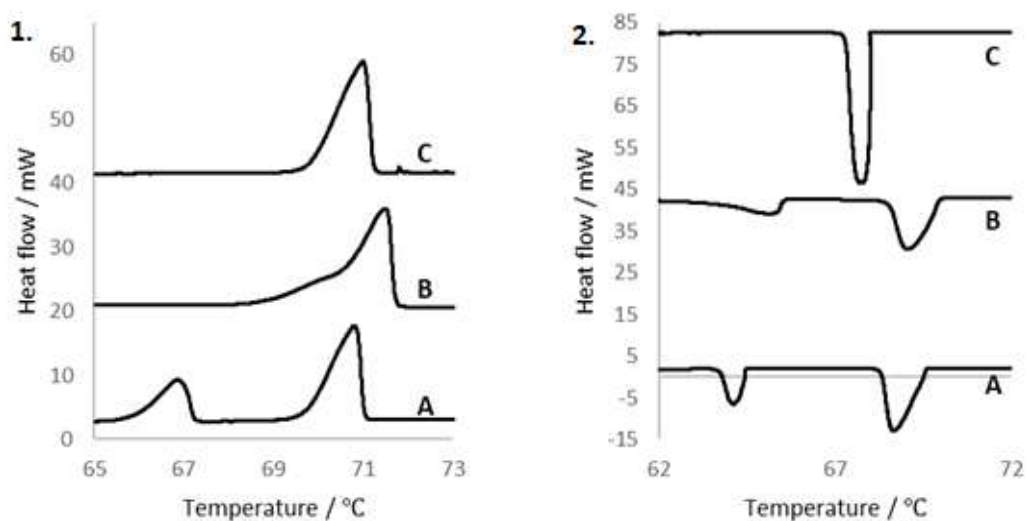


Figure 3-10 Overlaid DSC thermographs of the heating cycle, 1., and the cooling cycle, 2. For the three tested single component waxes: Dotriacontane (A), 1-docosanol (B) and stearic acid (C). All thermographs shown are taken from the second heating/cooling cycle.

It was observed that the thermographs of both 1-docosanol and stearic acid present only a single endothermic transition on heating, $T_M = 71.4$ °C and 70.9 °C, respectively, thought to be associated with melting. The 1-docosanol thermograph shows a shoulder peak ($T_{m1} \approx 70$ °C). Dotriacontane displayed two endothermic transitions: the first identified as a crystalline

phase change from an orthorhombic to a hexagonal phase¹⁹ ($T_{\text{RIII}} = 66.8\text{ }^{\circ}\text{C}$) and melting ($T_{\text{M}} = 70.5\text{ }^{\circ}\text{C}$). The enthalpies and temperature of each identified peak are given in Table 3-3.

Table 3-3 Observed enthalpies and temperature of the transitions within the tested single component waxes

Material	Phase transition	Temperature ($^{\circ}\text{C}$)	Temperature (K)	ΔH (kJ/mol)
Dotriacontane	Orthorhombic to hexagonal	66.8	339.91	32.75
	ΔH_{fus}	70.5	343.69	79.75
1-Docosanol	ΔH_{fus}	71.4	344.56	78.23
Stearic acid	ΔH_{fus}	70.9	344.08	50.79

Given the complex nature of the cuticle wax, using a single wax as a model film likely would be insufficient. Binary wax mixtures have provided helpful information permitting their use as simplistic models of more complex systems^{11,38}, including the cuticle. As such, the waxes were mixed to form samples consisting of dotriacontane: 1-docosanol (DOT: 1-D), dotriacontane: stearic acid (DOT: SA) and 1-docosanol: stearic acid (1-D: SA) at a range of compositions. Figure 3-11 shows the overlay of DSC thermographs for each of these binary wax mixtures, and Figure 3-12, Figure 3-13 and Figure 3-14 show the phase diagrams associated with these binary mixtures.

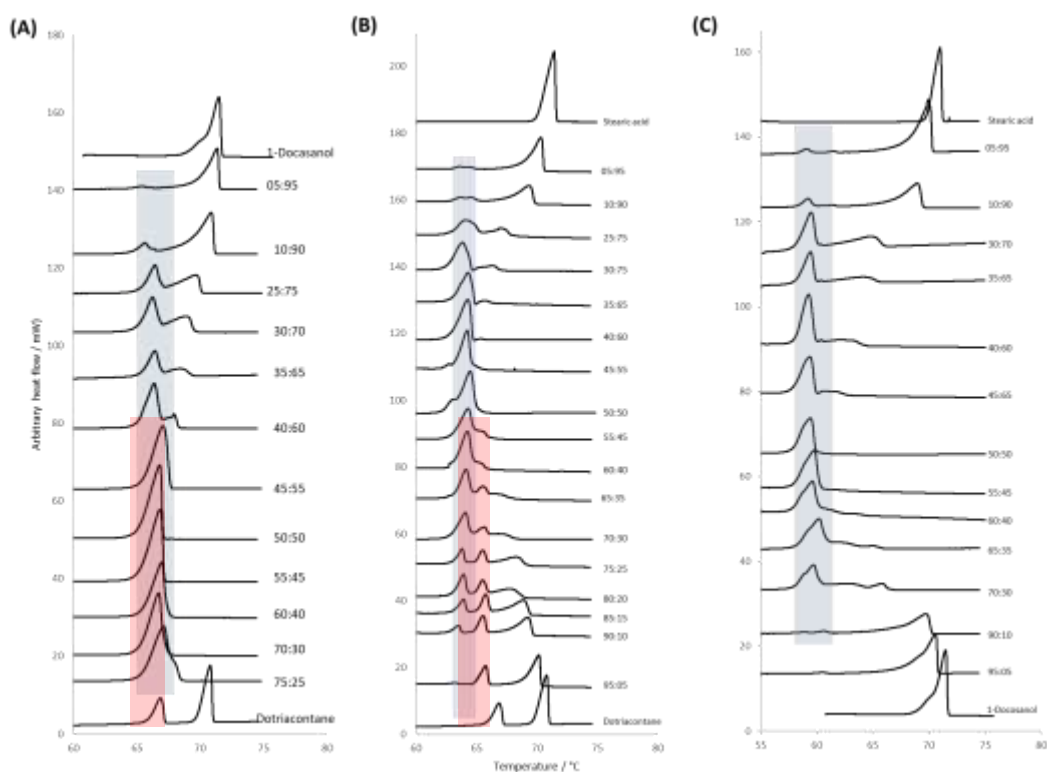


Figure 3-11 - Offset DSC scans of single component waxes and the intermediate binary mixtures: (A) DOT: 1-D, (B) DOT: SA and (C) 1-D: SA showing the eutectic melting peak in the grey box and the melting behaviour of excessive components in all tested mixtures. The red box over Figure 3-11 (A) and (B) show the shift in the dotriacontane orthorhombic to hexagonal phase transition overlapping with the eutectic peak.

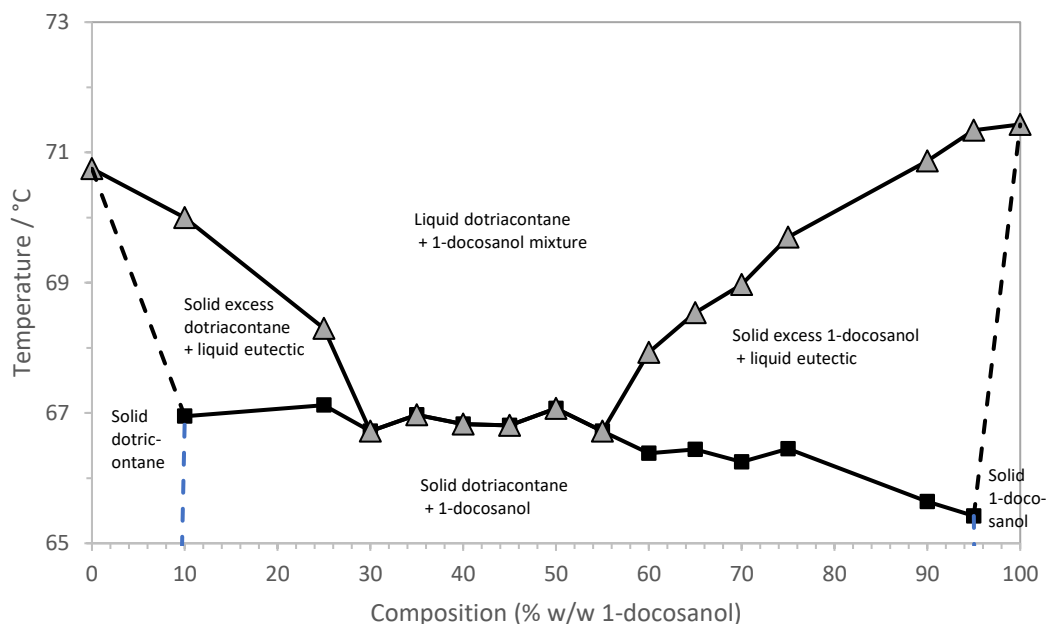


Figure 3-12 - Binary phase diagram of DOT: 1-D as obtained using DSC. Grey triangles represent the liquidus peak, and the black squares represent the solidus peak. The black dotted lines show the proposed solidus line, whereas the blue dotted line shows the proposed solvus line.

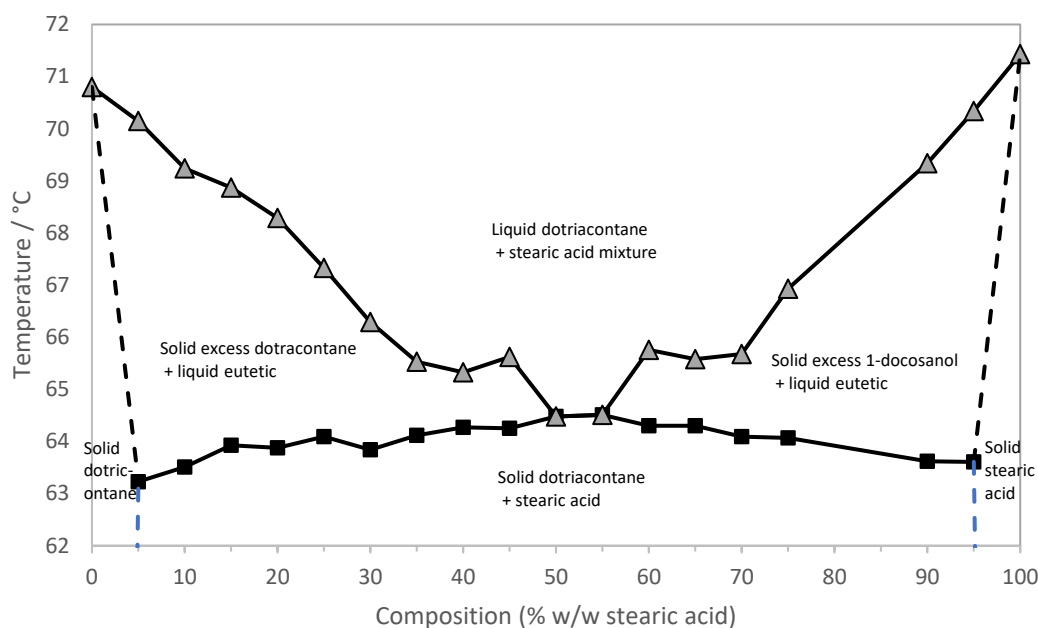


Figure 3-13 - Binary phase diagram of DOT: SA as obtained using DSC. Grey triangles represent the liquidus peak, and the black squares represent the solidus peak. The black dotted lines show the proposed solidus line, whereas the blue dotted line shows the proposed solvus line.

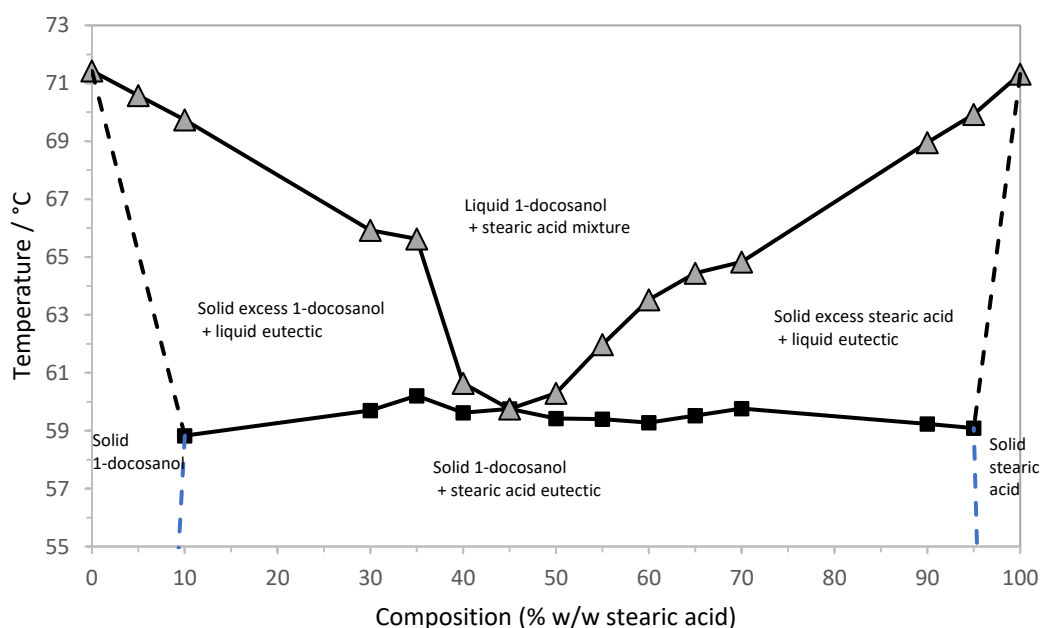


Figure 3-14 - Binary phase diagram of 1-D: SA as obtained using DSC. Grey triangles represent the liquidus peak, and the black squares represent the solidus peak. The black dotted lines show the proposed solidus line, whereas the blue dotted line shows the proposed solvus line.

As seen in Figure 3-11, following the addition of a small amount of the secondary wax, 5 or 10 % w/w, the melting peak of the bulk single component waxes (Table 3-3), now called the solidus peaks, begin decreasing in temperature. Moreover, a new peak (liquidus) appeared at a lower temperature that increased enthalpy as additional secondary wax was added to

the mixture. At the eutectic point, these two peaks merge as a single eutectic peak. The composition this occurs at is the eutectic point, signifying the composition of a eutectic.

Figure 3-12, Figure 3-13 and Figure 3-14 illustrate this behaviour as phase diagrams. In each phase diagram, a eutectic point can be seen at approximately 50: 50 % w/w mixture of each wax. Beyond this, clear trends can be observed regarding the liquidus and solidus peaks typical of a eutectic system. In all cases, the total enthalpy of the systems tested, solidus and liquidus peaks was invariable with the enthalpy of the single component waxes at these ratios, a common outcome in eutectic mixtures where no new compounds are formed.

Figure 3-14 will be used as an example to explain the similar behaviour of all three binary mixtures. Presented is a clear eutectic point when stearic acid consists of 45 % w/w of the mixture. Below 59.5 °C, the two components presented as separate solid crystalline phases. Above this temperature, three outcomes were observed depending upon the composition of the waxes and the ultimate temperature reached. When 1-docosanol was in excess, between 10 – 40 % w/w stearic acid, the binary wax mixture formed an isotropic liquid of both components with the excess 1-docosanol suspended as a crystalline solid. When stearic acid was in excess, between 50-95 % w/w stearic acid, the binary wax mixture formed an isotropic liquid of both components with the excess stearic acid suspended as a crystalline solid. As the temperature was increased above 59.5 °C, the excess solid crystalline material remains suspended in the liquid eutectic. The amount the temperature had to rise above 59.5 °C to induce the melt of the excess solid material depended on the composition. Smaller temperature changes were required to melt the excess solid in waxes with a composition close to the eutectic point. If the system's composition was at the eutectic point, 55: 45 % w/w 1-D: SA, no excess solid would remain after the initial melting event at 59.5 °C, forming an isotropic liquid of both components. This is shown by the curved set of points, called liquidus points, seen in each phase diagram.

This pattern is a common feature of phase diagrams seen in other binary wax systems^{19,39}. Unfortunately, the DOT: SA and the DOT: 1-D systems did not as clearly show this pattern. This is due to the peak associated with the phase transition of the dotriacontane from an orthorhombic to a hexagonal crystalline state ($T_{R_{III}} = 66.8\text{ °C}$) superimposing over the developing liquidus peak. This is highlighted in Figure 3-11 (A) and (B). As such, it is difficult to resolve the exact location of the eutectic point or draw the boundaries seen above the solidus temperature. This was less of an issue with the DOT: SA mixture (Figure 3-11 (B)). Dotriacontane's orthorhombic to hexagonal phase transition peak ($T_{R_{III}} = 66.8\text{ °C}$) occurred at

a higher temperature than the eutectic solidus peak (64.5 °C) and was sufficiently separated to resolve these two peaks. Prior literature did not observe this issue with a DOT: 1-D mixture with all peaks being observed several degrees lower and with more distinct peaks¹⁹. This was likely associated with the slower heating ramp used, 0.05 °C /min.

3.3.2.2. FT-IR

Eutectic waxes are known to arrange into a uniform microstructure of repeating molecules³⁹. The components of a eutectic mixture do not undergo chemical interactions, instead forming a type of alloy. For this reason, FT-IR is often used to assess the formation of such structures. It is expected that the obtained spectra from a binary wax will present peaks associated with the single component materials with an intensity determined by the relative molar quantity of the associated component. A eutectic mixture should display no new peaks, peak shifting or broadening³⁹.

To evaluate the chemical interactions involved in the formation of the binary waxes, FT-IR spectra of the three single component waxes and their binary mixtures were obtained (Figure 3-15, Figure 3-16 and Figure 3-17). As an aliphatic alkane, dotriacontane displayed no distinct peaks compared with the 1-docosanol (Figure 3-15) and stearic acid (Figure 3-16). 1-docosanol displayed a broad band from 3100-3450 cm⁻¹, associated with an H-bonded O-H alcohol stretch, a C-O stretch at approximately 1060 cm⁻¹ and a CH₂ waggle region at 1160-1390 cm⁻¹ with the number of peaks corresponding to the wax chain length. A CH₂ waggle was not identified in dotriacontane and was considerably larger in stearic acid (Figure 3-18). Stearic acid displayed a broad O-H stretch centred at 3100 cm⁻¹, a sharp peak between 1600-1810 cm⁻¹ associated with C=O carboxylic acid stretch, an O-H bending at 1430 cm⁻¹ and an unidentified peak centred between 940 and 890 cm⁻¹.

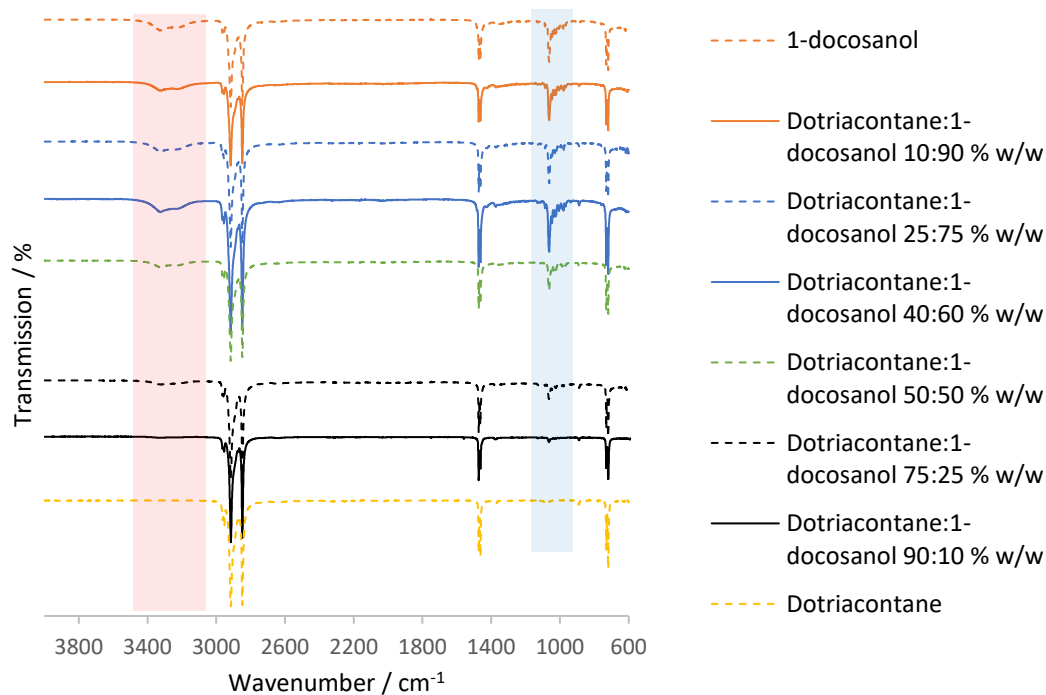


Figure 3-15 - FT-IR spectra of dotriacontane, 1-docosanol and associated binary waxes. The red box shows the O-stretch associated with alcohols, and the blue box shows the CH₂ waggle region found in spectra containing 1-docosanol.

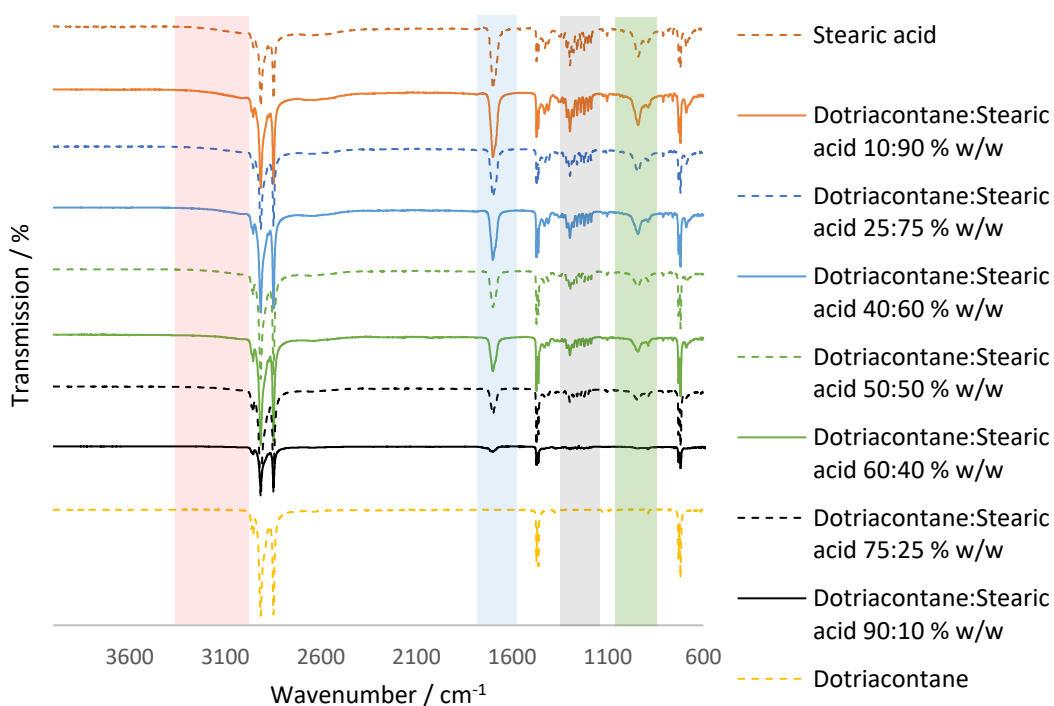


Figure 3-16 - FT-IR spectra of dotriacontane, stearic acid and associated binary waxes. The red box shows the O-H stretch associated with carboxylic acids, the blue box shows the stretch of a C=O associated with carboxylic acids, the grey box shows the O-H bending, and an unidentified peak centred between 890 and 940 cm⁻¹ is shown in the green box.

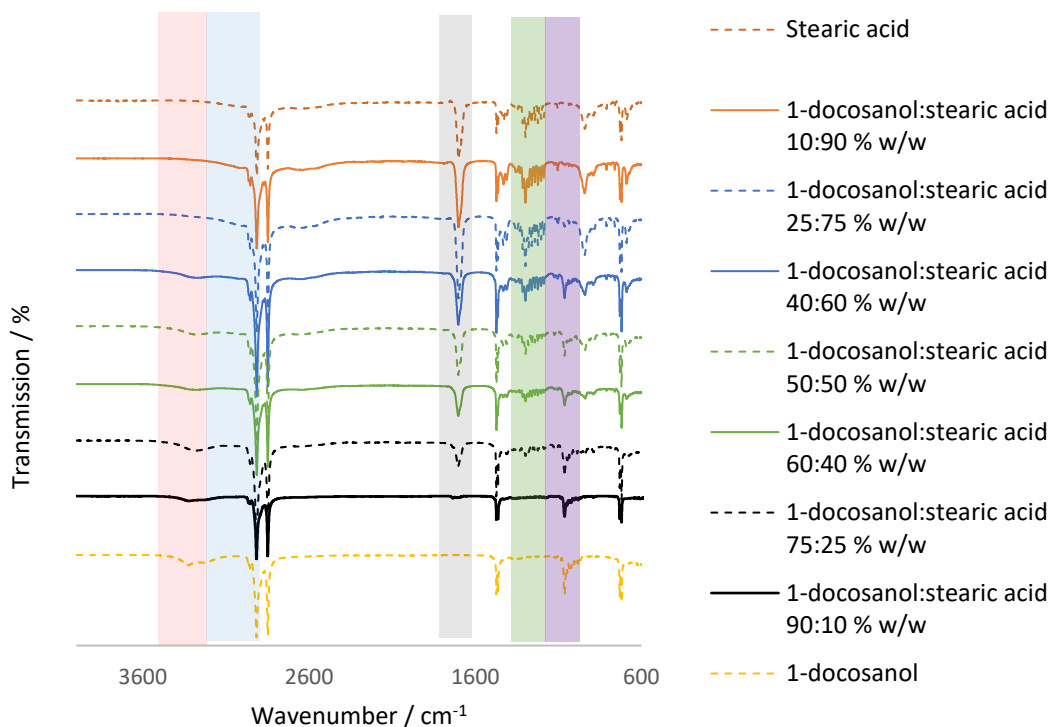


Figure 3-17 - FT-IR spectra of 1-docosanol, stearic acid and associated binary wax. The red and blue box shows the O-stretch associated with alcohols and carboxylic acids, respectively. The grey box shows the stretch of a C=O associated with carboxylic acid, the green box shows the O-H bending, and the purple box shows the CH₂ waggle region found in spectra containing 1-docosanol.

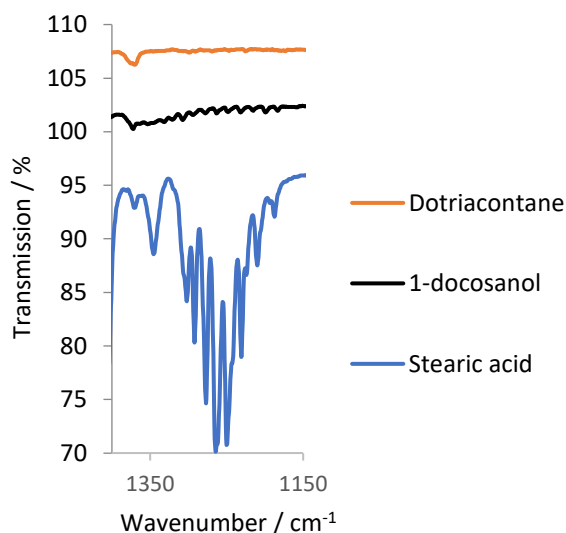


Figure 3-18- FT-IR spectra of the CH₂ wiggle region.

In all cases, the binary mixtures of waxes showed no new peaks, the disappearance of characteristic peaks, nor any peak shifting (Figure 3-15, Figure 3-16 and Figure 3-17). This suggests that the waxes formed a eutectic mixture and that no substantial chemical or physical reaction occurs between the two waxes. Further, no significant peak broadening was

observed, suggesting that the waxes retained their crystalline state and that minimal additional amorphous material was generated to produce the ink.

It is suggested by the DSC data that waxes transitioned back to their original crystalline phase during DSC analysis without annealing. However, if either 1-docosanol or dotriacontane are rapidly cooled from the molten state, they will arrange into a metastable hexagonal arrangement. After which, transition into the orthorhombic arrangement has been recorded to take several days¹¹. Within the DSC, waxes were cooled at 1 °C / min. The cooling of powders used for FT-IR was not controlled as accurately, potentially allowing the wax to become kinetically trapped in a hexagonal arrangement. This could result in the physical properties of the wax changing over time as the crystalline phase transitions from the hexagonal to an orthorhombic phase.

FT-IR was used to identify the crystalline arrangement of both compounds by observing the CH₂ bending region of 1-docosanol and the CH₂ rocking region of dotriacontane^{11,40,41}.

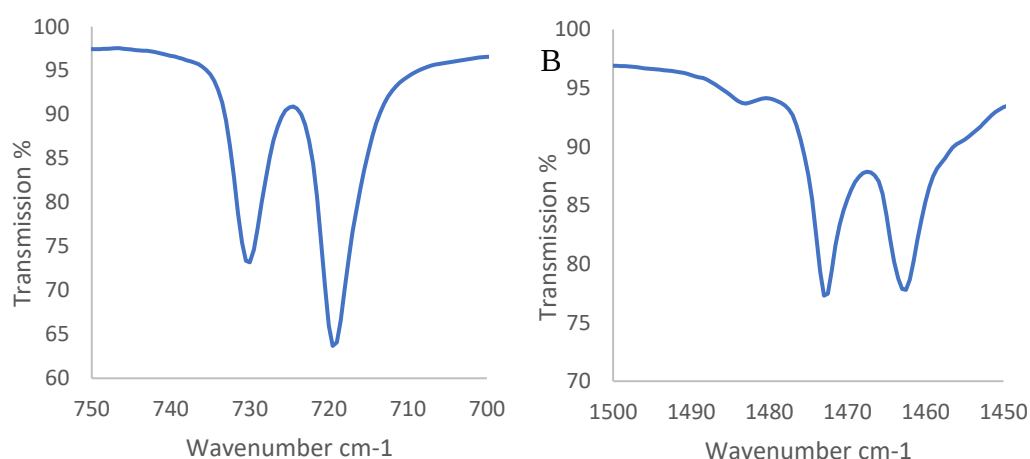


Figure 3-19 - FT-IR spectra of the (A) CH₂ rocking region of dotriacontane and (B) CH₂ bending region of 1-docosanol

Figure 3-19 shows that the FT-IR spectra produced in both cases match FT-IR spectra of the orthorhombic arrangements of both 1-docosanol and dotriacontane seen in the literature^{11,41}. The critical observation is that such regions present a doublet structure associated with orthorhombic phases. The second peak is not observed in the hexagonal phase. Similar structures were observed in each wax produced containing dotriacontane or 1-docosanol (Figure 3-20).

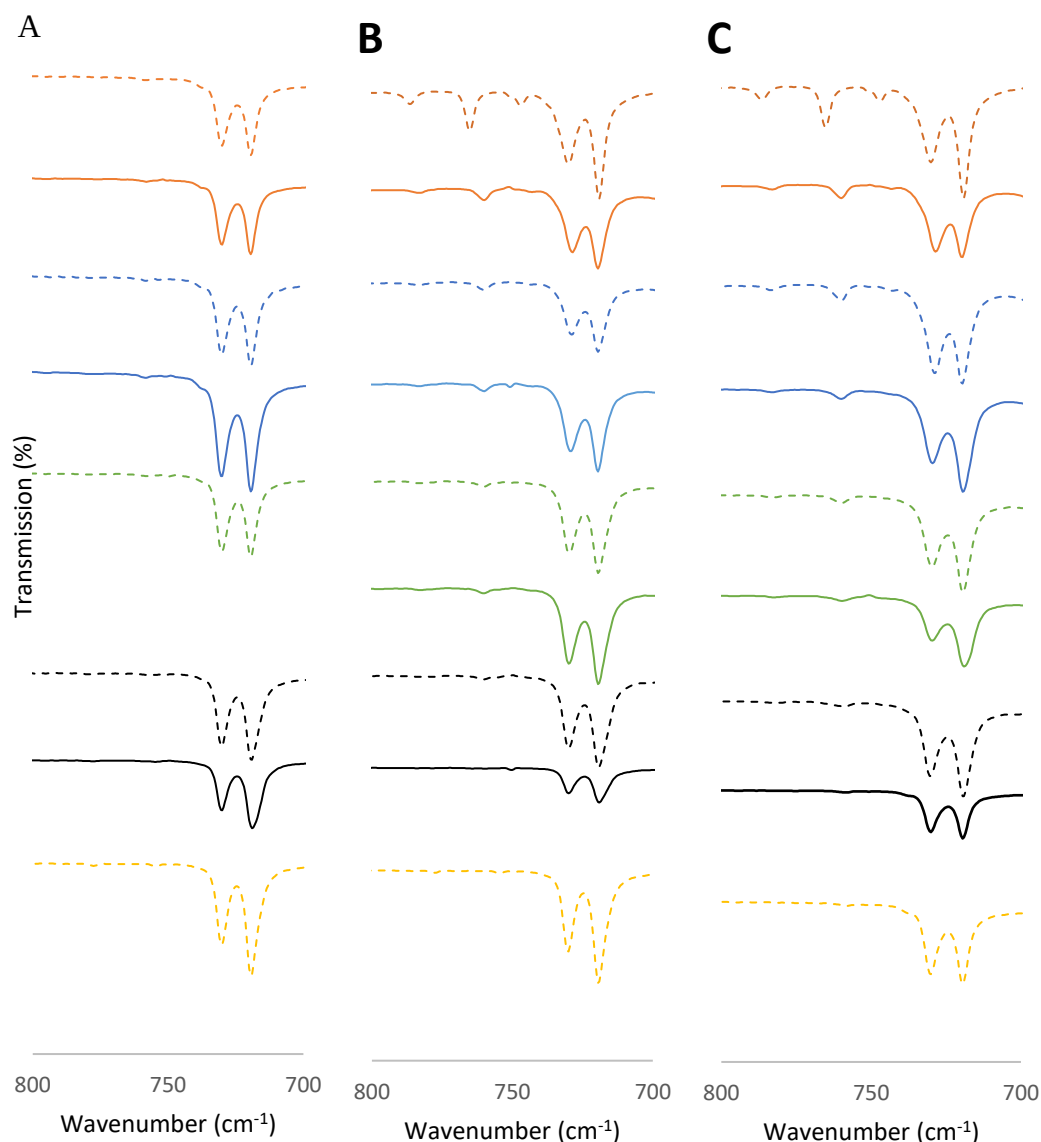


Figure 3-20 - FT-IR spectra of the CH₂ rocking region in (A) DOT: 1-D binary waxes, (B) DOT: SA binary wax and (C) 1-D: SA binary wax. For a legend, see Figure 3-15, Figure 3-16 and Figure 3-17 for (A), (B) and (C), respectively.

Further to this observation, the CH₂ rocking region (Figure 3-20) has been previously used to predict % crystallinity for Fischer-tropsch waxes, a type of synthetic long-chain aliphatic wax^{40,42}. The doublet peaks at 700 cm⁻¹ – 750 cm⁻¹ present with equal areas when the wax is 100 % crystalline, whereas, in a 100 % amorphous wax, only the 720 cm⁻¹ peak will be observed. This occurs because of the coupling of the CH₂ rocking vibrations within orthorhombic crystalline phases due to inference between nearby wax chains located at particular distances. Based on this, crystallinity can be predicted by measuring the area of each peak between 700 cm⁻¹ – 750 cm⁻¹ and identifying the ratio of areas⁴³. This method has been prior applied successfully to cuticle waxes. Similar methods of predicting crystallinity

have been conducted using the doublet peaks found at 1345 cm^{-1} , 1167 cm^{-1} , 1079 cm^{-1} and 960 cm^{-1} ⁴³.

Table 3-4 shows crystallinity of powder waxes, and Figure 3-21 shows crystallinity as a function of composition.

Table 3-4 - Predicted crystallinity of different waxes from evaluation of the CH_2 rocking region.

Dotriacontane:1-docosanol binary wax							
% w/w composition of 1-docosanol in dotriacontane	0	10	25	50	75	90	100
% crystallinity (of the CH_2 functionality)	73.546	56.005	66.293	84.220	89.980	88.573	86.240
Standard deviation	0.303	2.136	3.972	3.590	1.577	2.274	1.252
Dotriacontane:stearic acid binary wax							
% w/w composition of Stearic acid in dotriacontane	0	10	25	50	75	90	100
% crystallinity (of the CH_2 functionality)	73.546	79.934	84.376	75.272	70.187	57.924	73.546
Standard deviation	0.303	8.211	1.507	1.594	7.073	1.377	0.303
1-docosanol:stearic acid binary wax							
% w/w composition of Stearic acid in 1-docosanol	0	10	25	50	75	90	100
% crystallinity (of the CH_2 functionality)	86.240	84.719	93.137	56.536	83.397	87.575	96.945
Standard deviation	1.252	4.882	2.374	5.891	4.668	3.899	0.376

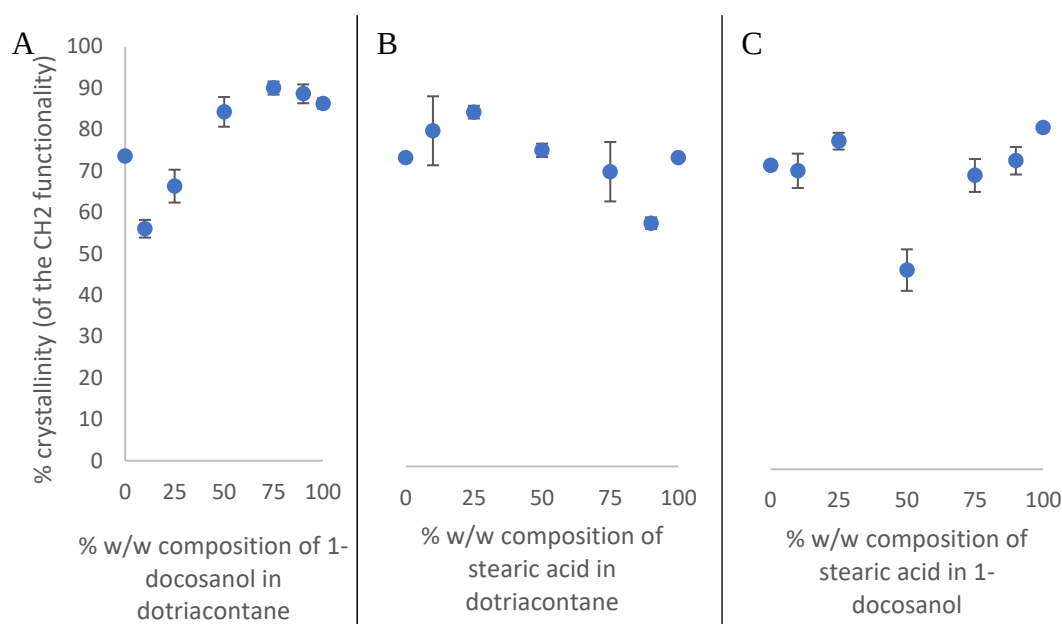


Figure 3-21 - Predicted crystallinity of different waxes from evaluation of the CH_2 rocking region.

Table 3-4 suggests that almost all the waxes were highly crystalline, often above 70 %. However, there was no identifiable trend between crystallinity and composition (Figure 3-21). It was observed that the addition of a small amount of secondary wax either results in a decreased or statically insignificant change in the degree of crystallinity. Further, the 50: 50 % w/w 1-D: SA binary wax (Figure 3-21(C)) was significantly less crystalline than all other waxes tested. This wax is close to the eutectic point and is the only wax close to the eutectic point to show a significant crystalline decrease.

It is likely that if the crystallinity of these wax were tested using nuclear magnetic resonance (NMR), the value measured would be lower. NMR assesses the entire wax, whereas FT-IR only considers the CH₂ group. Methyl groups and functional groups such as O-H are excluded⁴⁰. That functional groups are excluded from the evaluation, and the 1-D: SA binary wax system presents a functional group on all wax molecules that might partially explain the rapid drop in crystallinity of the 1:1 % w/w 1-D: SA binary wax.

3.3.2.3. Rheology

Rheological tests assess the effect of shear stress and rate on inks' physical properties and assess printing viability. Inks should not exceed the recommended viscosity of a printhead to avoid damage and should be above the minimum viscosity to avoid satellite droplet formation. This viscosity range is usually defined between 1 mPa s⁻¹ and 20 mPa s⁻¹⁴⁴. Each manufacturer also specifies a target viscosity range to minimise damage to the printhead and satellite droplets for their specific printhead.

Ideally, the shear rate applied to measure viscosity would match the sheer generated within the nozzle of approximately 10⁵ s⁻¹ – 10⁷ s⁻¹⁴⁵. However, this is outside the range of most rheometers. Instead, representative measurements were collected between 0.1 s⁻¹ – 1000 s⁻¹, assuming the wax would show Bingham plastic properties followed by Newtonian fluid behaviour like other waxes and straight-chain aliphatics⁴⁶. If so, the viscosity at 1000 s⁻¹ would represent the viscosity in higher shear environments.

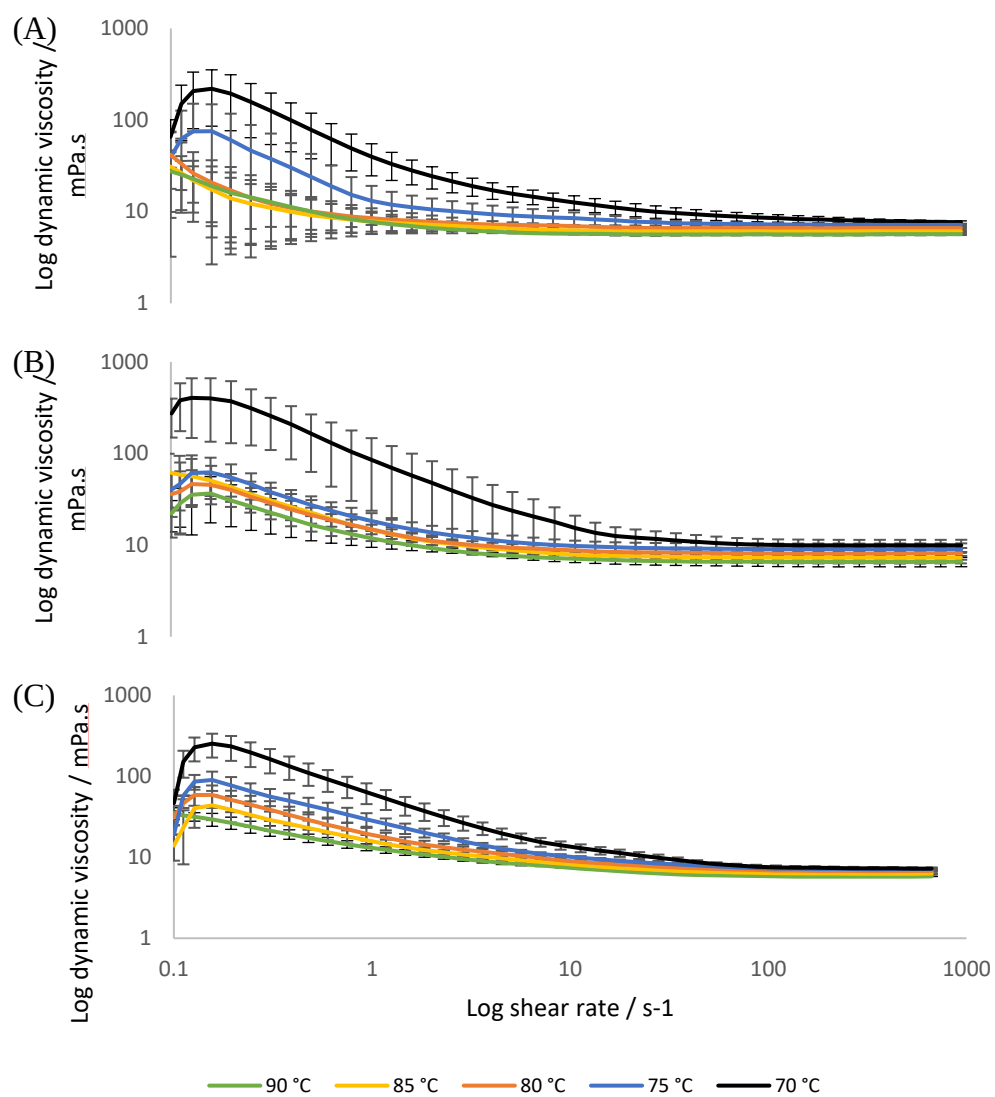


Figure 3-22- Sweep shear viscosity of (A) dotriacontane, (B) 1-docosanol and (C) stearic acid.

Table 3-5 - Shear viscosity of waxes between 70 - 90 °C at a shear rate of 1000 s⁻¹. SD represents the standard deviation.

Temperature / °C	Dynamic viscosity at 1000 s ⁻¹ / mPa.s					
	Dotriacontane		1-docosanol		Stearic acid	
	Average	SD	Average	SD	Average	SD
70	7.68	0.25	9.93	1.51	7.25	0.25
75	7.16	0.17	9.03	1.42	7.15	0.11
80	6.64	0.16	8.12	1.19	6.79	0.07
85	6.13	0.13	7.28	0.96	6.29	0.02
90	5.70	0.14	6.58	0.77	5.81	0.02
	25: 75 % w/w Do: 1-D		50: 50 % w/w Do: 1-D		75: 25 % w/w Do: 1-D	
	Average	SD	Average	SD	Average	SD
70	5.01	1.42	7.22	0.27	8.82	1.66
75	4.58	1.20	6.62	0.15	7.58	1.53
80	4.12	1.01	5.94	0.09	7.19	1.45
85	3.72	0.88	5.36	0.06	6.57	0.74
90	3.39	0.76	4.86	0.05	5.75	0.22
	25: 75 % w/w Do: Sa		50: 50 % w/w Do: Sa		75: 50 % w/w Do: Sa	
	Average	SD	Average	SD	Average	SD
70	5.62	0.04	3.62	0.03	6.92	0.10
75	5.20	0.02	3.43	0.01	6.54	0.05
80	4.77	0.01	3.18	0.00	6.02	0.02
85	4.37	0.00	2.96	0.00	5.54	0.01
90	4.02	0.00	2.76	0.01	5.12	0.01
	25: 75 % w/w 1-D: Sa		50: 50 % w/w 1-D: Sa		75: 25 % w/w 1-D: Sa	
	Average	SD	Average	SD	Average	SD
70	10.43	0.10	7.58	0.28	1.64	0.04
75	9.62	0.06	6.59	0.05	1.57	0.01
80	8.74	0.02	5.90	0.02	1.48	0.01
85	7.91	0.01	5.30	0.01	1.40	0.01
90	7.15	0.01	4.78	0.02	1.33	0.01

As the data in Figure 3-22 shows, the three single component waxes presented a high viscosity in the low shear region, around 0.1 s⁻¹, after which the wax underwent shear thinning, between 0.5 s⁻¹ and 100 s⁻¹ before stabilising and demonstrating a linear trend. Similar flow curves are typical for many aliphatic waxes and lipids called cross fluids, a general Newtonian fluid⁴⁷. The viscosity the fluid trends towards at high shear is called the infinite shear viscosity and is approximately the viscosity in high shear environments. Because the flow curve had converged towards a singular point, this is likely close to the infinite shear

viscosity. As such, the viscosity at 1000 s^{-1} is likely representative of the viscosity within the high shear environment of the printhead. Similar behaviour was seen in all tested waxes. Increasing the temperature of the wax from $70\text{ }^{\circ}\text{C}$ to $90\text{ }^{\circ}\text{C}$ resulted in a significant drop in viscosity in all cases (Table 3-5).

Interestingly, in all the waxes, except the 25: 75 % w/w 1-D: SA binary wax mixture, the viscosity at $90\text{ }^{\circ}\text{C}$ was either lower or insignificantly different than the main wax component present. However, no trends can be observed beyond this regarding the effect of composition on viscosity.

Unfortunately, all the tested waxes were below the recommended viscosity for the Spectra-s class printhead, $8\text{ cP} - 20\text{ cP}$, at all temperatures. Consequently, printing these inks would likely result in satellite droplet formation³⁶. However, it is worth noting that each wax is above the broader requirements for inkjet printing, $1\text{ cP} - 20\text{ cP}$, and thus are likely to be printable. No ink tested displayed a viscosity above the maximum limit, 20 cP , suggesting no wax is likely to damage the printhead.

3.3.2.4. XRD

XRD was performed to further confirm the eutectic nature of the wax mixtures and identify if the produced powders are crystalline. The crystallinity of waxes impacts the nature of agrochemical penetration due to its influence on tortuosity and path length⁴⁸.

It is expected that a eutectic mixture analysed by XRD will present a series of peaks that can be observed in the diffraction patterns of the component materials, with no new peaks being identified. However, in the diffractograms of hypoeutectic and hypereutectic materials, materials containing the eutectic's secondary material in insufficient or excessive quantity to form the eutectic, peaks predominantly found in the dominant component are expected⁴⁹.

Aliphatic long-chain molecules typically assemble to form parallel layered structures, wherein the length of the molecule equals the thickness of the layers formed. Due to the anisotropic nature of these long-chain aliphatic waxes, wax molecules are typically longer than their diameter, so a series of short and long spacing peaks will be observed⁵⁰. The long spacing peaks show the thickness of the molecular layers, typically comprising the entire molecule as a unit cell wherein molecules have arranged head-to-head, thus aligned in the C-axis. Short spacing peaks show the side-to-side spacing of parallel molecules. Such peaks are regularly observed when imaging epicuticle surface structures, reconstituted cuticle waxes and similar aliphatics. Critically the $21.4^{\circ} 2\theta$ and $24.5^{\circ} 2\theta$ peaks, representing 010 and 100 reflections respectively, as per literature, with basal spacing at $4.13\text{ \AA}$ and $3.63\text{ \AA}$

respectively, are seen in all the wax XRD diffractograms⁵⁰. This shows that the side to side distance between waxes in an orthorhombic arrangement does not change.

Figure 3-23 (B) shows that the stearic acid has a series of intense long spacing peaks, whereas the 1-docosanol and dotriacontane present lower intensity longer-range spacing peaks. That 1-docosanol does not present long spacing peaks agrees with the literature⁵¹. The lack of long spacing peaks is likely due to the heating process used to prepare the powdered dotriacontane. When aliphatic molecules, particularly plant waxes, lack intense long spacing peaks, it suggests that the crystalline material has formed a nematocrystalline order wherein the chains are aligned parallel but with no layer order (Figure 3-24 (C))⁵⁰. Alternatively, it can occur when layers of molecules with varied thickness are stacked upon each other⁵⁰. In this case, the initial long-range spacing peak shows a high intensity that rapidly decreases with each subsequent peak observed. The latter explanation is often used to explain why cuticles, which present broad distributions of chain lengths, very infrequently present well defined long spacing peaks^{50,52}. Regardless, structures lacking long spacing peaks are arguably less ordered with a less clear unit cell of the entire molecule. Dotriacontane and 1-docosanol wax powders show nematocrystalline order, whereas the stearic acid, due to its relatively small chain length and strong polar functional group, forms a more regular ordered head-to-head orientation with clear unit cells. Because the XRD was performed on powders, preferred sample orientations should not be present within the measured material with the crystal structures investigated showing a random distribution of crystal orientations.

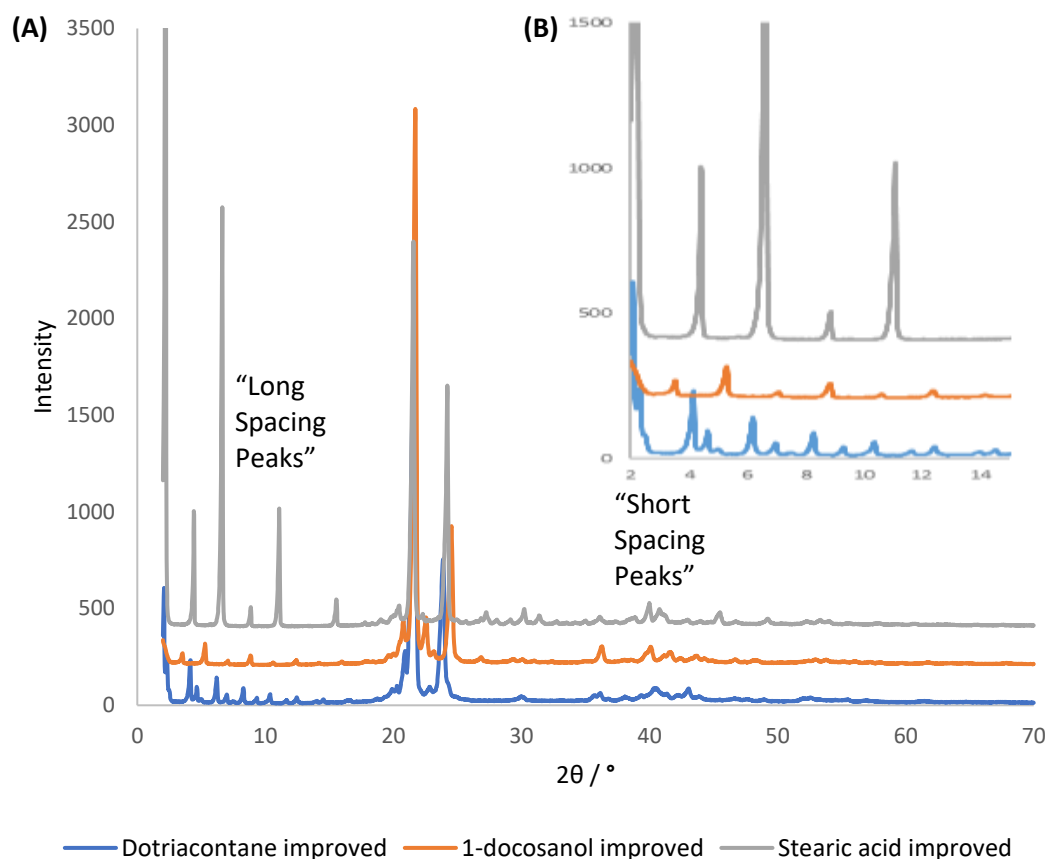


Figure 3-23 XRD diffractogram of single component aliphatic waxes materials that form the basis of the mixed waxes. The stearic acid powder shows a series of peaks at low 2θ values, indicative of regular ordering of the whole molecule unit cell. In comparison, 1-docosanol and dotriacontane show less significant peaks at low 2θ values. This graph has been cut off in the Y-Axis. (B) An insert between 2θ - 14θ shows a zoomed view of the long spacing peaks

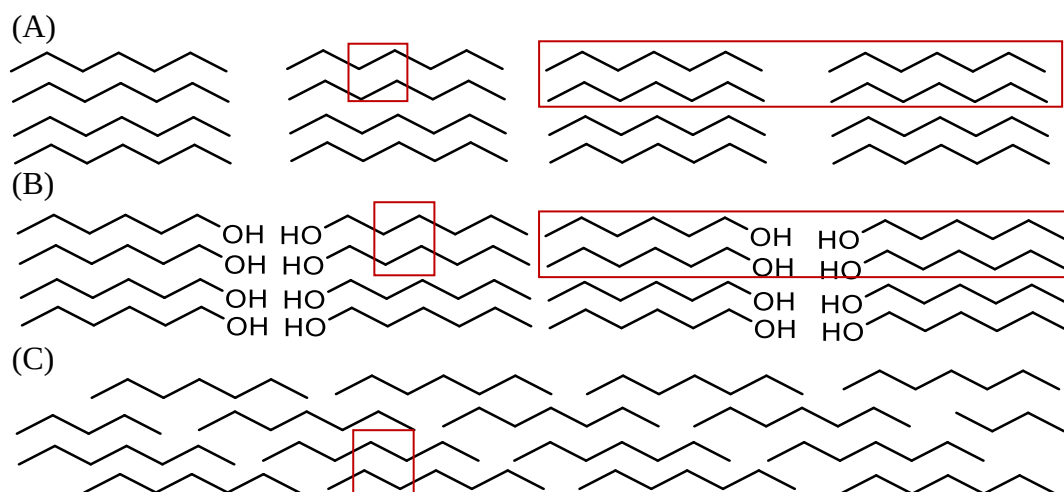


Figure 3-24 - Abstracted models of possible crystalline aliphatic molecules. (A) shows a regularly ordered alkane molecule, (B) shows a regularly ordered alcohol molecule wherein regularly spaced polar functional groups arrange head-to-head, forming a double layer structure and (C) shows a nematic structure with nematocrystalline order. Vertical red rectangles show proposed unit cells of methylene subcells between ordered chains responsible for the short spacing peaks, whereas the horizontal red rectangles show the unit cell of the whole molecule responsible for the long spacing peaks. These unit cells are not present in nematic structures.

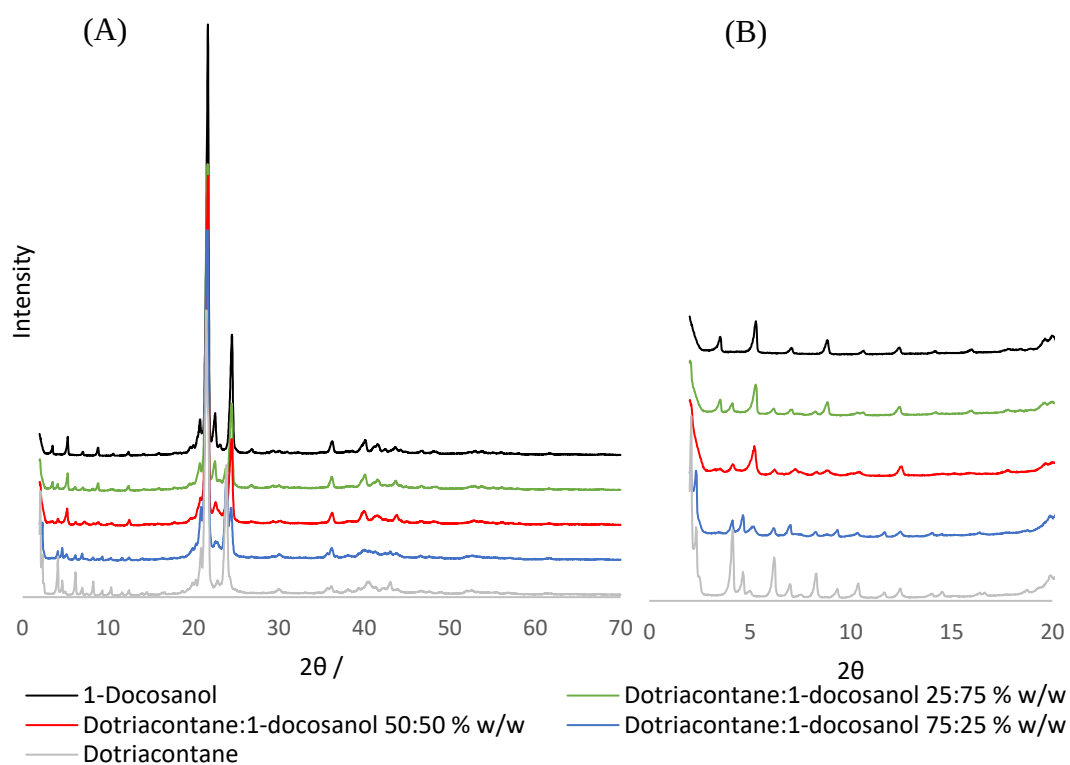


Figure 3-25 - XRD diffractogram of dotriacontane, 1-docosanol and associated binary mixtures. (A) shows the full diffractograms, cuts of at intensity 3000. (B) shows a zoomed-in view of 2 2θ - 14 2θ and intensity up to 2000.

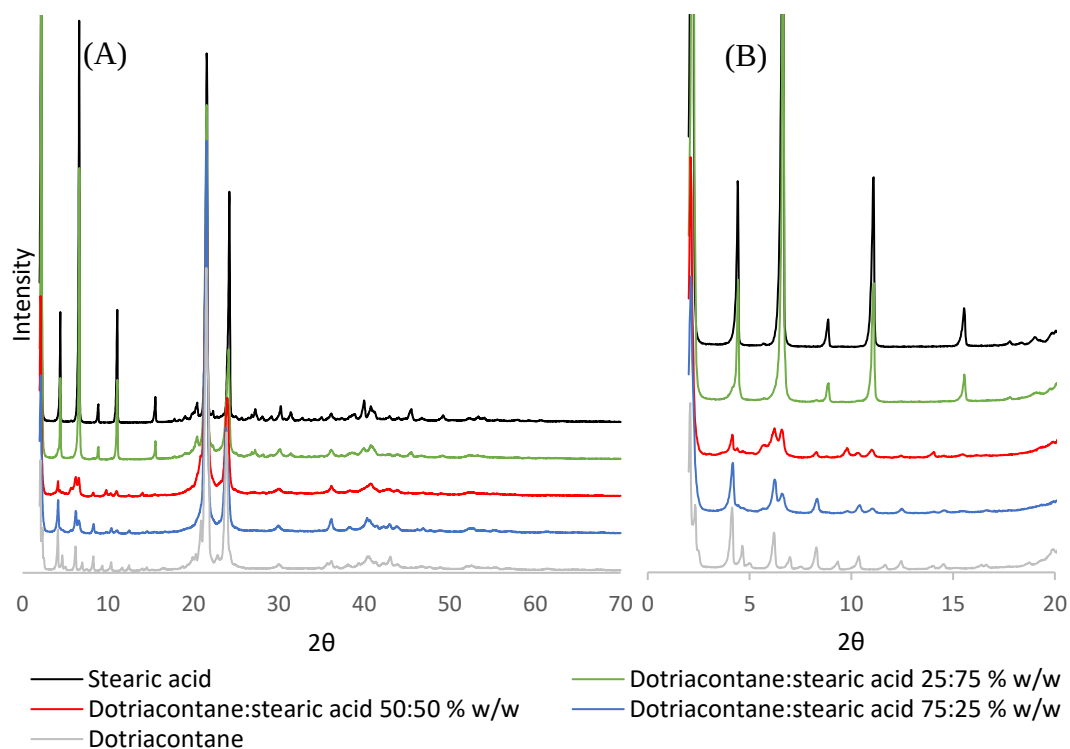


Figure 3-26 - XRD diffractogram of dotriacontane, stearic acid and associated binary mixtures. (A) shows the full diffractograms, cuts of at intensity 3000. (B) shows a zoomed-in view of 2 2θ - 14 2θ and intensity up to 2000.

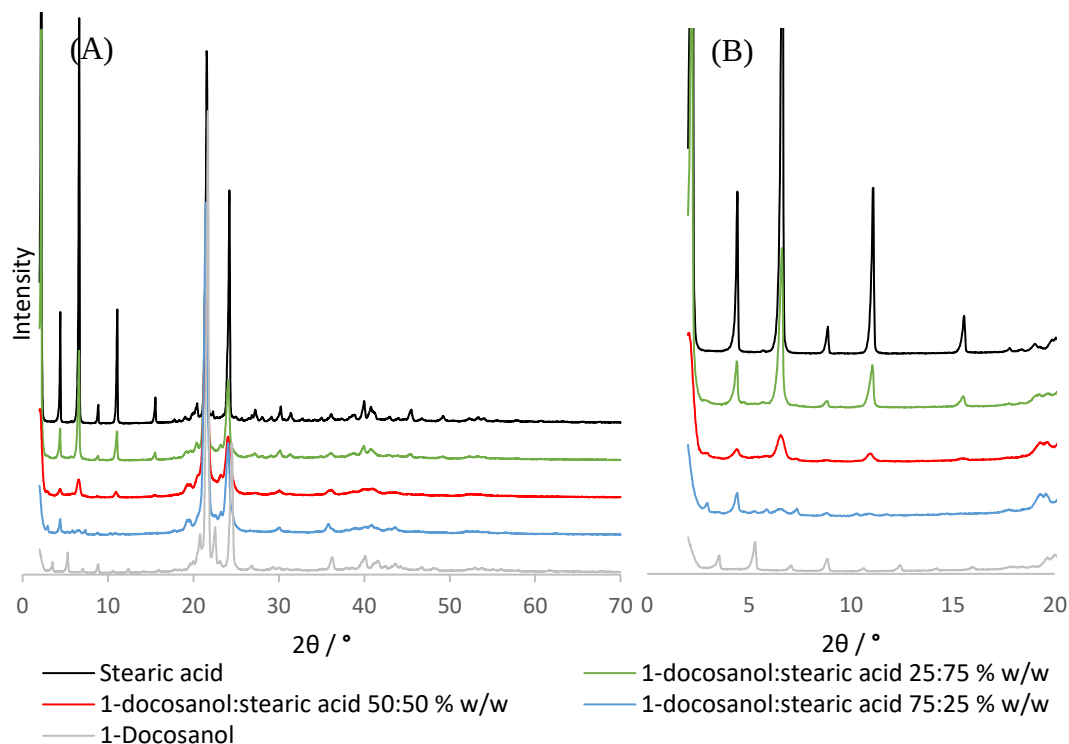


Figure 3-27 - XRD diffractogram of 1-docosanol, stearic acid and associated binary mixtures. (A) shows the full diffractograms, cuts of at intensity 3000. (B) shows a zoomed-in view of 2θ - 14θ and intensity up to 2000.

Figure 3-25, Figure 3-26, Figure 3-27 show diffractogram overlays of the single component waxes with a hypoeutectic, close to eutectic and hypereutectic mixtures. The 25: 75 % w/w and 75: 25 % w/w waxes only present peaks associated with the wax in higher concentrations. Because no chemical reactions occur between components within a eutectic material, the crystalline lattice structures of the parent components would remain largely unaffected. As such, structures are simply a microstructure of alternative platelets consisting of crystalline domains rich in a single parent component⁵³. This is a ubiquitous behaviour with hypoeutectic, eutectic and hypereutectic materials, strongly suggesting the material is eutectic. Furthermore, it excludes the possibility of the presented material being a co-crystal as all original component peaks are present with no addition peaks. It is likely, given the FT-IR, DSC and XRD data, that the wax has formed a phase-separated eutectic consisting of alternating platelets crystalline structures rich in the single component compounds⁵³.

Interestingly, the 50: 50 % w/w mixture of DOT: 1-D shows almost no dotriacontane peaks, whereas the 50: 50 % w/w mixture of DOT: SA shows almost no stearic acid peaks. This suggests these waxes are hyper- and hypoeutectic, respectively, but given the superimposing of the dotriacontane polymorphic change on the liquidus peaks (Figure 3-12 and Figure 3-13), it is challenging to know the exact location of the eutectic points.

Minimal or no amorphous halo could be identified in any wax imaged, and the peaks observed were relatively narrow. This suggests that the waxes are highly crystalline with relatively coarse grains. Other authors have reached similar conclusions concerning long-chain aliphatic compounds and cuticle waxes as measured by XRD⁵⁰. This suggests that the results observed in Table 3-4 might not represent the system's full complexity. 90: 10 % w/w DOT: SA and 50: 50 % w/w 1-D: SA, two waxes that present relatively low crystallinity values as per FT-IR (Table 3-4). However, neither wax show a clear amorphous halo, as might be expected based on the FT-IR results. It is unclear why this was found.

Interestingly the addition of secondary waxes wherein chain length and chemical functionality are varied does not result in the 010 and 100 reflections shifting. This has been seen prior in orthorhombic arranged waxes showing the crystalline arrangement, and basal side to side spacing of chains is unaffected by secondary waxes and is used to show waxes arranged into excluded crystalline grains wherein a stable structure is observed regardless of composition^{52,54}. This is observed in these tested waxes suggests that similar excluded crystalline structures have been formed.

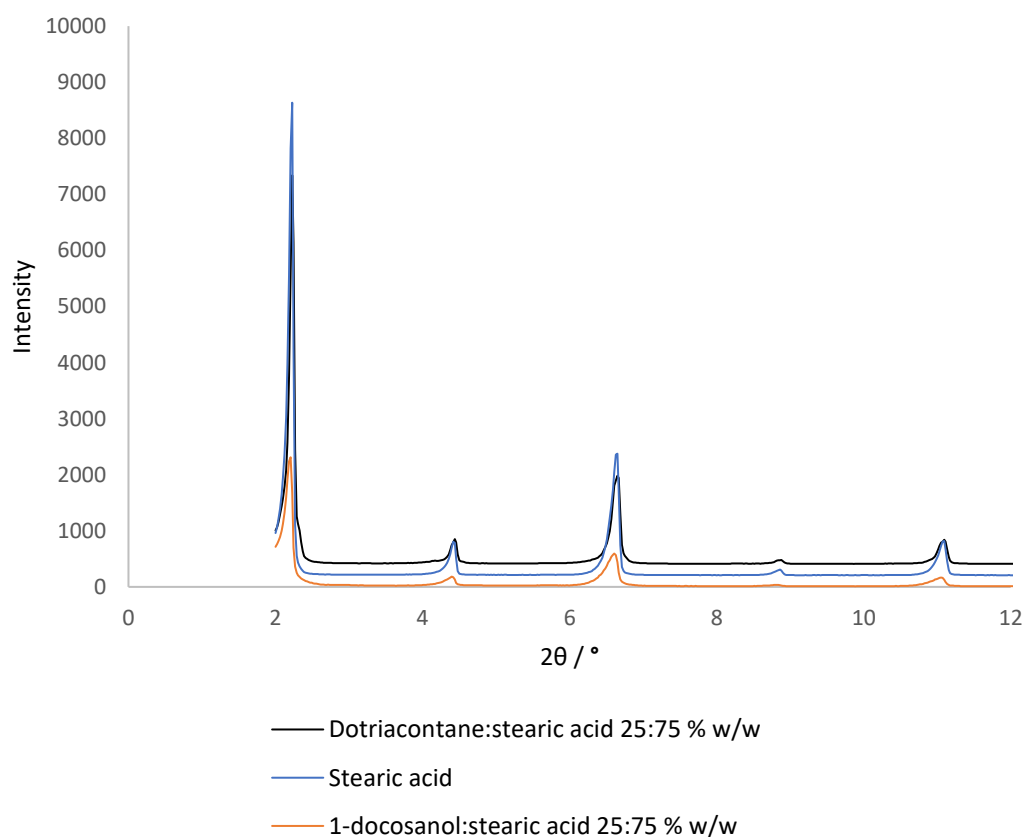


Figure 3-28 - XRD diffraction patterns showing the difference in intensity of long spacing peaks.

Only stearic acid and DOT: SA 25: 75 % w/w showed high-intensity long spacing peaks. Even though 1-D: SA 25: 75 % w/w appeared to have long spacing peaks when comparing against stearic acid and DOT: SA 25: 75 % w/w, Figure 3-28, the peaks are relatively small. NMR studies of similar aliphatic, lacking long-spacing peaks, have often shown a considerable amorphous component in the wax, directly contradicting the explicit crystalline behaviour as shown by XRD. This suggests that the models produced using NMR are too simplistic, a view reached by several authors^{40,55}. It is proposed that the measured amorphous component results from the disorder of the generated nematocrystalline order. This suggests that waxes lacking long-spacing peaks are all crystalline with a considerable head-to-head disorder, whereas waxes showing long-spacing peaks are crystalline in a long and short spacing order.

3.3.3. SEM imaging of drop cast waxes

Given that the waxes tested are likely printable, while suboptimal, it was essential to image the solidified wax surface to compare with printed surfaces. Based on a critical assessment of wax recrystallisation, it is likely that printed waxes will present similar surface morphologies as drop cast waxes. This is because waxes behave as a self-organising system that the following reconstitution from a solvent or recrystallisation from a melt will retain essential physical properties.

A total of 12 molten waxes: dotriacontane, 1-docosanol, stearic acid and the 25: 75, 50: 50 and 25: 75 % w/w mixtures of these waxes were drop cast onto a hotplate held at 90 °C before being moved onto hotplates held at lower temperatures to solidify before the surface was imaged using SEM.

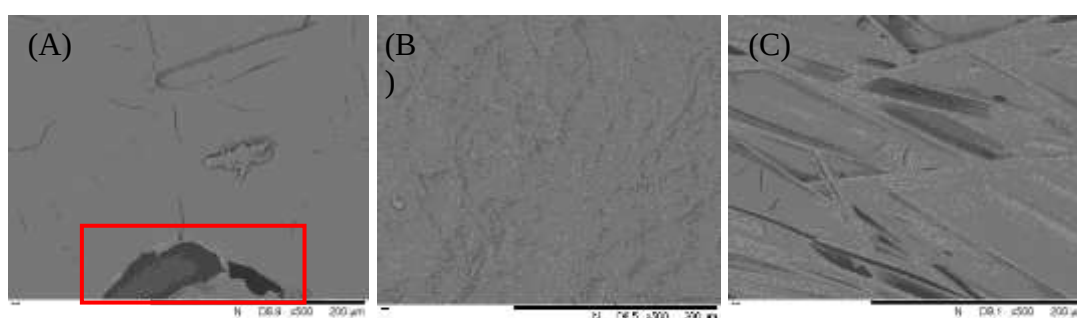


Figure 3-29 - (A) dotriacontane, (B) 1-docosanol and (C) stearic acid drop cast as molten wax on a PET substrate cooled at 25 °C. The red box on (A) signifies the location of a void on the wax film. The scale bar represents 200 µm.

Figure 3-29 shows that each wax produced a different surface topography depending on the wax composition. Dotriacontane (Figure 3-29 (A)) presents a relatively homogenous surface topography with small cracks throughout the film with no visible directionality. Striations are

observed in the void at the bottom of the image, suggesting that cooling might be different internally vs the air-wax interface, resulting in topography differences. 1-docosanol (Figure 3-29 (B)) shows a more heterogeneous surface with striations across the surface and lamellar structures, signifying the formation of ridges. Stearic acid (Figure 3-29 (C)) shows evident morphology, forming ridges and troughs across the surface. These structures show directionality, which suggests they are crystalline and ordered in nature. These ridge structures share some morphological similarities with either the crust or platelet structures found on the surface of a cuticle⁵⁶, Section 1.2. However, the structures are seen in Figure 3-29 (C) are much larger. No link to the defined families of wax crystalloids could be seen with dotriacontane or 1-docosanol.

The wax films in Figure 3-29 were all cooled rapidly. By modifying the temperature of the substrate, it was possible to change the wax film morphology as the rate of cooling was changed.

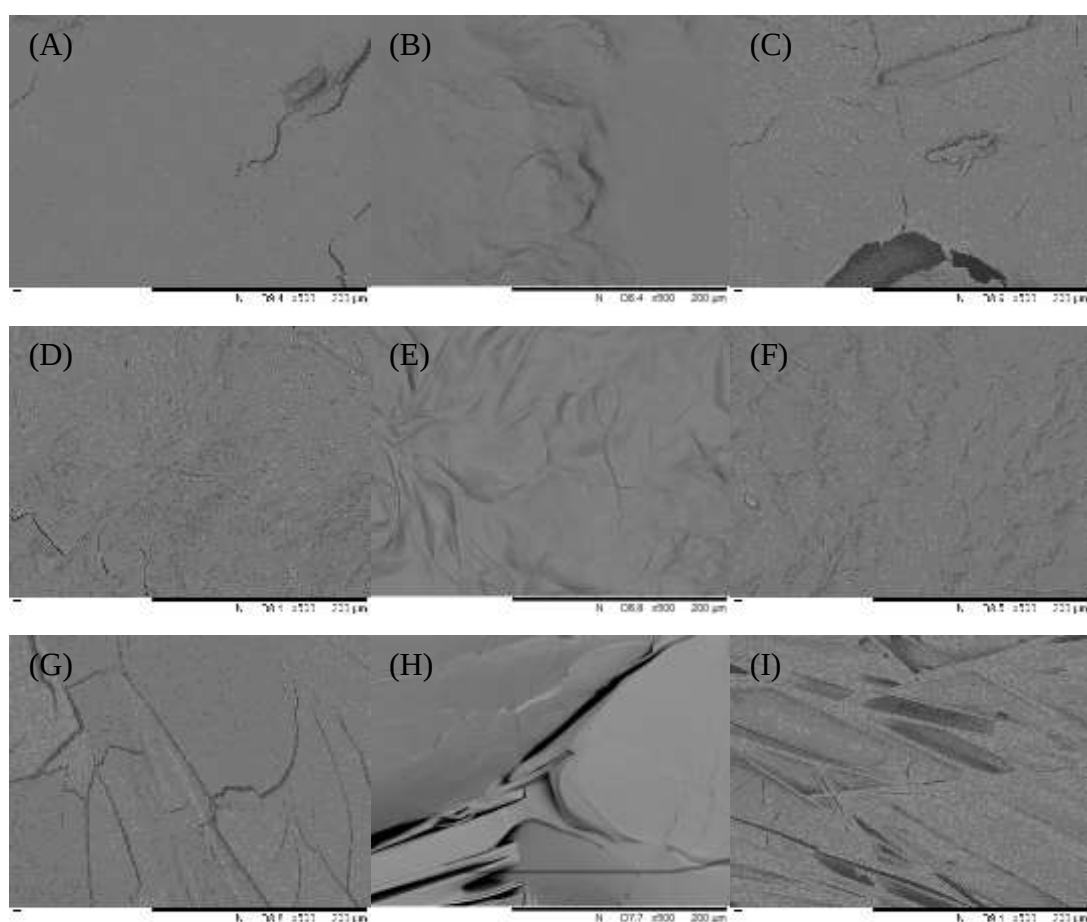


Figure 3-30 - (A) Dotriacontane drop cast from 90 °C to 60 °C, (B) dotriacontane drop cast from 90 °C to 40 °C, (C) dotriacontane drop cast from 90 °C to 25 °C, (D) 1-docosanol drop cast from 90 °C to 60 °C, (E) 1-docosanol drop cast from 90 °C to 40 °C, (F) 1-docosanol drop cast from 90 °C to 25 °C, (G) stearic acid drop cast from 90 °C to 60 °C, (H) stearic acid drop cast from 90 °C to 40 °C and (I) stearic acid drop cast from 90 °C to 25 °C. The scale bar represents 200 μm.

Figure 3-30 shows that as the cooling rate was decreased, the morphology of the wax film produced changed. Broadly, as the substrate temperature increased and the cooling rate reduced, the surface presented a more homogenous surface with fewer defining features. The images of 1-docosanol (Figure 3-30 (D)-(F)) clearly show this with increasing striations forming as the cooling rate was increased. The stearic acid film allowed to cool slowly (Figure 3-30 G) more closely resembles the fissured layer cuticle structure.

Such behaviour is well understood regarding metals⁵⁷. Similar work with waxes showed similar behaviour⁵⁸. As the cooling rate decreases, the recrystallisation is more uniform, and the crystals grow larger. Given this, it might be possible to control the printed film's morphology by modifying the cooling rate. This could be achieved via increasing or decreasing the difference in temperature of the droplets and the incident substrate, to increase or decrease the rate of cooling respectively.

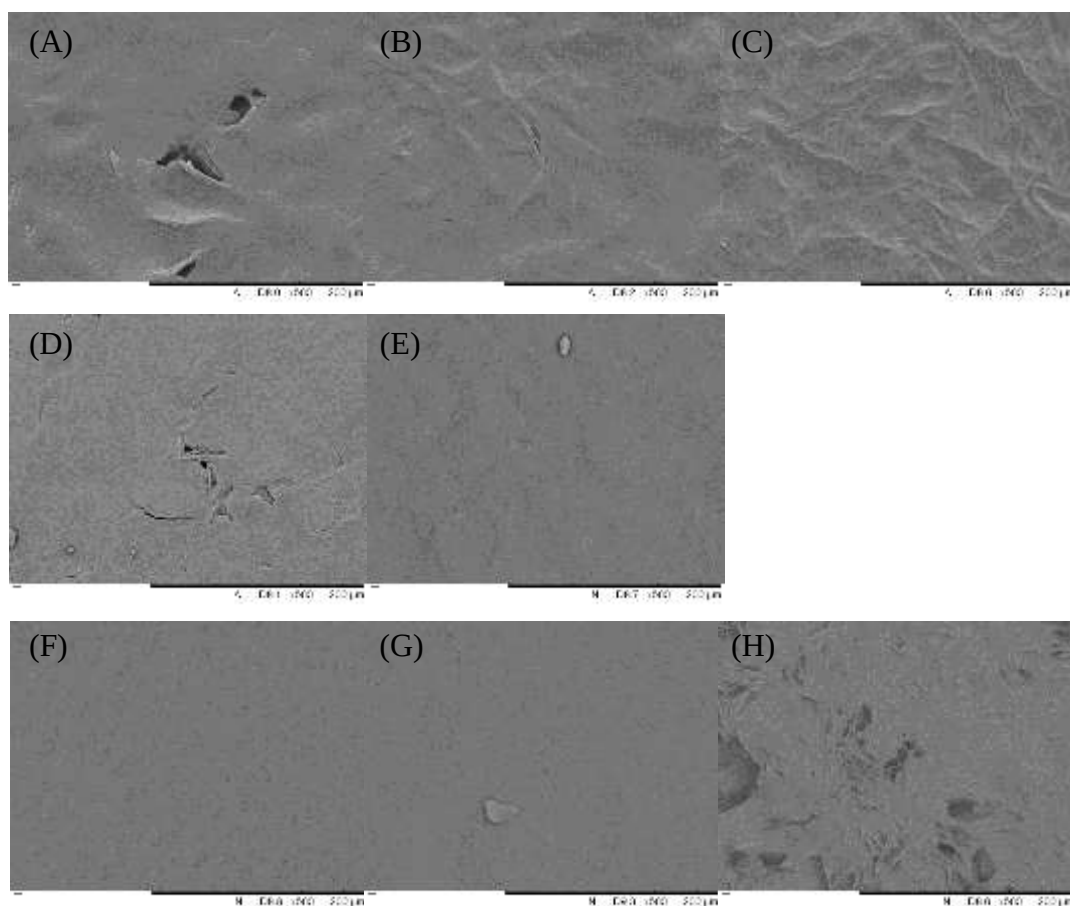


Figure 3-31 - (A) 75: 25 % w/w DOT: 1-D, (B) 50: 50 % w/w DOT: 1-D, (C) 25: 75 % w/w DOT: 1-D, (D) 75: 25 % w/w DOT: SA, (E) 50: 50 % w/w DOT: SA, (F) 75: 25 % w/w 1-D: SA, (G) 50: 50 % w/w 1-D: SA and (H) 25: 75 % w/w 1-D: SA. All drop cast waxes moved from a substrate held at 90 °C to 25 °C. Many surfaces lack prominent surface features, so images were taken at locations with contaminants to demonstrate the lack of features was due to the surface rather than poor focusing. The scale bar represents 200 μm.

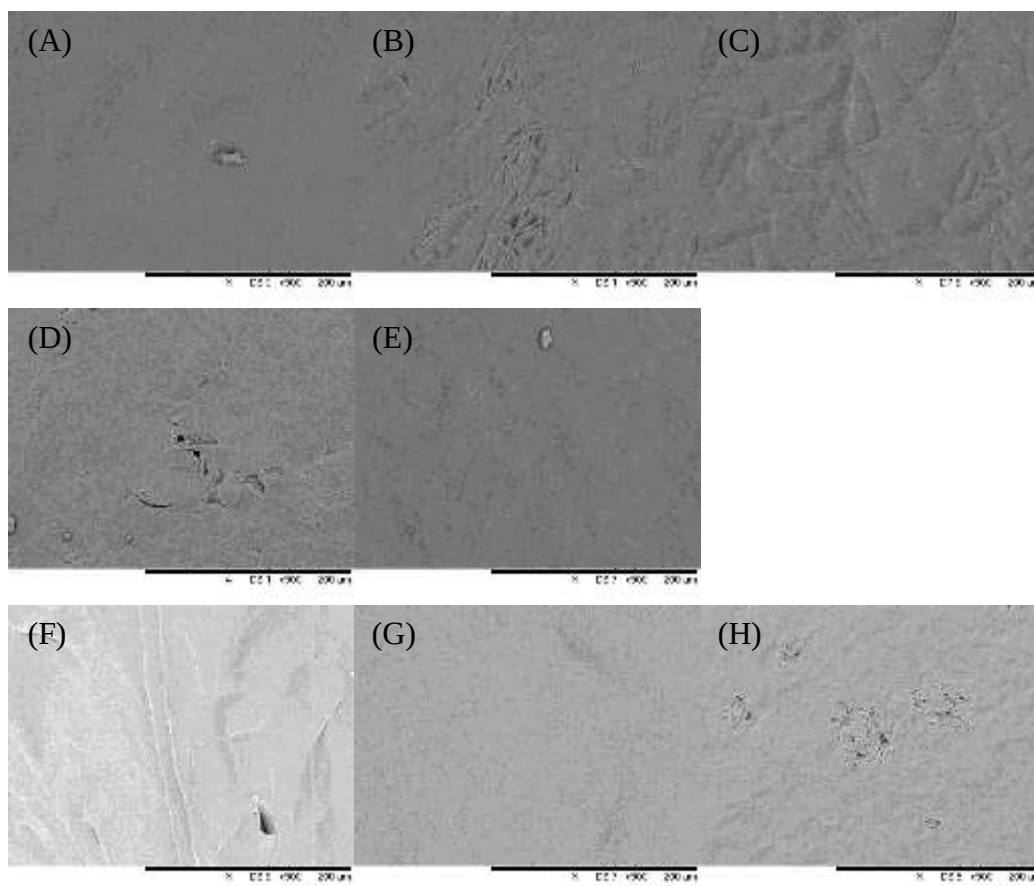


Figure 3-32 (A) 75: 25 % w/w DOT: 1-D, (B) 50: 50 % w/w DOT: 1-D, (C) 25: 75 % w/w DOT: 1-D, (D) 75: 25 % w/w DOT: SA, (E) 50: 50 % w/w DOT: SA, (F) 75: 25 % w/w 1-D: SA, (G) 50: 50 % w/w 1-D: SA and (H) 25: 75 % w/w 1-D: SA. All drop cast waxes moved from a substrate held at 90 °C to 60 °C. Many surfaces lack prominent surface features, so images were taken at locations with contaminants to demonstrate the lack of features was due to the surface rather than poor focusing. The scale bar represents 200 μm.

Similar to what is observed in Figure 3-30, the use of new compositions, in this case the binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid, resulted in morphology changes (Figure 3-31 and Figure 3-32). It can be readily observed that the morphology of these drop cast waxes shares morphological similarities with the components that comprise the bulk of the mass. As examples, Figure 3-31 (C) and Figure 3-32 (C) is comprised mainly of 1-docosanol and show striations very similar to Figure 3-29 (B). Additionally, samples comprised mainly of stearic acid typically showed the formation of platelet structures (Figure 3-31 (I) and Figure 3-32 (I)), while samples comprised primarily of dotriacontane were homogenous (Figure 3-31 (A) and (D) and Figure 3-32 (A) and (D)). 50: 50 % w/w waxes close to the eutectic point were expected to form a unique structure at the eutectic. As can be seen, the DOT: SA and 1-D: SA binary waxes formed a uniform homogenous surface, whereas the DOT: 1-D binary wax formed a more complex platelet structure not seen in either of the single component waxes.

Figure 3-31 and Figure 3-32 show that modifying substrate temperature resulted in changes to the morphology of the drop cast waxes similar to what was seen in Figure 3-30. By reducing the thermal gradient, the cooling rate is reduced, resulting in greater homogeneity.

Based on this, similar structures should be seen in the printed samples, provided the same substrate temperatures are used, and the temperature difference between molten wax and substrate remains the same. However, the smaller size of the printed droplets vs the bulk molten wax drop cast will likely result in more rapid cooling rates and, thus, more defined surface morphology such as platelet structures seen in the stearic acid samples.

3.4. Conclusions

ToF-SIMS analysis of the epicuticle and intracuticle *Solanum Lycopersicum* surfaces shows apparent compositional heterogeneity attributed to differing wax compositions. In particular, there is a trend for alcohol waxes to be associated with the epicuticle surface and for fatty acid waxes to be associated with the intracuticle surface. The presence of these two waxes agrees with bulk wax composition data for *Solanum Lycopersicum*, and the localisation of these waxes agrees with literature that has observed heterogeneous trends regarding wax distribution. This suggests that ToF-SIMS and associated MVA analysis might be a future avenue for spatial evaluation of cuticle composition, which has often been evaluated using Raman spectroscopy.

Subsequently, to model the waxes that comprise the bulk of the *Solanum Lycopersicum* cuticle waxes, dotriacontane, 1-docosanol and stearic acid were selected for further analysis. These three waxes were selected based on their chemical functionality and chain length being similar to the waxes found within the *Solanum Lycopersicum* cuticle while also being commercially viable for printing which can use multiple grams of material. Similar waxes have been evaluated before for eutectics when mixed. The mixing of waxes and potential generation of a eutectic should increase the scope of waxes for analysis and better model the cuticle, which is known to present many wax-wax interactions, including a large number of simultaneously forming eutectics.

Testing these waxes' thermal and rheological properties highlighted that all the single component waxes and associated binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid are suitable for printing. All waxes melt at temperatures below the maximum heating capabilities of the Spectra S-class printhead, and their viscosity does not exceed the printhead's capabilities, which could result in damage. All waxes, unfortunately, presented viscosities that fell below the recommended viscosity range, which might prove challenging to print. Thermal DSC, FT-IR and XRD analysis showed the mixed waxes formed crystalline eutectic structures with a small number showing long spacing order as per XRD. FT-IR showed that most waxes tested are predominantly crystalline, corroborating the XRD results. However, there is no clear trend regarding composition and crystallinity. The formation of a eutectic increases the scope of the available waxes beyond the three identified waxes.

SEM imaging of the tested waxes shows that all waxes display some degree of unique topography, which in some cases could relate to similar crystalloid structures found on the

epicuticle surface. Binary waxes wherein the mass composition are dominated by one wax showed morphology that largely resembled the dominant wax. However, waxes near the eutectic point showed an essentially unique morphology. This suggests that through modification of wax composition, it might be possible to induce specific morphologies. Similar results have been seen prior with reconstituted cuticle waxes and long-chain aliphatics. Waxes extracted from a cuticle retain the same structures before extraction and present unique surface morphology dependent on the wax composition and the cuticle the waxes were extracted from. Using different substrate temperatures and thus controlling the rate of cooling made it possible to modify the morphology of the wax further. Faster cooling rates result in homogeneous morphologies, consisting of evenly distributed crystalloids structures, with a greater propensity to form topographical structures. Therefore, by modifying aspects of the wax preparation during printing, it might be possible to induce the formation of specific surface features and potentially modify surface properties.

3.5. References

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4. Chapter 4 – Printing and analysis of wax films

4.1. Introduction

Plants regulate the ingress and egress of compounds through the plant surface by presenting a barrier to movement, the cuticle¹. Much of the research elucidating the processes involved in agrochemical permeation were published between the 1970s and 1980s^{2,3} and the early 2000s^{4,5}. It was determined that the cuticle is a compositionally and functionally complex structure⁶, wherein permeation of lipophilic agrochemicals is controlled by cuticle composition, particularly waxes⁷. Hence, developing a structure-function relationship is challenging due to this compositional complexity and species variability. However, it might be possible to elucidate more detail using models of the cuticle wax as is intended in this work.

Many models for cuticles developed to date use thin wax films as an analogue for the cuticle wax-polymer membrane⁸. The most common example of this is reconstituted cuticle waxes which allow the analysis of the provided waxes and thus wax behaviour on different cuticles^{9,10}. These films, however, do not provide information on the impact of specific waxes nor retain the original structure of the native cuticles^{4,11}. Only a small amount of work has been performed testing specific singular or binary waxes^{8,12,13}. Such models, it is hoped, would allow specific functions of different waxes to be elucidated regarding environmental interactions, agrochemical application or structural development.

Examples of studies using singular or binary wax models of the cuticle include investigating FT-IR spectra of wax mixtures⁸, surfactant-wax interactions¹² and wax crystalloid morphology¹³. An interesting idea is testing agrochemical penetration through model wax cuticles¹². Permeation of lipophilic compounds through the cuticle is controlled almost entirely by cuticle⁴, and as such, a wax film is likely a suitable model. Using singular or binary wax films, it might be possible to test the permeation of different agrochemicals and derive information of the role and specific waxes and wax-wax interactions on permeation. Such experiments cannot readily be performed with reconstituted waxes, as it is hard to selectively remove specific cuticle waxes from the cuticle¹⁴. It is also likely that a simple wax film could be scaled for high throughput penetration testing.

Additionally, by assessing the permeation of a formulation containing surfactants, it might be possible to identify the impact of surfactants on agrochemical permeation as a function of wax composition. The same surfactant responds differently to cuticles presenting unique

compositions, resulting in changes to permeation^{15,16}. However, the nature of these surfactant-wax interactions is not fully understood.

If the function of different waxes concerning cuticle properties can be understood, modifying formulations to maximise transport of agrochemicals to the site of action through evaluation of the chemical composition of the cuticle might be possible.

All leaves and isolated cuticles are currently used to test these properties, including permeation¹⁰. However, whole leaves often make analysis challenging due to their biological complexity¹⁷, and isolated cuticles often cannot be effectively extracted^{18,19}. Subsequently, the development of novel agrochemicals is slow and challenging.

Here it is demonstrated for the first time an inkjet-printed wax film to model the behaviour of the cuticle wax component using three different cuticle wax analogues: dotriacontane, 1-docosanol and stearic acid and a range of binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid. Inkjet printing allows around 50 μm resolutions to be achieved and facilitates the co-deposition of multiple inks without using a solvent (if a hot-melt approach is used). Inkjet-printing thus could enable replication of some of the compositional complexity observed in native isolated cuticles. As part of this process, a range of simple homogenous wax films was produced from 18 different waxes, characterised in Chapter 3. The tested waxes consisted of the three single component waxes: dotriacontane, 1-docosanol and stearic acid and 3 sets of 5 binary waxes consisting of various weights of the two single components waxes. Additionally, heterogeneous wax films were produced through co-deposition of different waxes within a single film. Modification of printing parameters: substrate temperature, drops per inch, droplet velocity and film thickness were investigated to assess the morphological effects this would induce.

All films were investigated using SEM, Water contact angle, XRD and FT-IR to study print induced modification regarding morphology, crystallinity and chemical composition. Raman spectroscopy was used to investigate potential phase separation on a subset of films.

This chapter's primary goal is to describe the development of homogeneous and heterogeneous wax films that could be printed consistently with minimal sample failure. These films could subsequently analyse surfactant-wax interactions, fungal attachment and agrochemical permeation.

4.2. Methods and materials

4.2.1. Materials

Wax materials used were powder 1-docosanol (98%), dotriacontane (97%) and stearic acid (98.5%) purchased for Sigma-Aldrich (Dorset, UK) and used without further purification.

Non wax materials used were Synperonic™ A20 (A20) and Synperonic™ A7 (A7). A20 and A7 are both alcohol ethoxylate surfactants with a 13 to 15 carbon chain and a 20 and 7 polyoxyethylene units respectively. A20 and A7 were kindly provided by Croda Crop care (Goole, UK).

4.2.2. Thermogravimetric analysis (TGA)

TGA measurements were performed using an SLA 6000 Instrument (Perkin Elmer, Waltham, USA). Approximately 5 mg -6 mg of wax powder was placed in a ceramic crucible on the sensitive analytical balance contained within the TGA furnace. Wax material was heated at a 20 °C /min rate between 50 °C to 600 °C under nitrogen purging, 20 mL/min. Gravimetric analysis of the material was continually captured and plotted as a function of temperature to generate TGA curves showing weight loss due to decomposition.

Differential thermogravimetric analysis (DTA) curves were produced by differentiating the TGA weight vs temperature curve, allowing accurate identification of the onset, peak and offset of thermal degradation events identified in the TGA curve

TGA was performed primarily to identify if wax powders would undergo any degradation at temperatures relevant to operational use, 70 °C – 90 °C, identify at what temperature 5% weight degradation could occur and identify the thermal degradation events for each wax. This was done to ensure the waxes used would be thermally stable for printing.

4.2.3. Inkjet Printing

Wax films were printed using a piezoelectric inkjet printer (PiXDRO LP50, Meyer Burger, Netherlands) equipped with a Spectra-S class printhead (Spectra® SE-128 AA, Fujifilm Dimatix, Santa Clara, USA). The S-class printhead presents 128 nozzles with a diameter of 35 µm each. Unless otherwise specified, printing was conducted using one nozzle at a firing voltage of 90 V and an applied voltage of 60 V. Waveforms were selected to maintain a droplet velocity of 3 m s⁻¹ 150 µs after droplet ejection.

The printer was equipped with a hot melt chamber, melting solid material before passing through the printhead. The hot melt chamber and printhead were maintained at 90 °C to minimise aberrant droplet ejection. A heated polyethylene terephthalate (PET) substrate

held at 50 °C was used. A heated substrate was used to control the formation of surface structures by controlling cooling rates. PET substrate was adhered to the heated bed using an underlying vacuum pump and tape around substrate edges. Prints were designed in Inkscape and imported as customised 2D designs in the .BMT file format.

All samples printed onto a heated substrate were tempered for at least 24 hours, after cessation of printing, at 50 °C. This was done because printing could take many hours to complete a large set of samples. Subsequently, samples printed at the start will present a different thermal history than droplets printed at the end of the print. Tempering the print was done to ensure the thermal history each wax was exposed to was as uniform as possible. Following tempering, the heated substrate was allowed to cool to room temperature. Samples were removed from the substrate using a razor to lift samples from the PET. Bending samples resulted in cracking and damage to the sample. Failure to adhere the PET to the heated substrate risks the PET bending during cooling, which would result in the prints likewise bending as they cool.

As performed in Section 3.2.2 waxes were mixed and printed at known ratios of w/w %. These ratios were: 10: 90 w/w %, 25: 75 w/w %, 50: 50 w/w %, 75: 25 w/w % and 90: 10 w/w %.

4.2.4. Scanning electron microscope (SEM)

Scanning Electron Microscope imaging of wax films was conducted using a Hitachi TM3030 tabletop SEM (Japan). For additional information see Section 2.9.

SEM was conducted to image the change in morphology induced by changing printing parameters and modification of wax composition. Additionally, it was used to image the change in wax surface morphology following surfactant drying.

4.2.5. Fourier-transform infrared spectroscopy (FT-IR)

FT-IR was performed on a Frontier FT-IR equipped with an ATR crystal (Perkin Elmer, Waltham, United States). Additional experimental information can be found in Section 3.3.2.2.

FT-IR was used to assess if printing waxes changed the IR spectra of these waxes relative to powder waxes. Potential peak broadening, peak shifting and novel peak formation were searched for evidence of chemical composition changes or changes to measured bonds, i.e. bond length. Additionally, the % crystallinity of printed wax was measured and compared against powder wax by evaluating the CH₂ rocking doublet. For further details, see Section 3.3.2.2.

FT-IR was performed before all printing. Excess material was purged from the printhead, and the FT-IR spectra were measured and compared with drop cast wax FT-IR spectra. This was performed to ensure no ink contamination within the printhead before beginning a print. If a spectra peak diagnostic of waxes not expected to be present in the ink, Section 3.3.2.2 was found it was assumed to be caused by a residual wax from prior prints contaminating the new ink. Additional ink was purged until an FT-IR spectrum showed no diagnostic peaks of unexpected waxes.

4.2.6. X-ray Diffraction (XRD)

XRD patterns were collected using a Bruker D8 Advance with DaVinci X-ray Diffractometer (Bruker, USA). Wax films were analysed by suspending a 15 mm x 15 mm wax film over a perspex sample holder using scotch tape to minimise interference in the XRD measurement. For further experimental details, see Section 3.2.7.

XRD was used to assess the changes in the crystalline structure of the printed wax films before and after printing. In addition, XRD was used to assess if a small selection of waxes would display variable crystalline arrangements as a function of temperature.

4.2.7. Water contact

Water contact angles on printed wax films were measured using a DSA100 (Kruss, Germany). 10 μ L of Milli-Q water was dosed using a 1 mL syringe and attached needle, external diameter 1.24 mm. The printed film sample was driven upwards to collect the droplet, minimising spreading due to impact before the substance was retracted, separating the droplet from the needle. Measurements began immediately after, with photos of the droplet taken at a rate of 1 Hz for 4 minutes. The contact angle of the left and right sides of the droplet was determined within each photo using a Young-Laplace fitting and were averaged to give a value of contact angle. The difference between the initial ($t = 0$ s) and final contact angle ($t = 240$ s) gives the change in contact angle over time. Between 2 - 5 droplets per surface were evaluated on three surfaces per measurement.

The top surface of 5 layer printed films was tested with Milli-Q water, A7 0.12 % w/v and A20 0.12 % w/v, whereas the top surface of 10 and 20 layer prints and the bottom surface of a printed sample was only tested with the water.

The Young's equation and contact angle measurements respectively were used to understand the shape of a stabilised liquid droplet, following impact with the substrate before the droplet froze, and to assess the spreading of water and the two surfactant

solutions on the printed wax films. The latter test was performed to understand if wax chemical composition affects spreading or if spreading effects are primarily a function of topographical features on wax surfaces.

4.2.8. Raman Spectroscopy

Confocal Raman spectroscopy was performed using a LabRAM HR confocal Raman microscope (Horiba) with a motorized XYZ stage. Spectra were obtained using a 785 nm laser at a power of approximately 20 mW and a 200 μm confocal pinhole. To simultaneously scan a range of Raman shifts, a 600 lines mm^{-1} rotatable diffraction grating along a path length of 800 mm was employed. Spectra were acquired using a Synapse CCD detector (1024 pixels) thermoelectrically cooled to $-60\text{ }^{\circ}\text{C}$. Before spectra collection, the instrument was calibrated using the zero-order line and a standard Si(1 0 0) reference band at 520.7 cm^{-1} . The spectral resolution was $<1.7\text{ cm}^{-1}$ in this configuration.

Three single-point spectra of 3D printed wax films containing a single component were acquired over the range 65 cm^{-1} - 3200 cm^{-1} with an acquisition time of 15 s and two accumulations to automatically remove spikes due to cosmic rays and improve the signal to noise ratio. These spectra were averaged to give a mean.

Multispectral images were obtained for binary wax 3D printed films by collecting spectra over the range 280 cm^{-1} – 1700 cm^{-1} in either 1 μm steps within a $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$ area with 10 μm steps within a $500 \times 500\text{ }\mu\text{m}$ area, a total of 2601 spectra each. Each spectrum was acquired for 1 s with 1 accumulation giving a total acquisition time of approximately 1 h. The spatial resolution was calculated to be approximately $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ (100x objective, $N_A = 0.75$) and $10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m} \times 30\text{ }\mu\text{m}$ (50x objective, $N_A = 0.55$). The DuoScanTM functionality was employed to increase this configuration's effective laser spot size. The extensive surface topography was corrected using AutoFocus and ViewSharpTM functionalities. Spectra were manually despiked to remove artefacts, extracted within the range 800 cm^{-1} - 1700 cm^{-1} , corrected by subtracting a linear baseline and fit with the component wax spectra using Classic Least Squares (CLS) regression analysis within Labspec 6.5 software. Images were generated by plotting the scores obtained from CLS analysis. CLS scores were normalised to a total of 100 %.

Raman spectroscopy was performed to evaluate the chemical composition of a subset of wax films across several print tracks to evaluate if the wax distribution was homogenous. Prior attempts had been made to achieve this using ToF-SIMs. However, the complexity of the spectra and similarity of wax materials made such analysis highly challenging.

4.3. Results and discussion

4.3.1. TGA

TGA allowed the determination of the thermal stability of waxes at relevant temperatures for printing and to understand the degradation process. Subsequently, mass loss at 70 °C, 80 °C and 90 °C, Δm_{70} , Δm_{80} , Δm_{90} respectively, and the temperature at 5 % weight loss, $T_{\Delta w 5\%}$, was measured. Additionally, the temperature at which degradation transition onsets, peaks and outlets, T_{on} , T_p , T_{out} respectively, and the peak of any secondary peaks, T_{s1} , T_{s2} , T_{s3} , ... est, was identified.

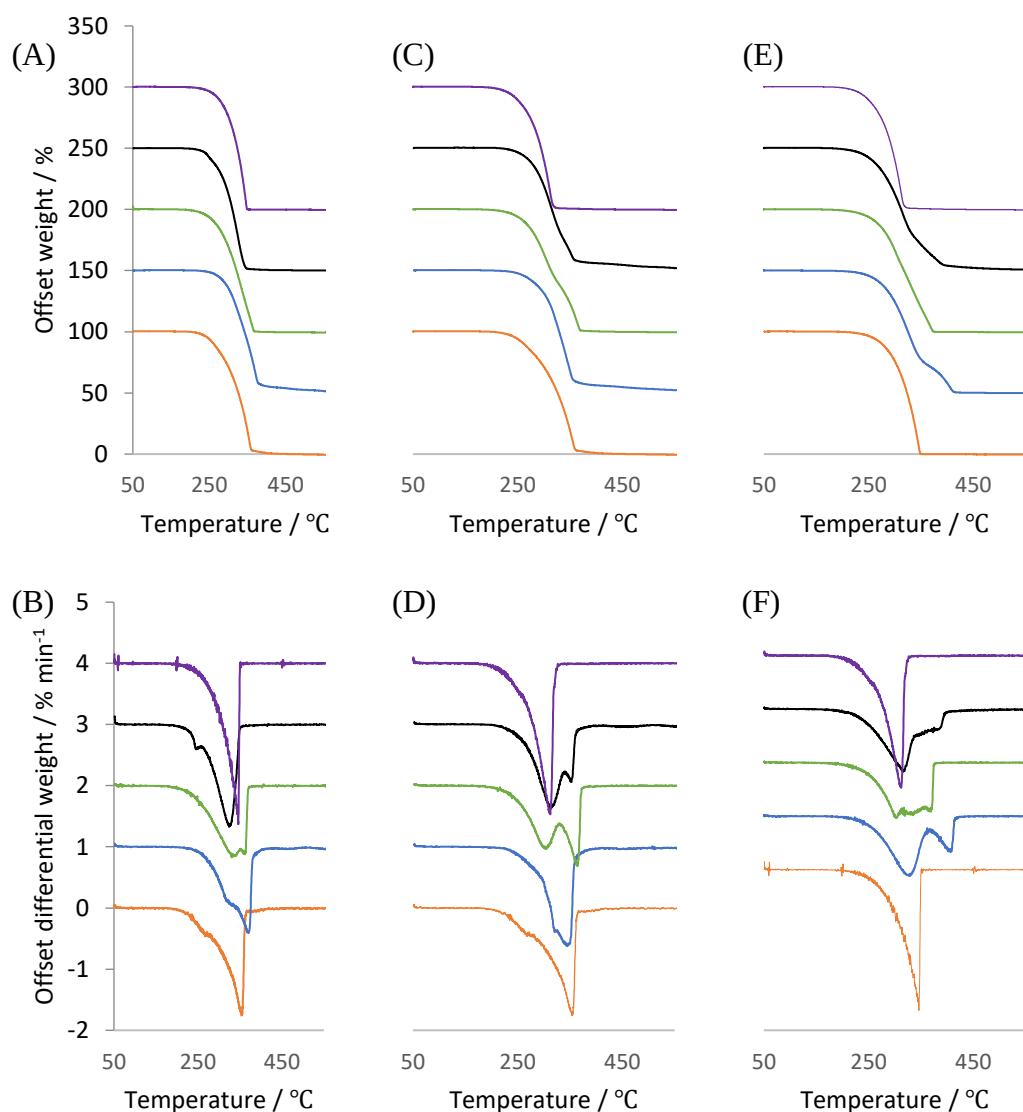


Figure 4-1 - TGA and DTA graphs of (A,B) dotriacontane, 1-docosanol and the associated 25: 75, 50: 50 and 75:75 % w/w mixtures, (C,D) dotriacontane, stearic acid and the associated 25: 75, 50: 50 and 75:75 % w/w mixtures and (E,F) 1-docosanol, stearic acid and the associated 25: 75, 50: 50 and 75:75 % w/w mixtures. (A, B and C) are TGA curves, and (B, D and F) are DTA curves. Orange lines represent the first single component wax: dotriacontane (A, B, C and D) or 1-docosanol (E and F), purple lines represent the second single component wax: 1-docosanol (A

and B) or stearic acid (C, D, E and F). The blue, green and black lines represent the 75: 25, 50: 50 and 25: 75 % w/w mixtures respectively of the first and second single component wax, respectively. $\Delta m_{70}/^{\circ}\text{C}$

Table 4-1 - Summary of all data acquired from TGA and DTA graphs.

Wax	$\Delta m_{70}/^{\circ}\text{C}$	$\Delta m_{80}/^{\circ}\text{C}$	Δm_{90}	$T_{\Delta 5\%}$	T_{on}	T_{s1}	T_p	T_{off}
Dotriacontane	99.9	99.9	99.9	252.8	172.0	273.3	354.1	419.9
DOT: 1-D 75: 25 % w/w	99.9	99.9	99.9	262.3	195.9	325.9	371.2	424.8
DOT: 1-D 50: 50 % w/w	99.9	99.9	99.9	253.8	179.9	334.4	362.1	381.5
DOT: 1-D 25: 75 % w/w	99.9	99.9	99.9	249.4	191.1	245.0	325.1	370.6
1-docosanol	99.9	99.9	99.9	233.5	187.3	-	346.2	357.6
Dotriacontane	99.9	99.9	99.9	252.8	172.0	273.3	354.1	419.9
DOT: SA 75: 25 % w/w	99.9	99.9	99.9	265.2	187.4	320.2	345.8	414.4
DOT: SA 50: 50 % w/w	99.9	99.9	99.9	245.3	174.1	301.8	364.4	384.1
DOT: SA 25: 75 % w/w	99.9	99.9	99.9	257.2	187.3	352.3	313.0	395.6
Stearic acid	99.9	99.9	99.9	233.1	172.5	-	312.4	334.2
1-docosanol	99.9	99.9	99.9	233.5	187.3	-	346.2	357.6
1-D: SA 75: 25 % w/w	99.9	99.9	99.9	246.0	178.4	407.1	326.8	441.3
1-D: SA 50: 50 % w/w	99.9	99.9	99.9	245.9	173.9	-	302.1	392.7
1-D: SA 25: 75 % w/w	99.9	99.9	99.9	243.9	171.4	384.5	315.7	421.9
Stearic acid	99.9	99.9	99.9	233.1	172.5	-	312.4	334.2

Figure 4-1 shows the TGA and DTA graphs of the three single component waxes and the associated binary wax mixtures tested. Critical derived data are summarised in Table 4-1.

Dotriacontane showed two peaks, the primary T_p at 354 °C and a shoulder at 273 °C. Studies of engine oils, which primarily comprise a range of long-chain alkanes, do not present a shoulder peak and present a T_p approximately 30 °C lower²⁰. This difference might result from the long-chain length of dotriacontane relative to the alkanes presented in the tested oils, increasing thermostability²¹. Samples degrade from 253 °C to 420 °C. This is similar to values in literature, shifted upwards²⁰. 1-docosanol presented only a single peak at 346 °C with a T_{on} of 233 °C and T_{out} of 358 °C. Compared with the literature, the peak shape and degradation rate are similar. However, 1-docosanol T_p is shifted upwards relative to literature. This is explained by the longer chain length of 1-docosanol relative to the tested waxes²¹. Stearic acid presented a single peak T_p = 312 °C with onset and outset at 233 °C and 334 °C, respectively. This is in close agreement with the literature²².

DOT: 1-D binary mixture (Figure 4-1 A and B) shows all three binary waxes and dotriacontane present a dual transition. This is clearest in the 50: 50 % w/w binary wax, wherein two peaks are found on the DTA curve, 334 °C and 362 °C, similar to the T_p of the component waxes,

354 °C and 346 °C for dotriacontane and 1-docosanol respectively. All DOT: 1-D binary waxes show a T_{on} between 172 °C and 196 °C and T_{out} between 425 °C and 358 °C. The T_{out} follows a relatively linear trend wherein the addition of dotriacontane increase the T_{out} . Interestingly, the T_p of the 25: 75 and 50: 50 % w/w correlated with the T_p of the dominant waxes present. However, 75: 25 % w/w binary wax showed a T_p larger than either dotriacontane or 1-docosanol. Why mixing these waxes in this w/w ratio produced a product with an increased T_{out} and T_{s1} is unknown. However, many eutectic materials show an increased T_{out} , with secondary peaks forming at temperatures exceeding the T_{out} of component materials²³. Subsequently, it is possible that the eutectic nature of the DOT: 1-D binary changes its thermal stability properties.

DOT: SA binary mixture (Figure 4-1 C and D) shows all binary waxes present dual transitions. The T_p of 25: 75 and 75: 25 % w/w waxes, 346 °C and 313 °C respectively, match the T_p of the dominant wax in the binary mixture, 354 °C and 312 °C for dotriacontane and stearic acid respectively. The 50: 50 % w/w mixture showed two peaks, 302 °C and 364 °C, with roughly similar areas. These peaks present at similar locations as the T_p of the component waxes. This suggests that mixing dotriacontane and stearic acid produced a homogenous dispersion with minimal modification of the thermostability properties. All DOT: SA binary waxes show a T_{on} between 172 °C and 265 °C and T_{off} between 334 °C and 420 °C. Interestingly, adding 25 % w/w dotriacontane to stearic acid resulted in a considerable increase in T_{off} relative to stearic acid. A similar phenomenon occurred with the 25: 75 % w/w DOT: 1-D wax, suggesting that adding dotriacontane can elongate the temperature window wherein degradation occurs.

Finally, considering 1-D: SA binary wax (Figure 4-1 E and F), the T_{on} varied from 233 °C to 246 °C, whereas the T_{out} varied from 334 °C to 441 °C. The addition of 25 % w/w 1-docosanol or stearic acid to form the 25: 75 or 75: 25 % w/w mixtures caused T_{out} to rise, relative to the bulk component wax, by approximately 80 °C - 90 °C, with minimal change to the T_{on} . This suggests that the addition of secondary waxes caused an elongation of the degradation window. The T_p of the 25: 75 and 75: 25 % w/w waxes, 327 °C and 316 °C respectively, was similar to the T_p of the bulk wax present, 346 and 312 °C, 1-docosanol and stearic acid respectively. However, both binary waxes displayed a secondary peak approximately 60 °C - 70 °C higher than the T_p of the bulk wax. This secondary peak is the cause of the elongated degradation window and is unlikely caused by a TGA operational issue as T_{on} remains relatively unchanged with any tested waxes, including the bulk waxes which match literature. If the location of peaks had shifted due to a potential heating issue, it would be expected that all thermal events would shift equally, including the T_{on} .

The 1-D: SA 50: 50 % w/w binary wax shows only one degradation event, unlike the DOT: 1-D and DOT: SA acid 50: 50 % w/w waxes tested, which show two peaks representing the two-component waxes. Instead, an elongated, linear degradation event is observed from 174 °C to 393 °C. Subsequently, the elongation of the degradation window seen in all 1-D: SA waxes does not always present as an aberrant secondary peak, seen in the 25: 75 and 75: 25 % w/w waxes.

As suggested prior, regarding the 75: 25 % w/w DOT: 1-D wax, eutectic mixtures have been shown to cause elongation of the degradation window²³. This is thought to be due to the eutectic material presenting greater thermal stability than the component material²³. It is possible, given the chemical functionality of both 1-docosanol and stearic acid, that the mixing of these materials has resulted in the generation of novel hydrogen bonds, increasing thermal stability.

All waxes tested showed inconsequential degradation at relevant operational temperatures, 70 °C to 90 °C, with the $T_{\Delta 5\%}$ occurring above 233 °C in all waxes. This highlights that each wax is stable at operational temperatures within the LP50 printer and are thus thermally compatible with the printing process.

4.3.2. Printing

4.3.2.1. Jetting parameters

Having confirmed the wax inks are thermally stable at relevant temperatures, see Section 4.3.1, and the inks are suitable for printing, see Section 3.3.2.3, attempts at inkjet 3D printing these waxes were made. Unlike many other inkjet printers, the LP50, and associated spectra S-class print heads, do not come equipped with standard jetting parameters. Instead, custom waveforms must be generated for each ink. In this thesis, to achieve uniform film formation, droplets should contact the substrate as a heated liquid and either contact adjacent liquid droplets or solidified adjacent droplets so the wax can form a film under capillary forces²⁴. Subsequently, the ideal droplet is spherical in the air with no satellite drop formation and minimal tailing. This is to ensure, upon contact of the printed droplet and substrate, a single sessile droplet will be formed with no aberrant droplets deposited. Additionally, the droplet should travel at an appropriate velocity to minimise splashing²⁵.

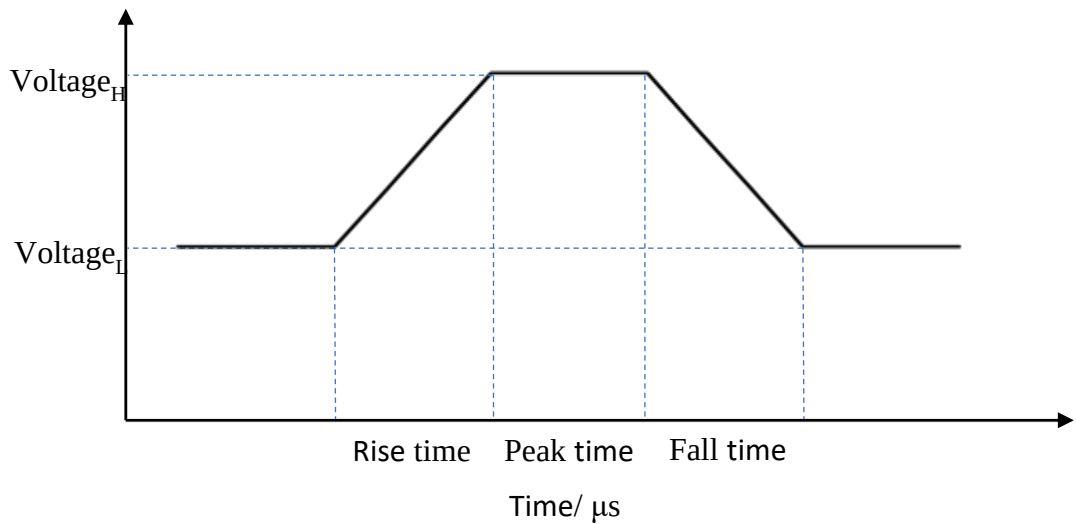


Figure 4-2 - Piezoelectric printing waveform pulse.

The generation of a droplet using a Spectra-S class printhead is controlled by modifying the waveform, Figure 4-2. By modifying the time taken to pulse the rise, peak and fall time and the difference between voltage_L and voltage_H, the applied voltage provides control over velocity²⁶. Controlling velocity can minimise tailing and satellite droplet formation, which occurs at high velocities, particularly with low viscosity inks^{27,28}.

Reducing the time taken for any one step, particularly the rise and fall period, increases velocity as the rate of contraction of the piezoelectric actuator required to eject the droplet decreases. Modification of the applied voltage changes the force exerted upon inks within the piezoelectric actuator with greater applied voltages resulting in greater applied force and thus velocity²⁶.

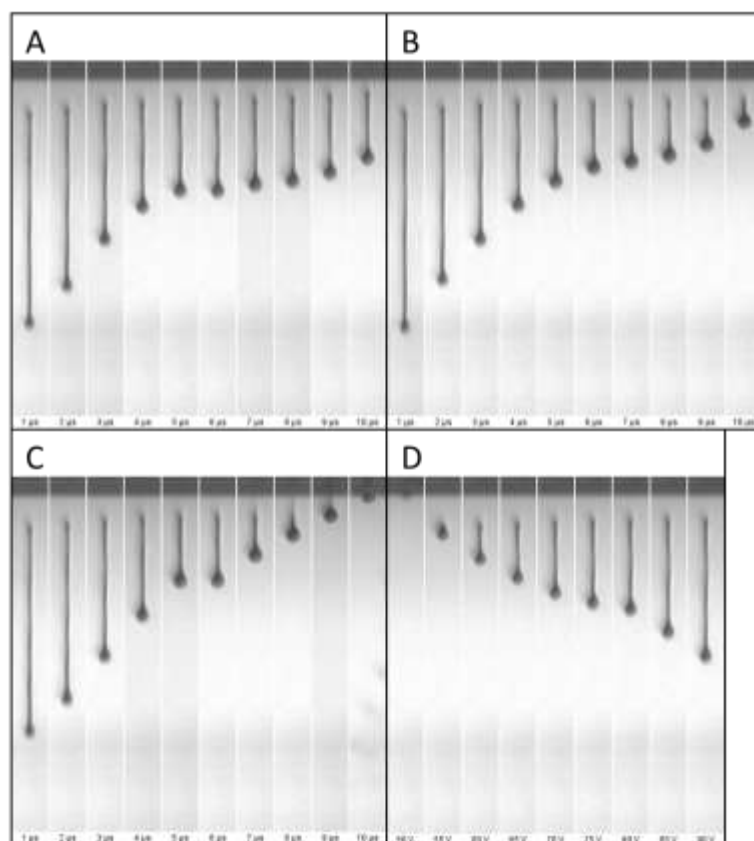


Figure 4-3 - Jetting test showing the effect of varying the (A) rising edge, (B) peak time, (C) falling edge and (D) applied voltage on jetted 1-docosanol. Images were taken using the Advance Drop Analysis software integrated with the LP50 printer 50 μ s following the ejection. In all tests, the rising edge, peak time, falling edge and applied voltage were maintained at 3 μ s, 3 μ s, 3 μ s and 60 V bar, the parameter being tested. Printhead and jetting temperatures were maintained at 90 $^{\circ}$ C.

Figure 4-3 shows how modifying the waveform affects the shape and velocity of ejected droplets. As the impulse time of rising, peak or falling time is increased, the velocity of the droplet is decreased. As peak time is increased, a linear decrease in velocity is seen. However, a plateau in velocity can be seen at 5 μ s – 7 μ s and 5 μ s – 6 μ s rise and fall time, respectively. Through modification of the impulse time, cessation of printing could not be induced. However, Figure 4-3 (C) shows that a further increase of fall time might cause printing to fail. Through increasing the applied voltage, a continuous increase in velocity can be seen. Voltages approximately 50 V and lower caused printing to cease and ink to pool on the printhead nozzle plate. This test was performed for all printed waxes to achieve a velocity of approximately 3 m s⁻¹. Table 4-2 shows the final waveform parameters used for all waxes printed.

Higher velocities were attempted; however, this resulted in a greater propensity of satellite droplet formation. Given that the surface properties and permeation of agrochemicals through these films are to be tested, droplets deposition must be precise and accurate. This is to minimise void formation and produce a uniform surface topography. A slower droplet

velocity and associated slower printing speed were deemed a suitable compromise to ensure uniform printing. With no wax or jetting parameter, was droplets' direction observed to consistently drift more than 4/5 degrees from the ideal vertical direction. Because of this, it was deemed relatively possible to deposit droplets uniformly with minimal risk of intra-print variability.

It should be noted that in all cases, printing was conducted at 90 °C. Printing at this temperature decreases the viscosities to very low values, Section 3.3.2.3. Printing at lower temperatures, 75 °C - 80 °C in particular, often resulted in the ejection of aberrant droplets, showing extensive drift in printing angle. It is possible that at lower temperatures, wax temporary re-solidified in the nozzles or viscosity increased sufficiently to disrupt ink flow.

Table 4-2 - Printing waveform parameters used to achieve 3 m s⁻¹ velocity jetting at 90 °C.

	Composition / % w/w 1-docosanol in dotriacontane						
	0	10	25	50	75	90	100
Rise time / μ s	3	3	3	3	3	3	3
Peak time / μ s	7	7	7	7	7	7	4
Fall time / μ s	3	3	3	3	3	3	3
Applied voltage / V	60	60	60	60	60	60	60
	Composition / % w/w stearic acid in dotriacontane						
	0	10	25	50	75	90	100
Rise time / μ s	2	2	2	2	2	3	3
Peak time / μ s	4	4	4	5	5	6	6
Fall time / μ s	2	2	2	2	2	2	2
Applied voltage / V	60	60	60	60	60	60	60
	Composition / % w/w stearic acid in 1-docosanol						
	0	10	25	50	75	90	100
Rise time / μ s	2	2	2	2	2	2	2
Peak time / μ s	6	6	6	5	5	5	5
Fall time / μ s	2	2	2	2	2	2	2
Applied voltage / V	60	60	60	60	60	60	60

4.3.2.2. Substrate temperature and Drops per inch (DPI)

Given that inks could be printed, it was critical to optimise the printing of the wax film. Many parameters can be changed to achieve this, two being of interest: substrate temperature and Drops per inch (DPI) (100 drops per inch = 3.93 drops per mm).

It has already been shown in Section 3.3.3 that increasing the difference between the molten wax and substrate temperature, the so-called subcooling, modifies surface morphology

through modification of substrate temperature. Increasing the rate of subcooling resulted in greater homogeneity of print morphology.

The waxes imaged in Section 3.3.3 were large deposits compared to a printed droplet, 1 mL and approximately 50 μL ²⁹, respectively. It is expected that solidification will occur more rapidly and be more influenced by substrate temperature within the droplet. Thus, this more rapid cooling might amplify the influence of cooling on morphology.

Figure 4-4 shows the influence of substrate temperature on droplet morphology. As substrate temperature increases from 30 °C to 50 °C little visible change is induced, but a moderately increased droplet spreading.

Figure 4-4 (C and D) shows that between 55 °C - 60 °C droplets become aberrant, showing irregular protrusions from the droplet resulting in the droplet losing uniformity. The uniform homogenous droplet shape is lost at higher temperatures, possibly due to heterogeneous cooling. Subsequently, it is challenging to print a continuous, homogenous film at temperatures exceeding 50 °C. Furthermore, it is unclear if films printed at higher temperatures would solidify within the time taken to print each layer, potentially preventing subsequent drop deposition onto a solidified film.

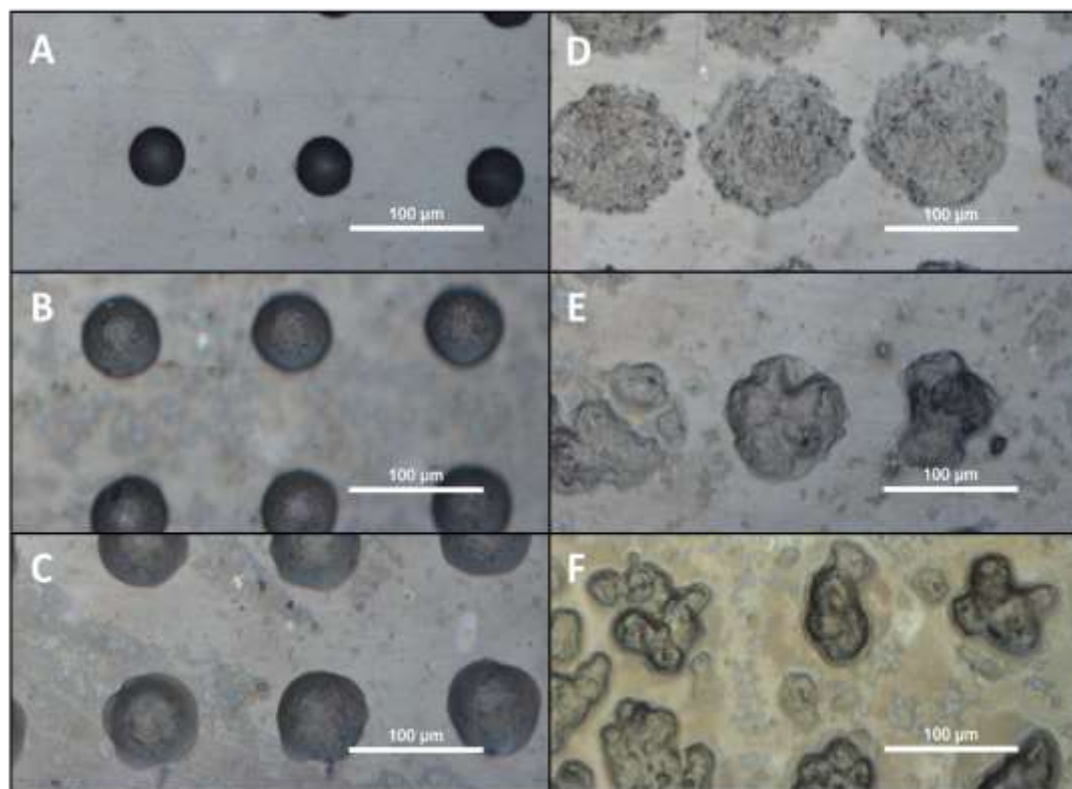


Figure 4-4 - 200 DPI sessile droplets of 1-docosanol/stearic acid 50: 50 % w/w printed onto (A) 30 °C, (B) 50 °C, (C) 55 °C, (D) 60 °C, (E) 65 °C and (F) 70 °C substrate.

1-D: SA 50: 50 % w/w was selected for these images, Figure 4-4, as this wax presented one of the lowest melting points of all tested waxes, Section 3.3.2.1 If a uniform was used with all printed waxes, this temperature cannot exceed the maximally tolerated substrate temperature observed here. Given that substrate temperatures above 50 °C were unsuitable, further tests were conducted between 25 °C and 50 °C.

Interestingly, the increased spreading between 30 °C and 50 °C has implications on printing. Molten droplet spreading is a consequence of the interfacial tension between solid, substrate and air, rather than an effect of the impact of the droplet on the substrate^{24,30}. This is because, predicted based on prior literature, solidification time is greater than the time of impact and associated recoil. As such, solidified droplets will have fully recoiled and will form a stable liquid droplet before solidifying. As a result of this, the shape and the final contact angle is determined by an equilibrium between the interfacial tensions of the air, substrate and liquid phase rather than through any impact borne splashing, spreading or recoil. This can be best expressed by saying the final contact angle of the droplet is driven by the interfacial tensions as determined by the physical properties of liquid and substrate as per the Young's equation³¹. Prior work has shown waxes that present similar physical properties, and are of similar volumes, solidify significantly slower than the process of droplet spreading and recoil following impact with a substrate. Subsequently, at higher substrate temperatures, given that it is likely the solidification will still take longer to occur than droplet spreading and recoil upon impact, it should be possible to print films at lower DPIs wherein droplets can still form a thin continuous bead. This is because droplets were shown to spread further at higher temperatures as the hotter liquid droplet will have a lower viscosity, see Section 3.3.2.3.

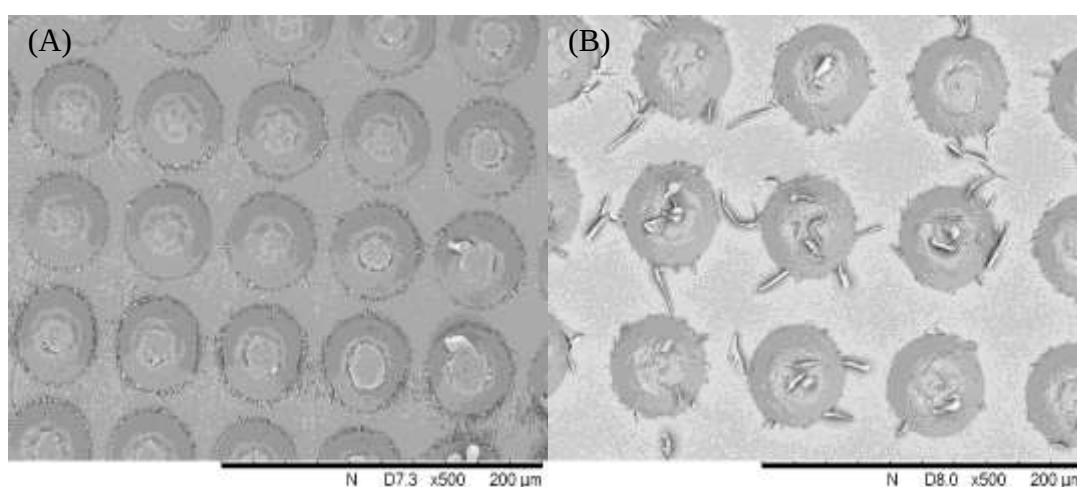


Figure 4-5 - Sessile droplets of 1-docosanol printed onto PET substrate held at (A) 25 °C and (B) 50 °C.

Figure 4-5 shows that in addition to affecting droplet spreading, substrate temperature affected surface crystal structure formation. On a 25 °C substrate, Figure 4-5 (A), these structures that show crystalline-like morphology are relatively small and confined to the droplet edge contacting the substrate or the top surface of the droplet. These structures show clear directionality and form structures between droplets. On a 50 °C substrate, Figure 4-5 (B), crystals are more prominent, are distributed less uniformly and display no directionality. It is possible substrate temperature, and thus the cooling rate is responsible, as seen in Section 3.3.3.

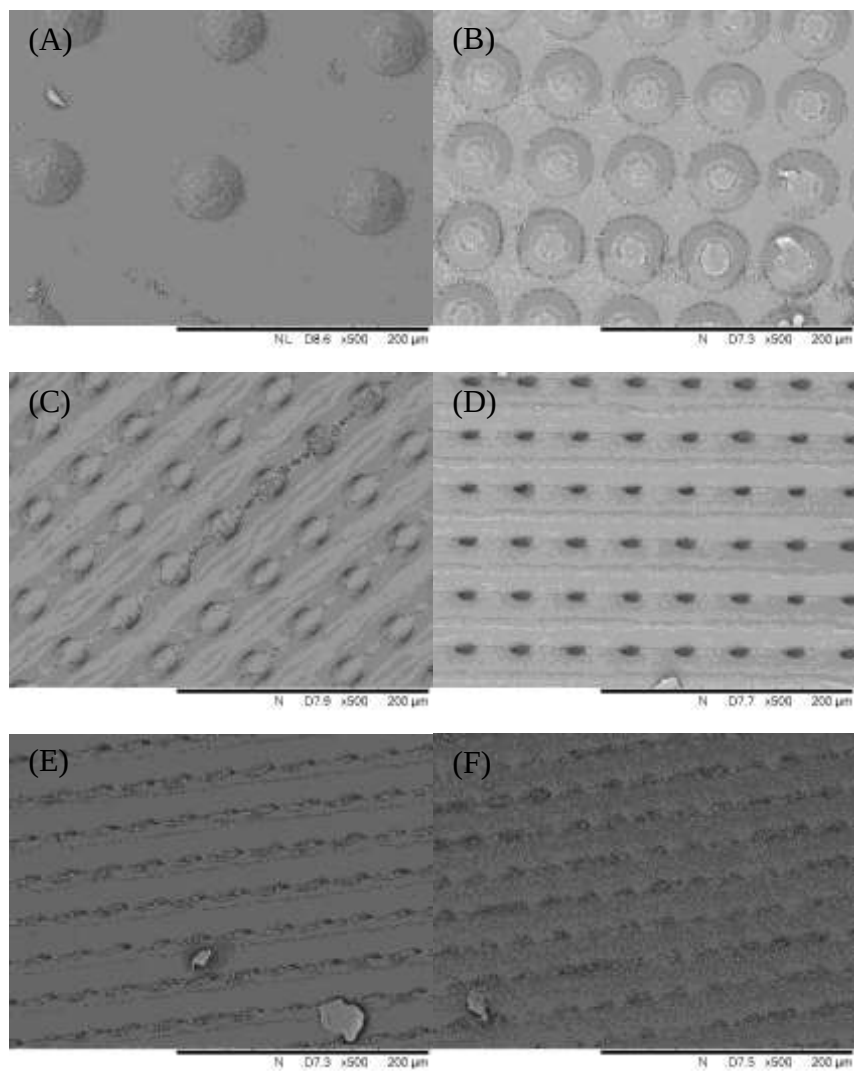


Figure 4-7 - 1-docosanol printed on a 25 °C substrate at 200, 400, 500, 600, 700 and 900 DPI (A, B, C, D, E and F respectively).

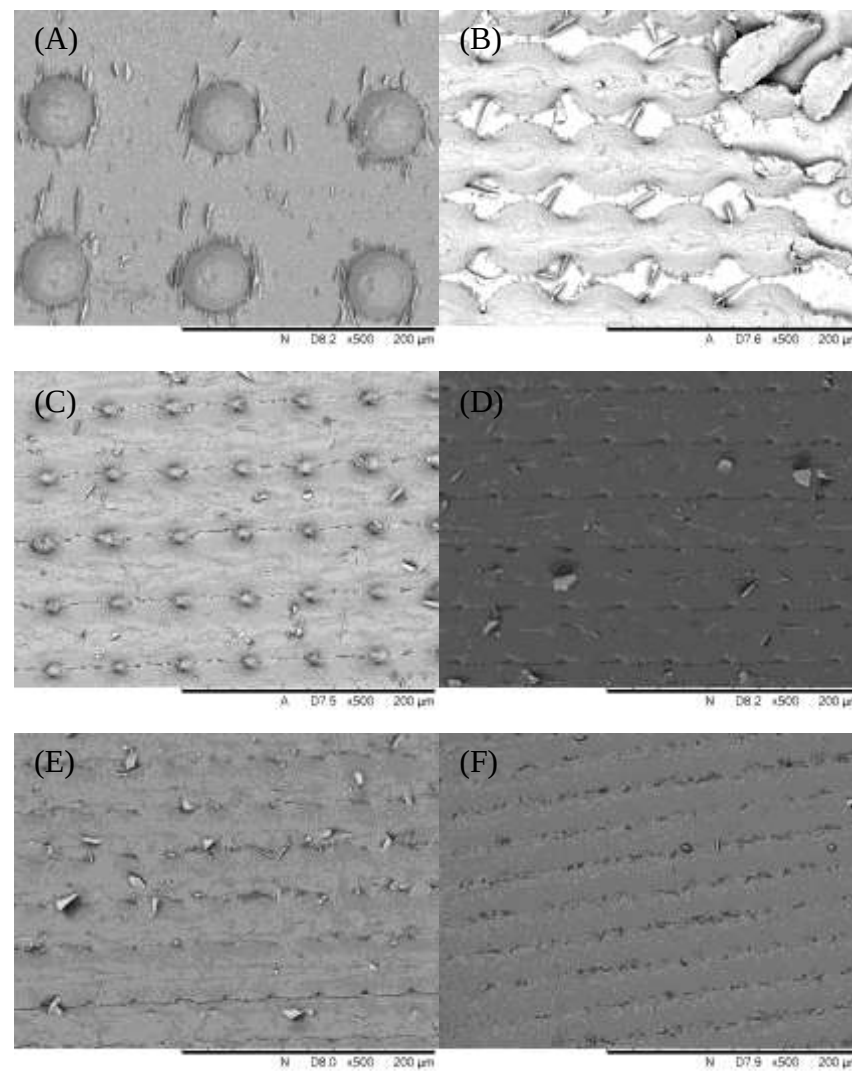


Figure 4-6 - 1-docosanol printed on a 50 °C substrate at 200, 400, 500, 600, 700 and 900 DPI (A, B, C, D, E and F respectively).

Figure 4-7 and Figure 4-6 show the impact of DPI and substrate temperature on film formation. At low DPIs, sessile droplets are generated regardless of substrate temperature. However, unlike in Figure 4-5 (A), no crystals are spread between droplets in Figure 4-7 (A). This suggests crystals on the substrate primarily form between droplets spaced closely together. These structures thus may be residue from droplet-droplet interactions occurring as droplets spread on impact. As DPI is increased (Figure 4-7 B-F and Figure 4-6 B-F), at higher substrate temperatures, a continuous bead is formed at lower DPIs (Figure 4-6 B), likely a consequence of increased spreading. Droplet coalescence initially occurs only parallel to the printing direction. This is likely due to droplets printed in quick succession, being liquid and simultaneously spreading upon impact, allowing interaction and coalescence before droplets retract. Coalescence perpendicular to print direction requires coalescence between a solid and liquid droplet, only one of which is actively spreading, so droplets need to be more closely distributed to drive coalescence. This phenomenon has been reported previously and is termed Drawback³¹.

At DPIs of 600 and greater, the droplets coalesce parallel and perpendicular to print direction regardless of substrate temperature. However, large voids can be observed at lower substrate temperatures (Figure 4-7 D). Interestingly, clear print track interfaces can be observed where solid and molten droplets interact. For the droplet to form a continuous bead, wherein no interface is apparent, the molten droplet must be capable of re-melting the solidified droplet at the point of contact to form a continuous bead³¹. This is not occurring at either substrate temperature, as shown by the clear print track interfaces. Instead, it is likely that when a molten drop interacts with a solid droplet, molten wax flows over the solid droplet by capillary action before rapidly solidifying²⁴.

It should be noted, in almost all cases, crystalloids are only seen at the drop-substrate interfaces and between interfaces between layers (Figure 4-8 B). This is possibly a consequence of induced localised purification at the print track interfaces and droplet-substrate interfaces which leads to the formation of short and long-range ordering crystalline structure formation. One reason this might occur is explored in Section 4.3.6 and postulates in a binary wax the wax with the higher freezing point in the binary wax phase separates once the temperature falls below this freezing point. Subsequently this wax is heterogeneously distributed at the drop-substrate and print track interfaces. It has been shown in literature thermal energy is more rapidly transferred into another material at the material-material interface compared with the material-environment interface resulting more rapid cooling at these locations³¹. If a similar effect is occurring in these printed films, it is possible the

heterogeneous crystalline formation occurs at these locations as they more rapidly cool. Additionally, it would explain why crystalloid structures are denser on surfaces printed on a 25 °C substrate than a 50 °C substrate as cooling rate throughout the droplet will be greater, including at the print track interfaces. Intriguingly, as shown in Figure 4-8 (A), these crystalloids are also present across almost the entire interface between two droplets printed on top of each other. The crystalloids may be artefacts of the droplet pillar breaking. However, because only limited regions present a smooth topography suggests only a limited amount of subsequently deposited droplets within a column coalesce to form a continuous bead. Instead, most of the molten droplets, when they interfaced with a solid droplet, might have failed to remelt the prior droplet precluding any coalescing and thus cooled rapidly, forming the crystalloid structures that do not fuse with the prior printed droplet as a single bead.

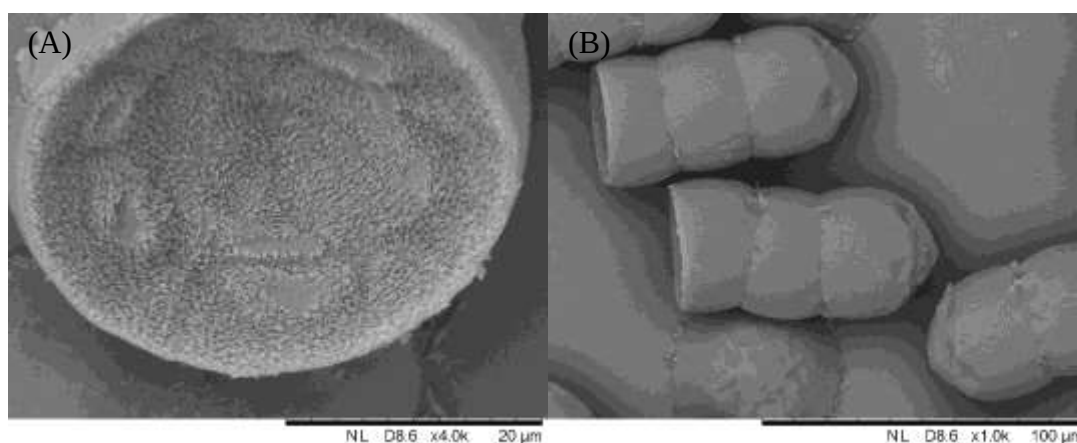


Figure 4-8 - Crystalloid structures at the interface between droplets. (A) shows crystalloids present on the surface that interfaced directly with the underlying droplet in the pillar. The pillar broke away from the underlying droplet during samples preparation. (B) shows the side of similar broken pillars.

From this, the parameters: velocity 3 m s⁻¹, substrate temperature 50 °C, the print head temperature 90 °C and a DPI of 900 were identified as suitable for printing a selection of waxes. The use of 900 DPI prints onto a 50 °C substrate minimises print-induced topography and crystalloid structure formation. These printing parameters were subsequently applied to all 18 printed waxes.

4.3.2.3. Composition and number of printed layers

There is significant literature showing that wax composition affects surface topography and morphology. This is seen on the cuticle wax surface^{14,32}, deposited reconstituted cuticle waxes on artificial surfaces^{33,34} or deposited waxes derived from alternative sources¹³. The printed wax composition may affect the surface, as seen in Section 4.3.2.2.

Figure 4-9 and Figure 4-10 demonstrate that the printing parameters selected, Section 4.3.2.2, print films broadly showing minimal print induced topography. Additionally, films could be consistently printed with minimal sample failure, including cracks, delamination or aberrant droplet deposition.

As a function of composition, the variety of morphologies is due to the various physical properties and recrystallization processes affecting droplet spreading, droplet-droplet interaction, and droplet solidification. Prior work suggested composition and the nature of recrystallisation being the driver of modifying the morphology¹³. In this work, droplet-droplet interactions must also be considered.

Dotriacontane (Figure 4-9 A) presents a homogenous film lacking crystals. However, print induced topography, as seen by shadowed regions, is visible, suggesting considerable variance in topography. 1-docosanol (Figure 4-9 D) shows crystalloids localised heterogeneously at the printed track interface with minimal coverage across the track width. 1-docosanol print tracks are difficult to identify if not for the presence of the crystalloids. This suggests wax printing can be optimised to generate homogenous prints with minimal print induced topography. The stearic acid film (Figure 4-9 G) presents relatively clear print induced topography. Print tracks can be observed however, these tracks do not protrude, as seen in dotriacontane, and present no crystalloid structures, as seen upon 1-docosanol. A novel feature is observed in striations across the print track, a location that presented a low density of structures in dotriacontane and 1-docosanol prints. In Section 3.3.3 drop cast, stearic acid presented similar striations. Printed stearic acid may be recrystallizing similarly, thus presenting similar morphologies. The size variation in these striations on printed and drop cast stearic acid may be a consequence of the cooling rate affecting the size and density of these structures. Why printed dotriacontane and 1-docosanol do not show similar features as drop cast waxes is unknown however, this is likely a consequence of the small volume of 3D printed droplets. As shown in Section 4.3.2.2, even within the space of a single droplet, approximate volume 50 pL, there is heterogeneous distribution of surface crystalloids as a function of location i.e., at the print track interfaces. Within the drop cast waxes imaged it is expected that the larger volume led to a slower cooling rate and subsequently different arrangements of crystalline structures compared with the smaller volume, and thus more rapidly cooling, printed droplets.

Additionally, it should be noted that sample-to-sample variation can occur within the 3D printed wax films. Figure 4-9 D and Figure 4-7 F were printed with identical print parameters;

however, they display variance in morphology. This is likely a consequence of challenging to control variables, induced by printing, influencing the conditions experienced by the droplet that can change on a print-by-print basis. Any change in the arrangement or calibration of the printhead, any change in the location on the print bed which may be unevenly heated or even a change in the thoroughness of printhead cleaning, which can impact the formation of stable droplets, as shown in Section 4.3.2.1, may lead to variation in the morphology of the printed film. In Figure 4-9 D and Figure 4-7 F however it is clear that the general morphological features were consistent such as the structure of the protruding crystalline structures and the heterogeneous distribution of these structures at the print track interface.

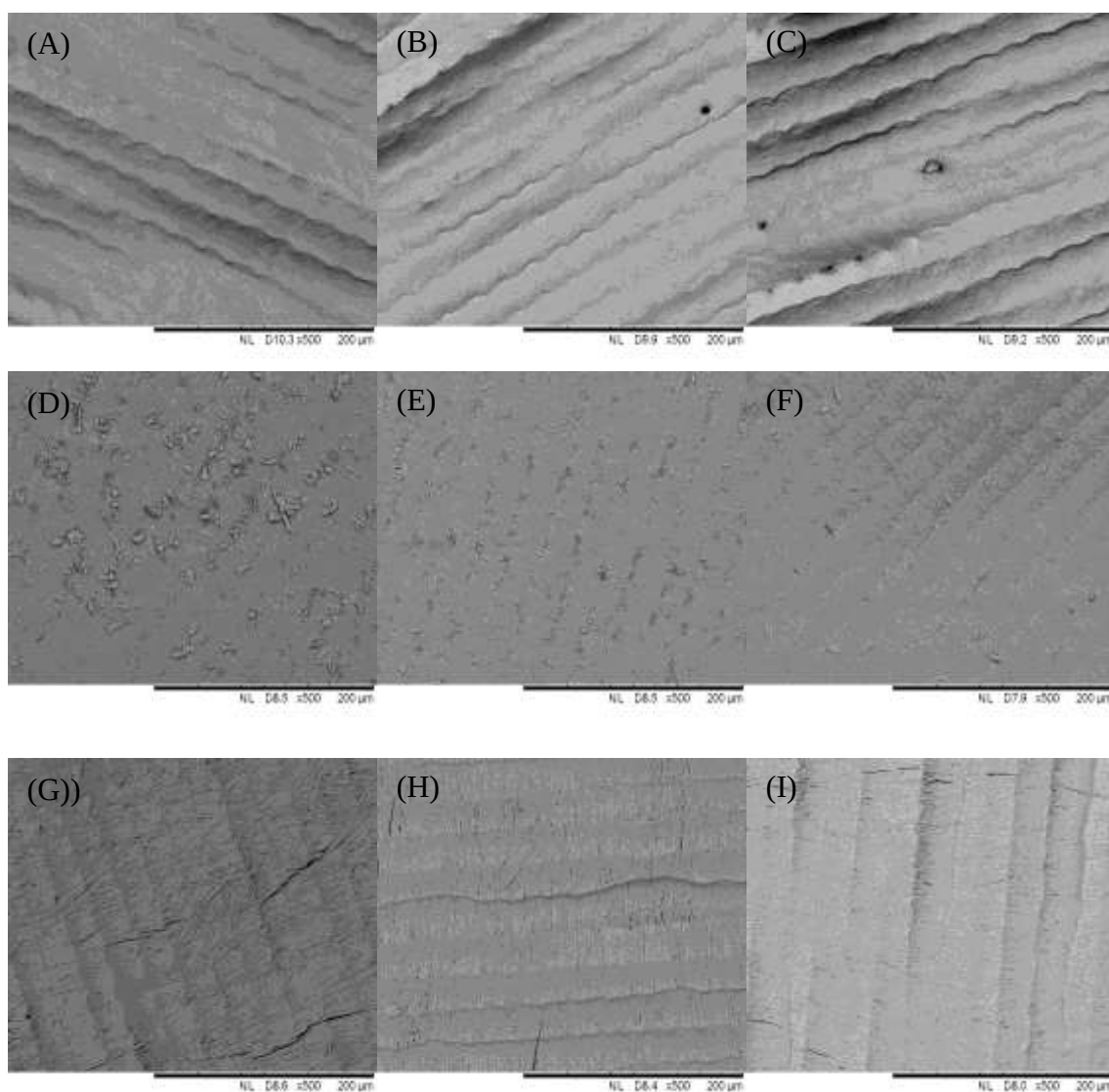


Figure 4-9 - Printed films of dotriacontane, 1-docosanol and stearic acid showing the effect of varying the number of printed layers and thus film thickness. (A) dotriacontane 5 layers, (b) dotriacontane 10 layers, (C) dotriacontane 20 layers, (D) 1-docosanol 5 layers, (E) 1-docosanol 10 layers, (F) 1-docosanol 20 layers, (G) stearic acid 5 layers, (H) stearic acid 10 layers, (I) stearic acid 20 layers.

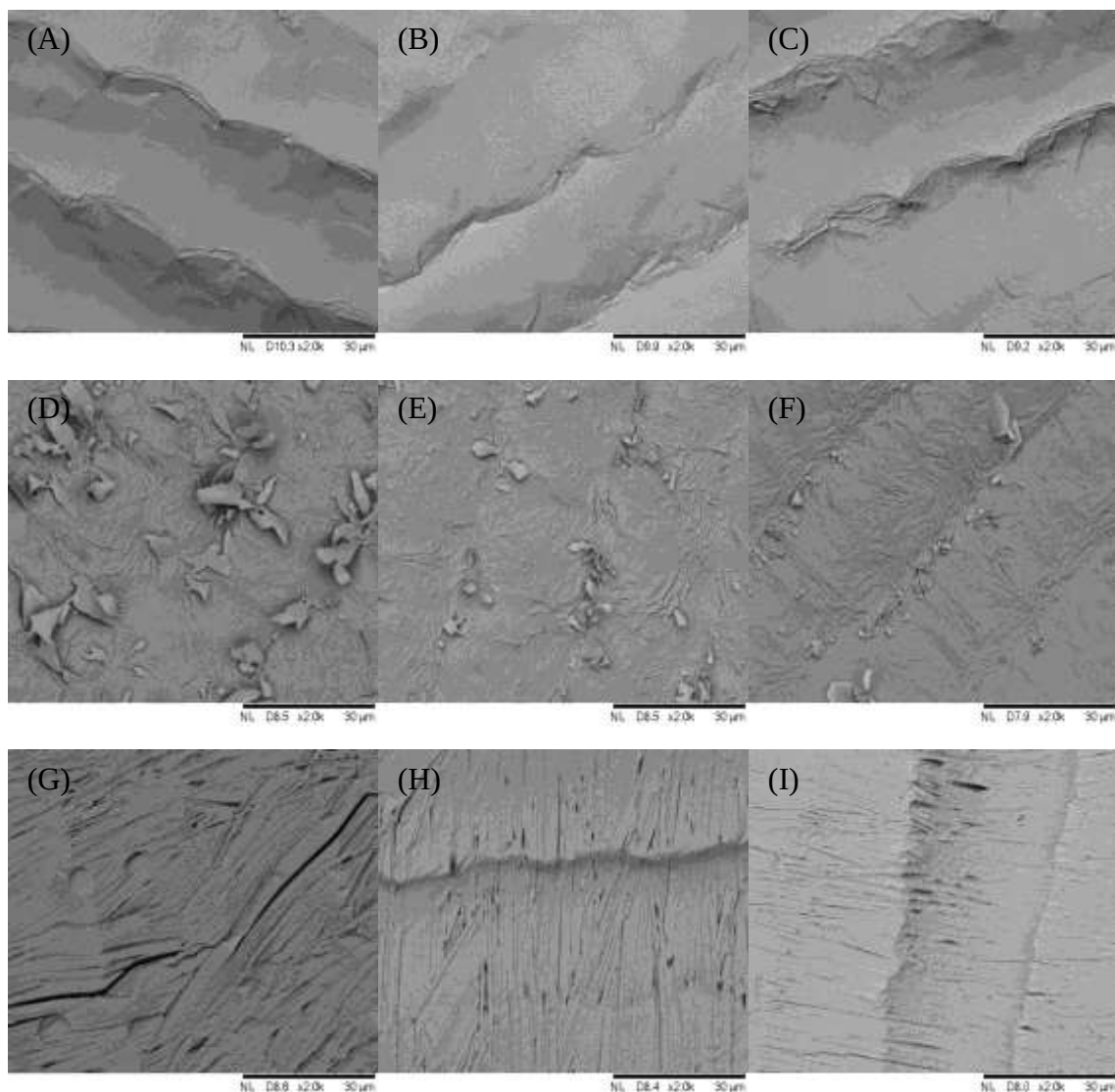


Figure 4-10 - Printed films of dotriacontane, 1-docosanol and stearic acid showing the effect of varying the number of printed layers and thus film thickness. (A) dotriacontane 5 layers, (b) dotriacontane 10 layers, (C) dotriacontane 20 layers, (D) 1-docosanol 5 layers, (E) 1-docosanol 10 layers, (F) 1-docosanol 20 layers, (G) stearic acid 5 layers, (H) stearic acid 10 layers, (I) stearic acid 20 layers.

Figure 4-11 shows print track interfaces at a higher magnification. It is evident that print induced morphology is a consequence of an interaction between print tracks, whereas the structures present upon the surface result from the composition. Beyond observing changes induced by composition, modifying film thickness modified surface morphology. Similar results have been seen prior wherein as the thickness of an FDM print was increased, increasing void formation was observed³⁵. As Figure 4-9 and Figure 4-10 show, 5, 10, and 20 layer wax films are visually different. Figure 4-9 (C) and (I) shows the topography buckling to a greater extent, whereas the 1-docosanol films, Figure 4-9 (F), present visible print tracks not visible on 5 layer films and a lack of crystalloids.

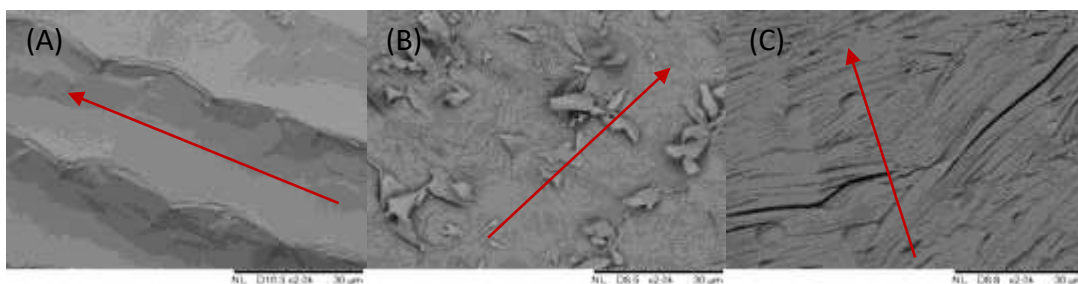


Figure 4-11 - Printed dotriacontane (A), 1-docosanol (B) and stearic acid (C). The red arrow shows the direction of printing.

In inkjet printer films, bulges (Figure 4-9 C and I) often occur where excessive material is deposited, resulting in inappropriate coalescence^{24,31}. However, if these bulges are a consequence of inappropriate DPI, their number should not be linked with film thickness. That is to say the same topography should be seen on a five-layer print as a 20 layer print. It is suggested that topographical changes are a consequence of cooling. It is possible that as the print thickness increases, the top surface cools more quickly compared to thinner films relative to the heated substrate. This would reduce droplet spread and coalescence as the print progressed, increasing the presence of abnormal bulges caused by the coalescence of ink, similar to the data in Figure 4-6 and Figure 4-7. This, however, cannot explain the images in Figure 4-10 D-E showing a decreased density of crystals as print layer thickness increased. Figure 4-6 and Figure 4-7 show that crystalloids formation was more prominent on lower temperature substrates, and thus it would be expected that as print thickness is increased, crystalloids would become more prominent. Reduced droplet spreading at lower temperatures may reduce droplet-droplet interactions limiting crystalloid formation.

Furthermore, a thermal gradient across only 20 layers is unlikely to cause such aberrant morphological changes. Morphological changes might be induced by printing onto successively less smooth surfaces, resulting in droplet spreading and subsequently droplet coalescence changes. Surface morphology becomes visually rougher as the film thickness is increased. The lack of clear coalescence of droplets printed upon one another (Figure 4-8 A) suggests this hypothesis may be correct as it shows droplets are not forming a continuous bead with prior layers.

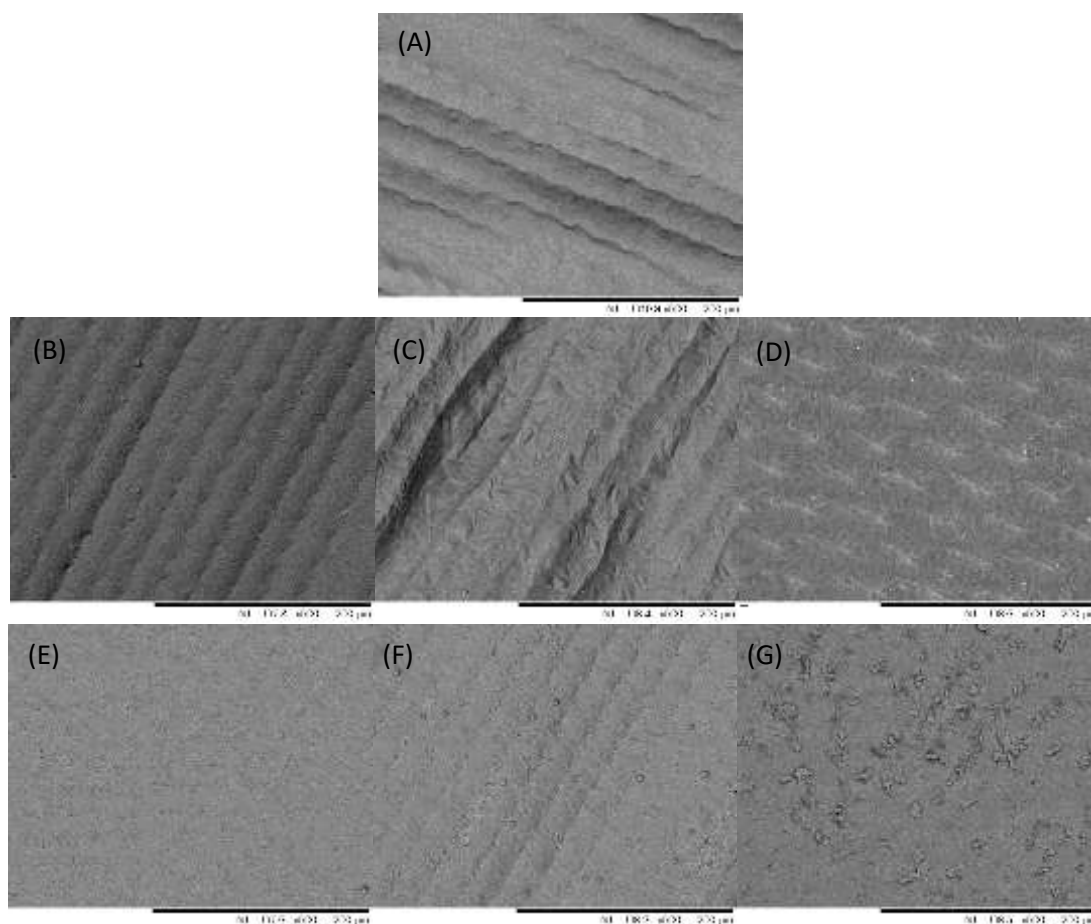


Figure 4-12 - 5 layer printed wax films of (A) dotriacontane, (B) DOT: 1-D 90:10 % w/w (C) DOT: 1-D 25: 75 % w/w (D) DOT: 1-D 50: 50 % w/w (E) DOT: 1-D 75: 25 % w/w (F) DOT: 1-D 10:90 % w/w and (G) 1-docosanol.

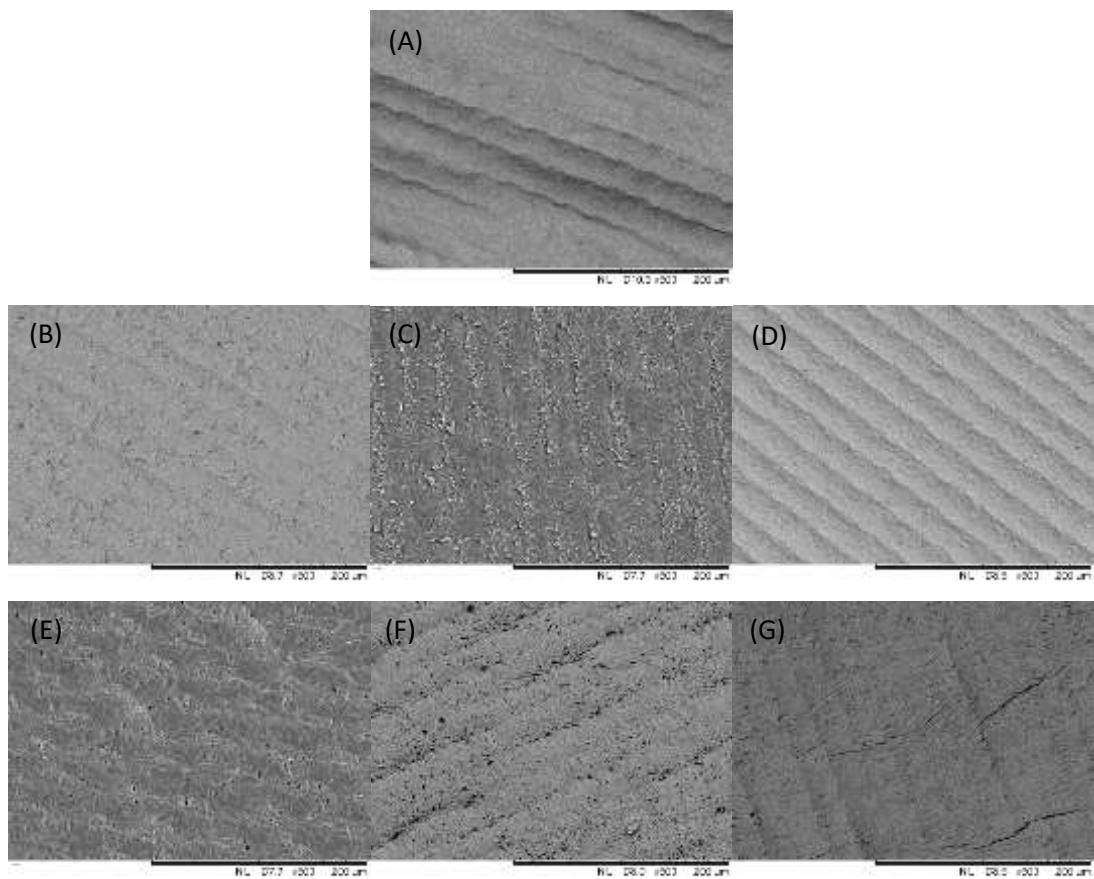


Figure 4-13 - 5 layer printed wax films of (A) dotriacontane, (B) DOT: SA 90:10 % w/w (C) DOT: SA 25: 75 % w/w (D) DOT: SA 50: 50 % w/w (E) DOT: SA 75: 25 % w/w (F) DOT: SA 10:90 % w/w and (G) stearic acid.

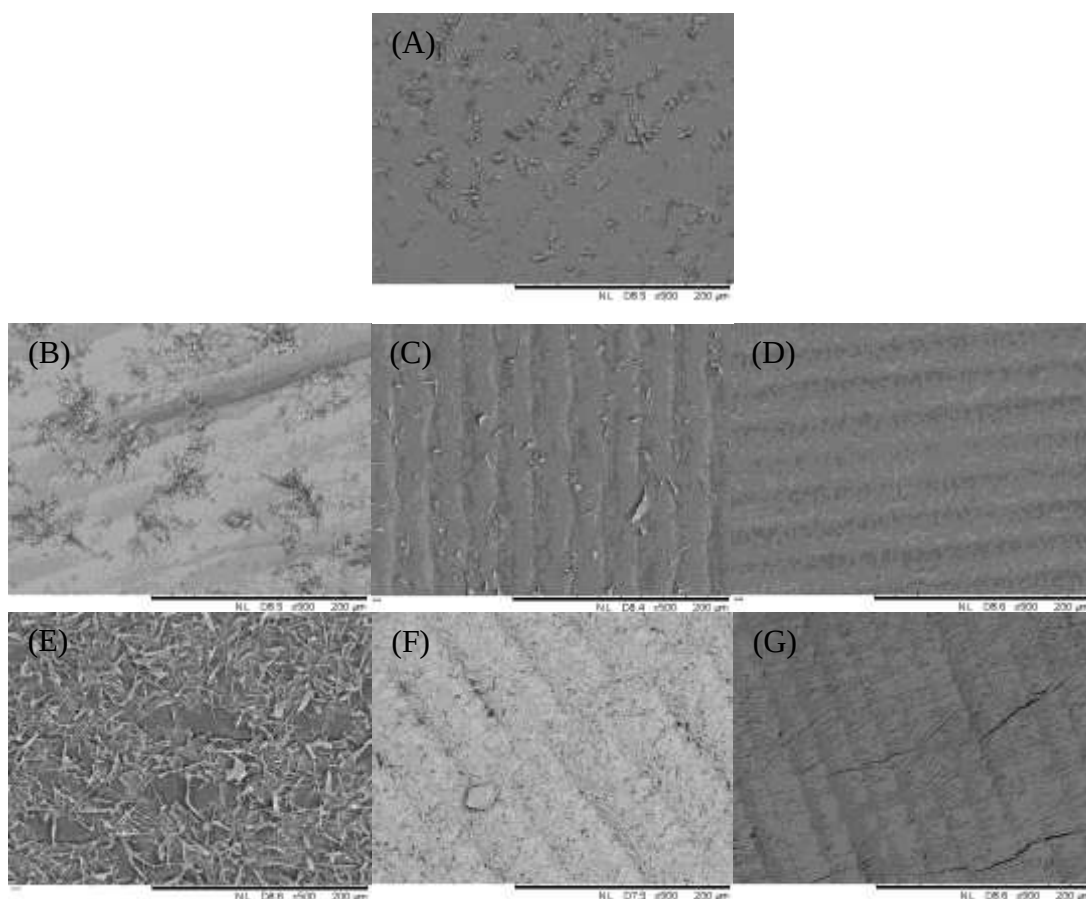


Figure 4-14 - 5 layer printed wax films of (A) 1-docosanol, (B) 1-D: SA 90:10 % w/w (C) 1-D: SA 25: 75 % w/w (D) 1-D: SA 50: 50 % w/w (E) 1-D: SA 75: 25 % w/w (F) 1-D: SA 10:90 % w/w and (G) stearic acid.

Considering mixed waxes, Figure 4-12, Figure 4-13 and Figure 4-14 show that composition affected surface morphology. Furthermore, the printing parameters used whilst not optimised for each wax printed successfully, DOT: 1-D 75: 25 % w/w (Figure 4-12 C), being an example.

Frequently, the surface morphology of printed binary wax films corresponded to the morphology of the dominant constituent wax. As the % w/w of the secondary wax increases, the morphology becomes more homogenous, meaning the morphology was changed towards a uniform surface free of crystalloids or presented unique features, such as Figure 4-14 E, not seen in either constituent wax. All 50: 50% w/w waxes presented homogenous surfaces presenting only print induced morphology with no diagnostic feature of either component waxes.

Many binary wax films presented crystal structures resembling structures found on native cuticle surfaces. These structures do not follow the patterns observed upon single component waxes wherein crystalloids were restricted to 1-docosanol print track interfaces or across the print track on stearic acid films. Instead, the opposite occurred. Waxes

comprised primarily of 1-docosanol displayed homogenous surface structures, not confined to print track interfaces. Waxes mainly comprised of stearic acid either presented high-density features within print track interfaces or homogeneously. Figure 4-14 E shows a dense, uniform formation of surface features not seen in either component wax. That the morphology is not a simple reflection of wax composition agrees with literature¹³.

It is proposed that the imaged crystalloids may be similar to efflorescence structures on the surface of beeswax. These structures form during cooling and in greater density at higher cooling rates^{36–38} through phase separation of the different waxes based on their physical properties, particularly lipophilicity and volatility³⁹. This would aid in understanding crystalloid formation here and support the theory that cooling rate dictates crystalloid density. Phase separation induced through this process might explain the structures seen within Figure 4-13 and Figure 4-14, where phase separation of binary waxes is proposed to be observed. However, it cannot explain the crystalloids found on the single component 1-docosanol films (Figure 4-9, Figure 4-10).

Interestingly, while composition affects surface morphology, these results do not align with prior literature. Figure 4-12 shows wax comprised of either an alkane or alcohol. Many species of wheat are primarily comprised of alkane and alcohol waxes and develop to present a range of spike and rosettes crystalloids⁴⁰. Such structures are not observed on the printed films. This suggests the composition of the printed film did not replicate the compositional complexity of the native wheat cuticle, resulting in the film failing to present similar crystal structures. Alternatively, the printing process influenced surface morphology. Given that the wheat cultivar presents many waxes, in addition to alkanes and alcohols⁴⁰, and removing waxes induces cuticle morphological changes¹⁴, the former is the likely cause of the lack of surface structures.

4.3.2.4. Multimaterial printing

Printing multi-material films was attempted as part of a test to layer different wax materials. Figure 4-15 and Figure 4-16 shows films containing layers of two waxes, dotriacontane and 1-D: SA 10: 90 % w/w, that had prior shown to present unique surface features. The 1-D: SA 10: 90 % w/w wax was printed upon a variable number of dotriacontane layers to assess if mixing occurred at the print interface and if the amount of underlying dotriacontane affected print morphology.

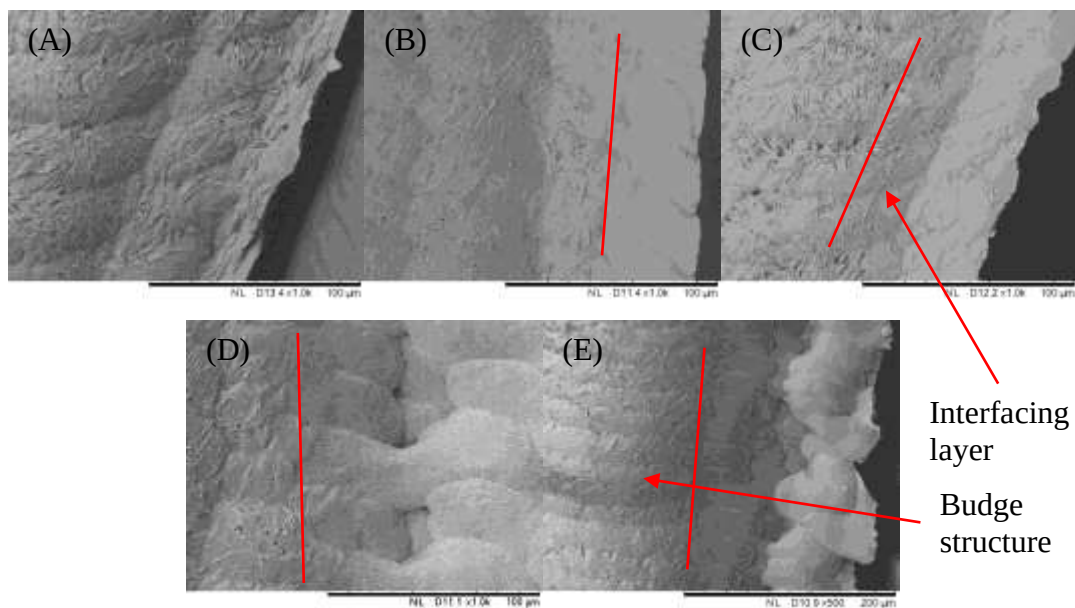


Figure 4-15 - The profile of a print consisting of 1-D: SA 10:90 % w/w printed onto (A) 0, (B) 1, (C) 2, (D) 3 or (E) 4 layers of dotriacontane. All images were captured at 1000x magnification. (D) was captured in a low-quality mode as extensive image drift occurred, blurring high-quality images. The red lines highlight the interface between the initially printed dotriacontane and 1-D: SA 10:90 % w/w.

At the interface between layers of different waxes (Figure 4-15 C), mixing has occurred at the interfacing layer where material from solidified printed layers is moving into the molten droplets following deposition. This is indicated by the reduced density of crystalloids unique to the 1-D: SA 10: 90 % w/w surface (Figure 4-14 F, Figure 4-15 A and Figure 4-16 A), at the interface between dotriacontane and 1-D: SA. This is clear in Figure 4-15 B, where the different layers present a gradient of crystal structure density in the first three layers particularly. Figure 4-15 E, presenting a single layer of 1-D: SA 10: 90 % w/w printed on 4 layers of dotriacontane, shows bulging of the top layer, not seen prior. It is clear from the observed interfaces between layers of unique waxes printed on top of each other that the potential mixing only occurs between the top layer of deposited material and the first layer of alternative waxes printed onto another wax. This is seen as morphology changes are only observed as a gradient of crystal structure immediately around the interface of two different waxes. That is to say, the printing of a second layer of the secondary wax will not be affected by the underlying initial wax substrate.

Considering morphology changes are only induced within the first layer of molten droplets of 1-D: SA 10: 90 %, this is assumed the only location where mixing can be observed in a profile view. The deposition of molten droplets may cause underlying dotriacontane to flow into the molten droplet of 1-D: SA 10: 90 %. Interestingly, this might occur without forming a continuous bead if these droplets are interfacing with each other in a similar manner as

seen in Figure 4-8 A. Mixing of the material shown in Figure 4-16 B-D only occurs in the layer of 1-D: SA 10: 90 % w/w contacting dotriacontane. No change can be observed in the underlying dotriacontane, suggesting the flow of 1-D: SA 10: 90 % into the underlying dotriacontane does not occur.

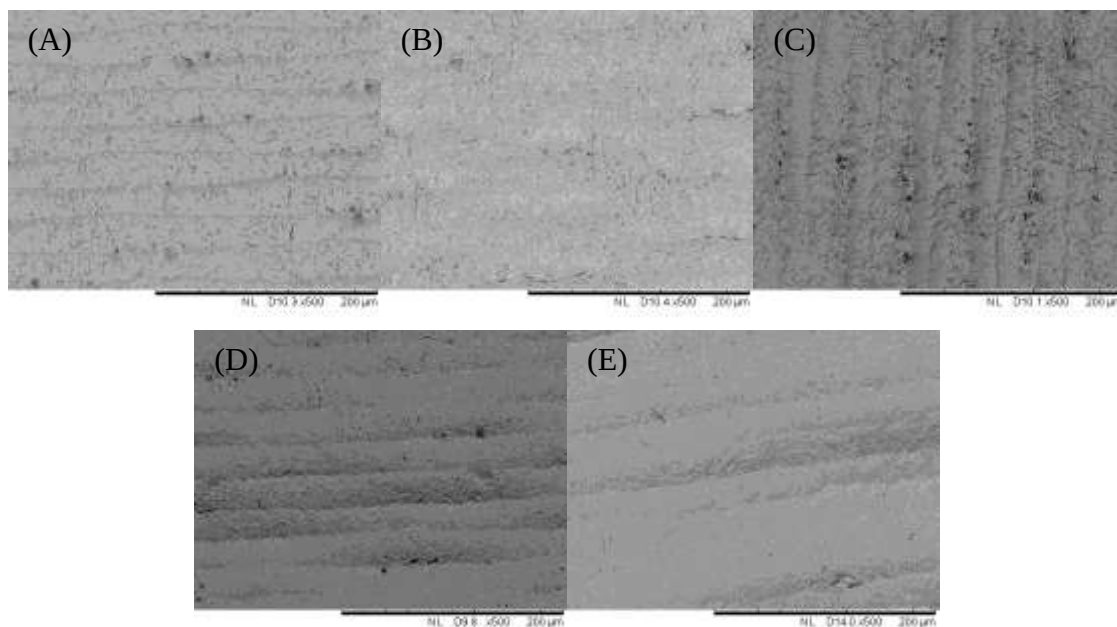


Figure 4-16 - The top surface of a print consisting of 1-D: SA 10: 90 % w/w printed onto (A) 0, (B) 1, (C) 2, (D) 3 or (E) 4 layers of dotriacontane. All images were captured at 500x magnification.

Figure 4-16 further shows the impact of printing multi-material films. Figure 4-16 B and C show no visible difference compared with Figure 4-16 A. However, Figure 4-16 D and E, wherein 2 and 1 layers of 1-D: SA 10: 90 % w/w are printed upon dotriacontane, respectively, show a reduction in striated lamella and pores density, relative to Figure 4-16 (A), occurs suggesting a composition change similar to Figure 4-15. That morphological changes can be seen in Figure 4-16 D and E relative to A, suggesting impingement of dotriacontane can occur through up to 2 layers of printed wax.

This loss of compositional control at the interface, potentially affecting up to two subsequent layers, highlights the difficulties of printing films consisting of multiple layers of variable composition as the composition at layer interfaces would be challenging to control.

4.3.3. Contact angle

Surface wettability is often measured by evaluating the contact angle between a liquid and the surface of interest. Small contact angles occur when droplets spread extensively across the surface, whereas larger contact angles occur when droplets bead on a surface, minimising contact⁴¹ (Figure 4-17 and B, respectively). As per the Young's equation the contact angle of

a droplet is determined by the equilibrium obtained between the interfaces of the environment which is often the air, the substrate and the liquid droplet⁴¹.

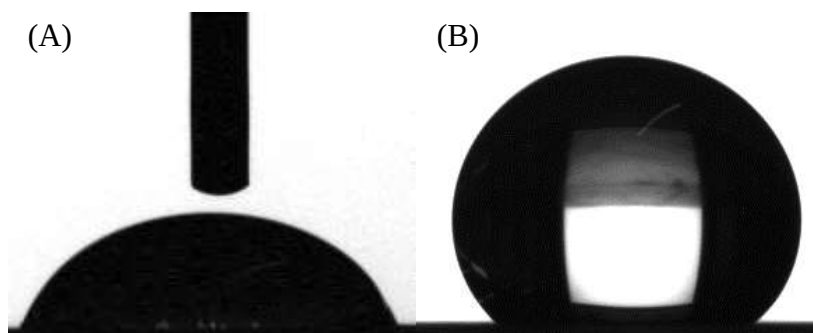


Figure 4-17- Dotriacontane surfaces with a droplet of (A) A7 displaying hydrophilic properties and (B) water displaying hydrophobic properties.

Plant cuticles are often hydrophobic due to the hydrophobic properties of waxes⁴¹ and the presence of surface crystalloids on many cuticles that introduce roughness at the nanoscale^{42,43}. This serves to decrease the surface energy and the interfacial tension between the liquid droplet and substrate, resulting in the droplet remaining as a bead on the substrate.

Subsequently, follicular applications of agrochemical solutions are prone to run off⁴³, potentially contaminating soil and groundwater and failing to reach their target. Surfactants can minimise runoff by decreasing the contact angle of the droplet, thus increasing spreading through a reduction of surface tension relative to the surface energy of the cuticle⁴⁴.

Two essential parameters controlling contact angle are solid surface morphology and chemical composition. Subsequently, a modification of crystal feature density and wax composition can change the contact angle of a cuticle surface⁴⁵. For example comparison of wild type and cuticle mutants showed a 3 % - 24 % reduction in contact angle resulting from reduced epicuticle crystalloids⁴⁵, whereas modification of wax composition, induced by growing the plant in a low humidity environment, induced decreased wetting⁴⁶. However, the relationship between composition and contact angle is poorly understood outside a few broad conclusions.

It might be possible to determine the relationship between wax composition, hydrophobicity, and surfactant spreading using 3D printed wax films. Understanding these relationships might allow the development of a wetting model as a function of composition. Subsequently, based on wax composition, controlled spreading based on surfactants

selection might be possible. Furthermore, its known permeation of hydrophilic compounds and cuticle hydrophobicity are correlated, so understanding this relationship is critical⁴⁷.

Figure 4-18, Figure 4-19 and Figure 4-20 shows the contact angle, over 4 minutes, following Milli-Q water application to wax films of varied composition. For graphs with 1, 2, and 3 minutes data points, see Figure 4-35, Figure 4-36 and Figure 4-37.

Modifying wax composition resulted in significantly different contact angles. After spreading for 4 minutes, the 5 layer 10: 90 % w/w DOT: 1-D film presented the highest contact angle, $138.4^{\circ} \pm 0.8^{\circ}$, whereas the lowest contact angle was observed on 5 layer 50: 50 % w/w 1-D: SA, $87.1^{\circ} \pm 2.8^{\circ}$. In most cases, the addition of 10 % w/w of a secondary wax resulted in a significant rise in contact angle, partially following the addition of dotriacontane or no significant change. Only one wax showed a significantly decreased contact angle following mixing of a single component wax with 10 % w/w of a secondary wax, 5 layer DOT: 1-D 90: 10 % w/w.

Interestingly, adding 1-docosanol to bulk dotriacontane increased the contact angle linearly with 1-docosanol % w/w composition. This matches literature showing a correlation between increased alcohol wax content and contact angle upon cuticles containing large amounts of alkanes⁴⁷. However, adding alcohol groups to a wax comprised mainly of stearic acid resulted in a significant reduction in contact angle as the 1-docosanol % w/w was increased.

The literature hypothesises a correlation between alcohol content and contact angle results from 1-docosanol promoting dense layers of crystalloid structures, increasing roughness and thus contact angle. This trend is not seen in the data in Figure 4-12 and Figure 4-14, which show that increasing alcohol content does not promote crystallisation. However, only a single alcohol wax was tested, and the printed films present a substantially lower compositional complexity than cuticles waxes. It is possible that the addition of alcohols, or the addition of alternative alcohols, would induce crystalloid formation in compositionally complex films, as seen in wheat cultivars³⁴.

The contact angle significantly decreased for many waxes as the number of layers of printed wax increased from 5 to 10. This is seen particularly in waxes containing large quantities of dotriacontane and pure dotriacontane, which showed a decreased contact angle in all cases as film thickness increased. Interestingly, the difference in contact angle on a 10 and 20 layer film is often insignificant despite the film further increasing in thickness as seen with 90: 10 DOT: 1-D, 75:25 DOT: 1-D and 50: 50 DOT: 1-D w/w films, Figure 4-18 B and C.

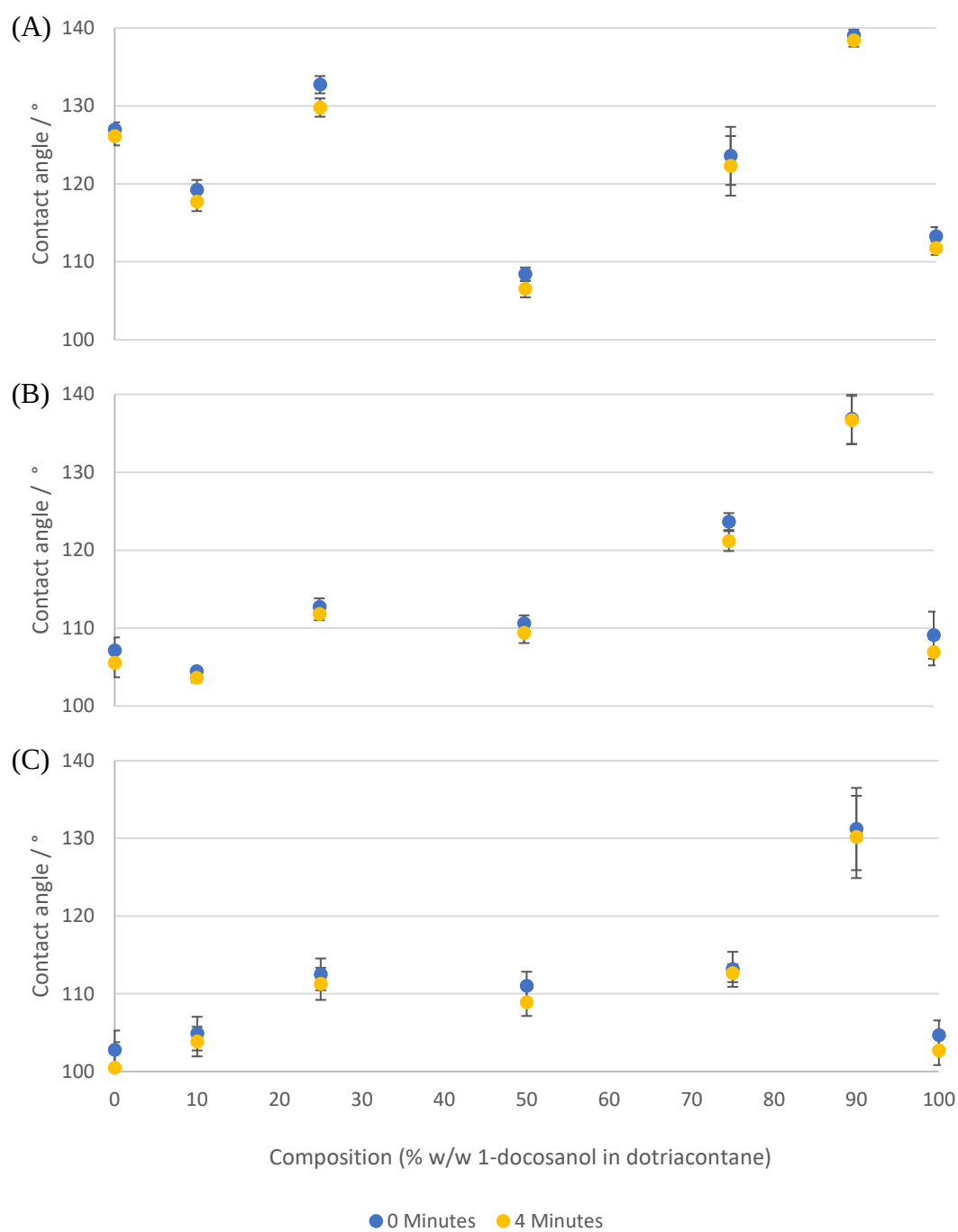


Figure 4-18 - Contact angle of water over 4 minutes on (A) 5 layer, (B) 10 layer and (C) 20 layer wax films of DOT: 1-D.

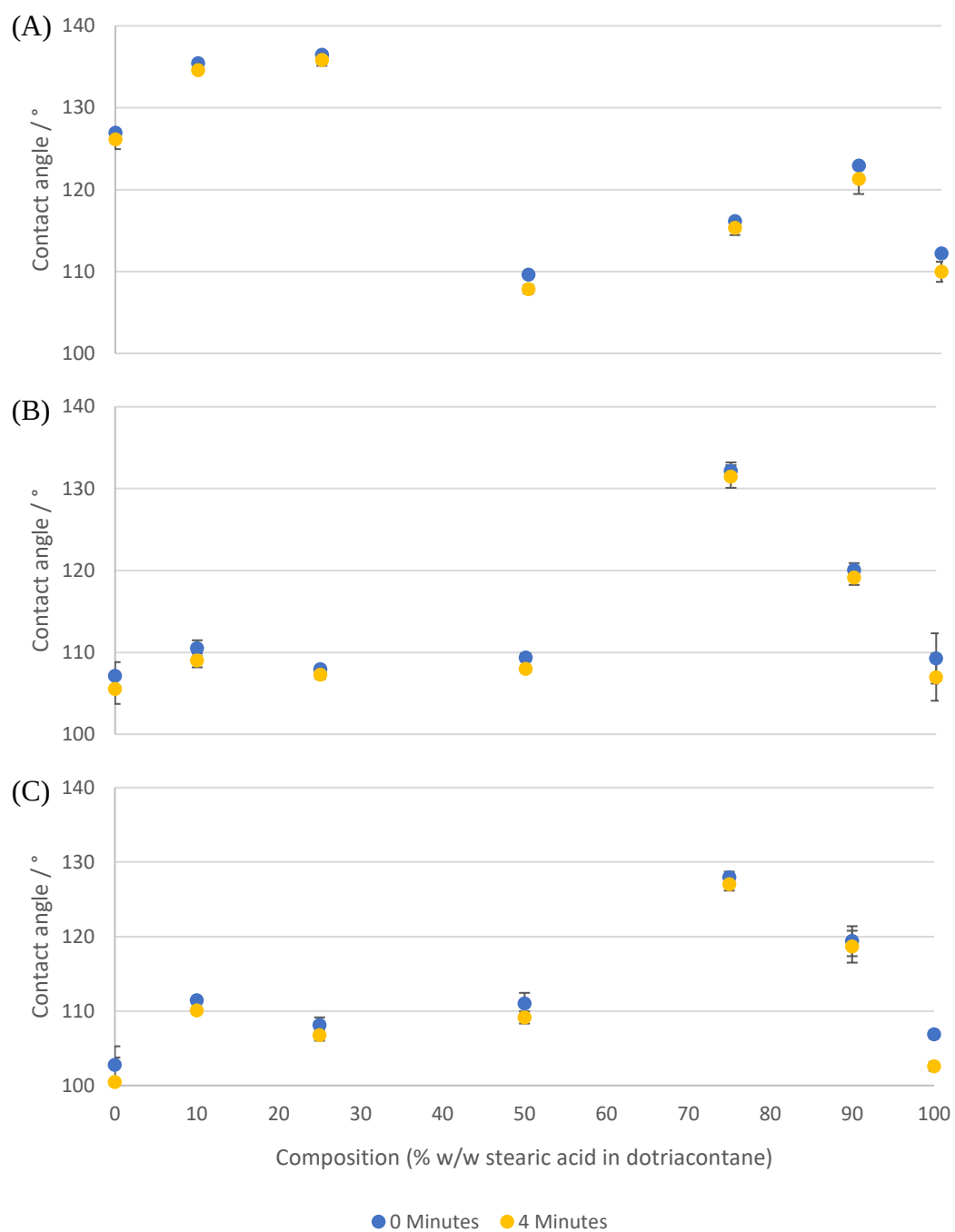


Figure 4-19 - Contact angle of water over 4 minutes on (A) 5 layer, (B) 10 layer and (C) 20 layer wax films of DOT:SA.

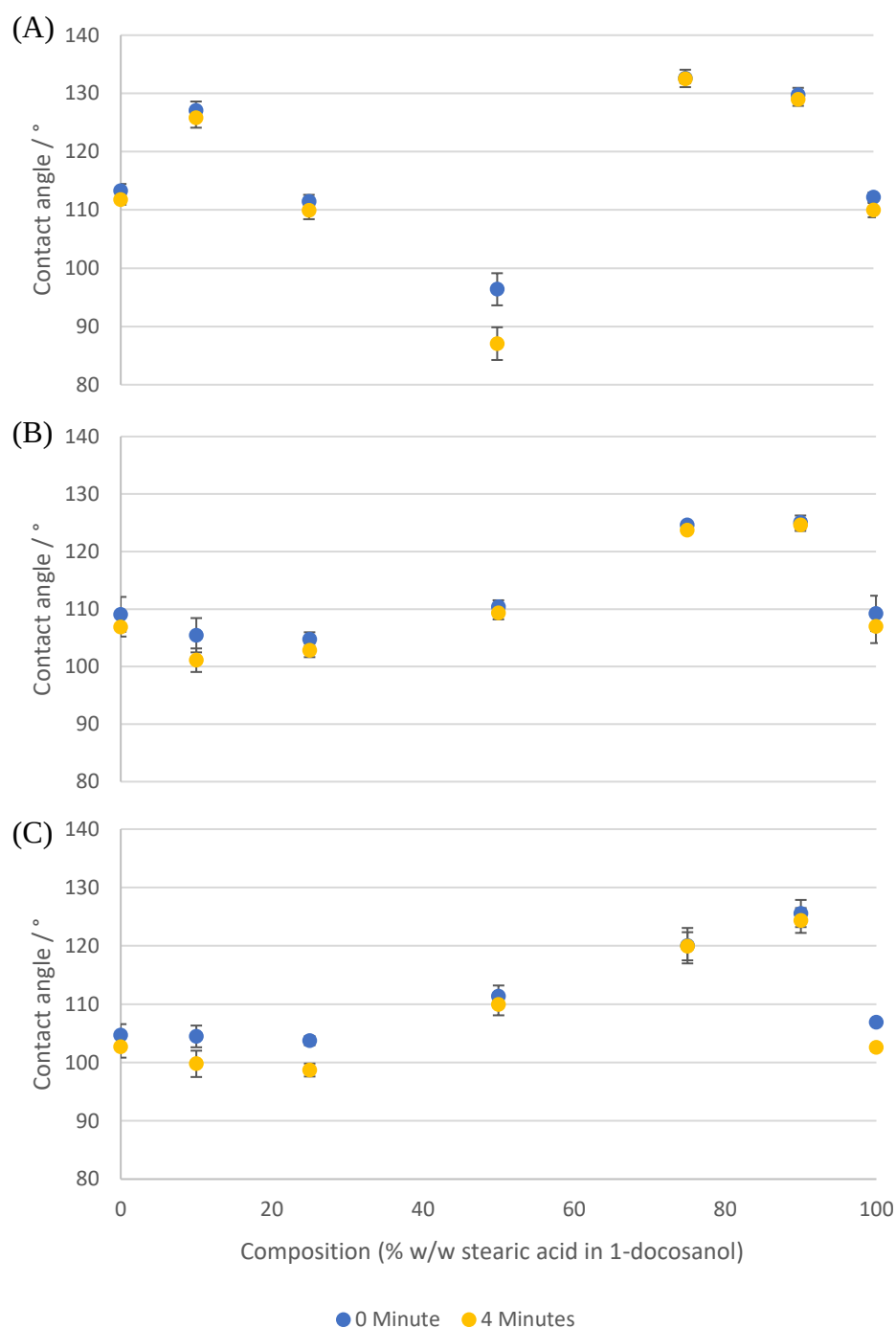


Figure 4-20 - Contact angle of water over 4 minutes on (A) 5 layer, (B) 10 layer and (C) 20 layer wax films of 1-D: SA.

It is possible that much of the variance in contact angle, as a function of film thickness and composition, is induced by changes in print induced topography and the heterogeneous distribution of crystalline structures

To evaluate the impact of topography on contact angle and to elucidate the relationship between contact angle and wax composition, the water contact angle on the bottom surface of printed films was measured. Such surfaces present little topography or crystalloid

structures as these surfaces form in conformal contact with the underlying flat substrate. Subsequently, the significant factors determining contact angle are interfacial tensions as per the Young's equation, as topography has limited impact.

The contact angle of water on the bottom surface of the printed wax was, for the majority, either significantly lower or effectively the same as the contact angle on the top surface. Only two waxes showed a significant increase in contact angle: 25: 75 % w/w and 75: 25 % w/w 1-D: SA. Considering that all these samples lack extensive topographical features, the primary component affecting the air-surface and surface-liquid interfacial energies should be chemical composition. Subsequently, observing chemical compositions' effect on contact angle should be possible to resolve. Unfortunately, as Figure 4-21 shows, no trend linking composition and contact angle was observed, with many waxes presenting insignificantly different contact angles. Many of the trends seen in Figure 4-18, Figure 4-19 and Figure 4-20 were not present. This includes the increased contact angle as 1-docosanol content in bulk dotriacontane increases or decreases contact angle as 1-docosanol content in bulk stearic acid increases seen on the top surface of the printed film but not the bottom surface. This suggests that the topographical features, not composition, dominate observed trends. This conclusion has been tentatively reached by other studies evaluating a wide range of cuticle contact angles, partially regarding the density of crystalloids^{45,47}.

Further, the spread of water over the bottom surfaces over 4 minutes was often significant. However, when water was deposited on the top surface of 5, 10 or 20 layer films, most films presented no significant change. This suggests that the topographical features presented on the top surface pin the droplet, minimising spreading. Such behaviour is commonly seen upon cuticle surfaces due to interaction with crystalloid structures⁴⁵ and trichomes⁴⁸. Further, it suggests the water caused limited or no rearrangement of the surfaces over the 4 minutes that would enable further spreading of the droplet.

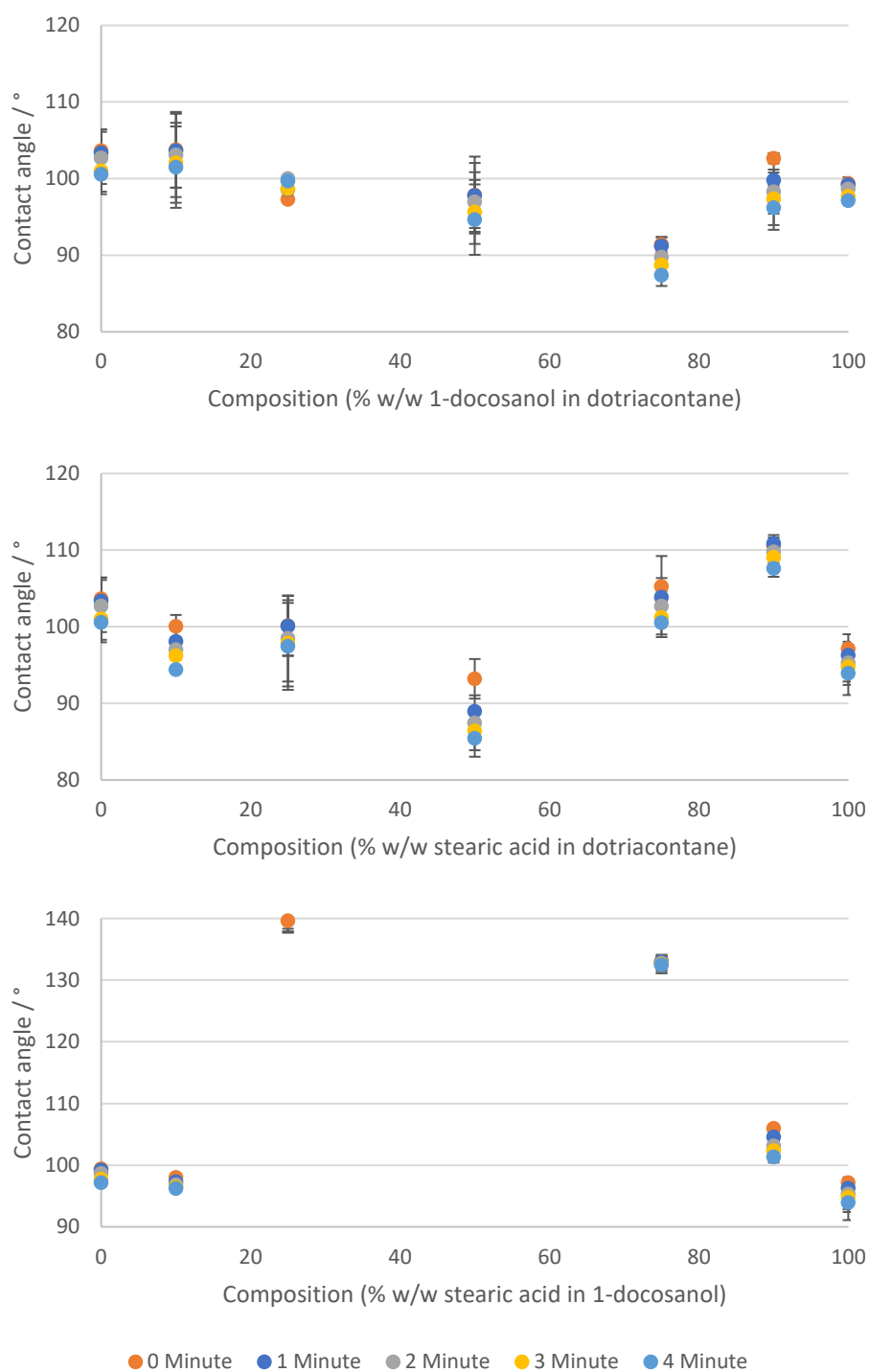


Figure 4-21 - Contact angle of water over 4 minutes on the bottom surface of (A) DOT: 1-D, (B) DOT: SA and (C) 1-D: SA.

Having seen that contact angle is principally associated with topographical features rather than chemical composition, droplets of 0.12% w/w A20 and A7 were deposited on wax films to assess if a surfactant would present similar trends see Figure 4-22 and Figure 4-23, respectively.

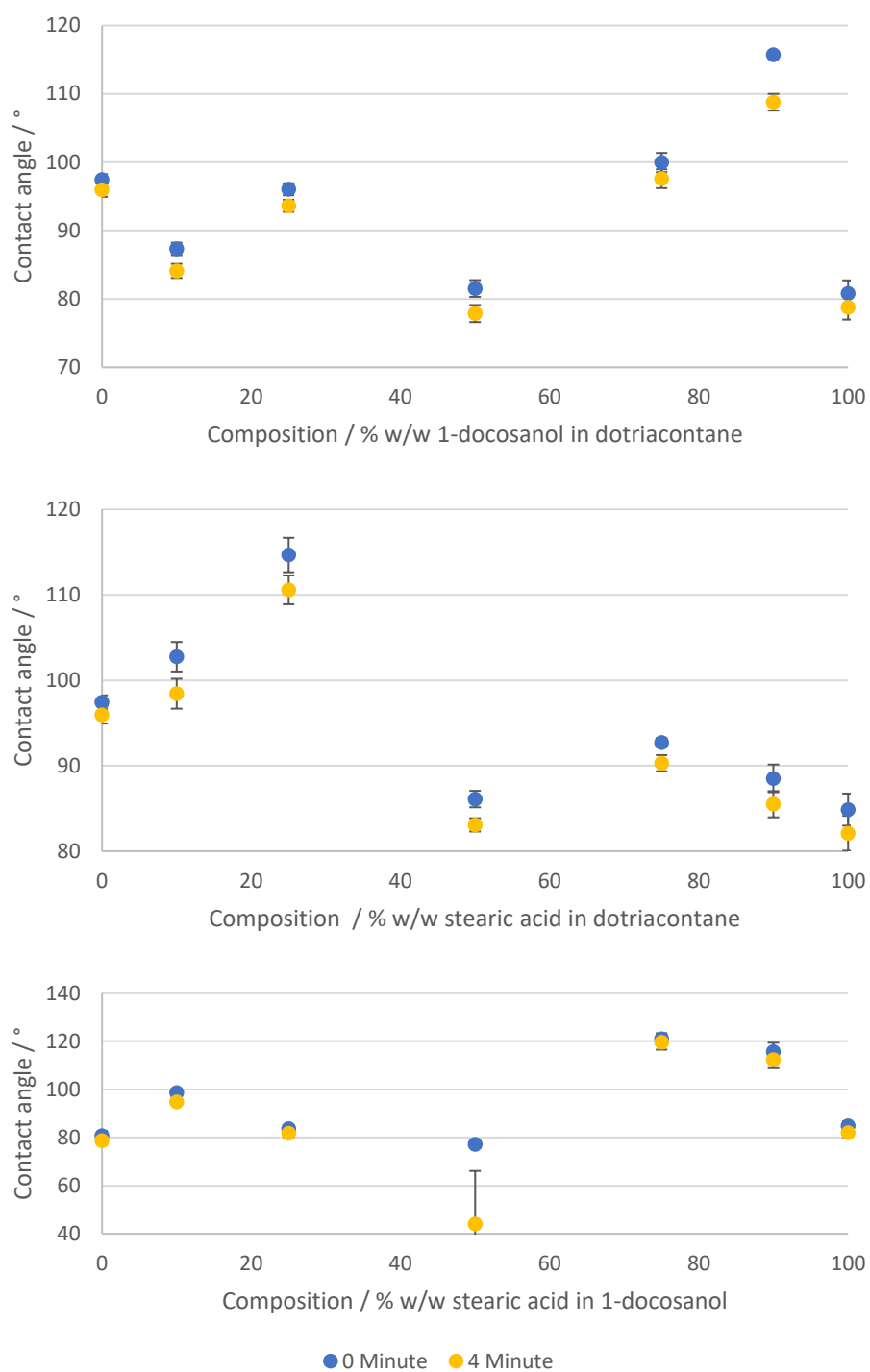


Figure 4-22 - Contact angle of A20 over 4 minutes on 5 layer printed films of (A) DOT: 1-D, (B) DOT: SA and (C) 1-D: SA.

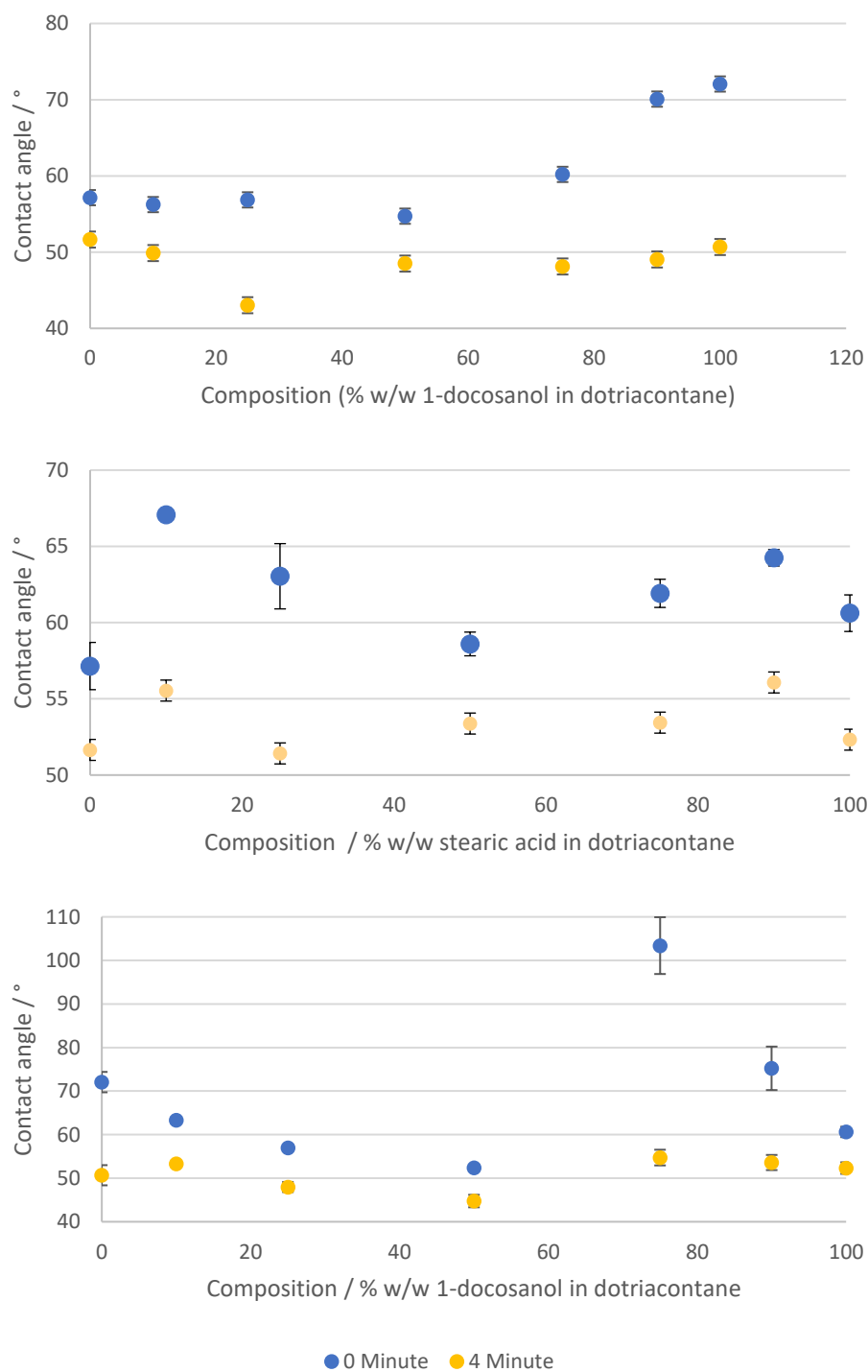


Figure 4-23 - Contact angle of A7 over 4 minutes on 5 layer printed films of (A) DOT: 1-D, (B) DOT: SA and (C) 1-D: SA.

Table 4-3 - % change in contact angle following application of either A20 or A7 compared with water.

		% change in contact angle / water vs A20									% change in contact angle / water vs A7						
		Composition / % w/w 1-docosanol in dotriacontane									Composition / % w/w 1-docosanol in dotriacontane						
Time / mins		0	10	25	50	75	90	100	Time / mins		0	10	25	50	75	90	100
0		23.3	26.8	27.6	24.8	19.1	16.8	28.7	0		55.0	52.8	57.2	49.5	51.3	49.6	36.4
1		23.4	27.4	27.6	25.8	19.4	20.1	28.7	1		56.6	55.1	63.9	51.9	58.1	60.9	48.7
2		23.6	27.8	27.7	26.3	19.8	20.8	29.0	2		57.7	56.0	65.2	52.5	59.0	62.4	52.2
3		23.7	28.1	27.6	26.6	19.9	21.1	29.2	3		58.0	56.6	66.1	53.6	59.9	63.7	54.4
4		23.9	28.6	27.9	26.9	20.2	21.4	29.5	4		59.1	57.6	66.8	54.5	60.7	64.6	54.7
		Composition / % w/w stearic acid in dotriacontane									Composition / % w/w stearic acid in dotriacontane						
		0	10	25	50	75	90	100			0	10	25	50	75	90	100
0		23.3	24.1	16.0	21.4	20.2	28.0	24.4	0		55.0	50.5	53.8	46.5	46.7	47.7	46.0
1		23.4	25.8	17.8	22.4	21.1	28.4	24.8	1		56.6	56.2	59.7	48.4	50.9	51.8	49.9
2		23.6	26.3	18.0	22.5	21.2	28.9	24.8	2		57.7	57.4	60.4	49.1	52.0	52.6	50.5
3		23.7	26.5	18.3	22.8	21.4	29.2	25.2	3		58.0	58.2	61.2	49.8	52.8	53.2	51.5
4		23.9	26.9	18.6	23.0	21.7	29.5	25.3	4		59.1	58.7	62.1	50.5	53.7	53.8	52.4
		Composition / % w/w stearic acid in 1-docosanol									Composition / % w/w stearic acid in 1-docosanol						
		0	10	25	50	75	90	100			0	10	25	50	75	90	100
0		28.7	22.3	24.9	19.9	8.7	10.8	24.4	0		36.4	50.2	48.9	45.7	22.0	42.0	46.0
1		28.7	23.3	24.7	23.0	9.1	11.9	24.8	1		48.7	55.2	55.1	42.8	45.3	54.3	49.9
2		29.0	23.7	25.2	29.4	9.4	12.3	24.8	2		52.2	56.2	55.7	44.1	52.6	55.9	50.5
3		29.2	24.2	25.4	33.6	9.6	12.6	25.2	3		54.4	56.9	56.0	46.2	56.2	57.4	51.5
4		29.5	24.6	25.6	49.4	9.7	12.8	25.3	4		54.7	57.6	56.4	48.6	58.7	58.4	52.4

It was readily observed that following the application of A20, each wax presented a significant decrease in contact angle compared with the contact angle of water on the same surface, Figure 4-22. Furthermore, the contact angle significantly decreased after 4 minutes for many wax films due to spreading upon many waxes tested, i.e. 50: 50 % DOT: 1-D w/w (Figure 4-22 A). This had not been extensively observed following water application. Interestingly spreading of A20 was not observed upon 1-D: SA binary waxes (Figure 4-14). It is thought this is because these waxes presented crystalline structures that could pin water droplets, but the reduced surface tension of A20 droplets and possible surface rearrangement effects allowed many A20 droplets to overcome this pinning effect.

Interestingly the contact angle of A20 presented a similar trend as a function of contact angle against compositions as found when testing the relationship of water against composition, i.e. the addition of 1-docosanol to dotriacontane increased contact angle. In contrast, adding 1-docosanol to stearic acid decreased the contact angle.

Table 4-3 shows this clearly as the contact angle of A20 compared with water was decreased between 20.2 % and 29.5 % upon 14 of 18 waxes suggesting the general trends relating contact angle and composition were retained. Given this, it is suggested A20 reduces contact angle by reducing the water tension of the droplet, not through surface modification. This is in agreement with prior literature⁴⁹. Given this, many of the trends seen between wax composition and water contact angle are still visible.

Considering A7, similarly to A20, all waxes presented a significant decrease in contact angle relative to the contact angle of water (Table 4-3). Additionally, all waxes showed a significant spread of the A7 droplet over 4 minutes, including 1-D: SA binary waxes that had not shown

significant spreading of A20 droplets over 4 minutes (Figure 4-22 C and Figure 4-23 C). This suggests that the A7 solution could overcome the pinning induced by the wax surface. Reducing the ethoxylated alcohol chain's length increases the surfactants' capability to wet hydrophobic surfaces⁴⁴, which agrees with these results.

Like A20, as shown in Table 4-3, A7 contact angles after 4 minutes decreased uniformly across most tested waxes. The contact angle of A7 was 51 % and 61 % smaller than the contact angle of water following application to 14 of the 18 tested waxes. As shown following A7 or A20 application, Table 4-3, the droplet rapidly spread within the first 1 minute before spreading relatively slowly. Prior literature has suggested that A7 can induce irreversible morphological changes that further the surfactants capability to spread⁵⁰. Droplets were deposited and allowed to spread before being dried and imaged.

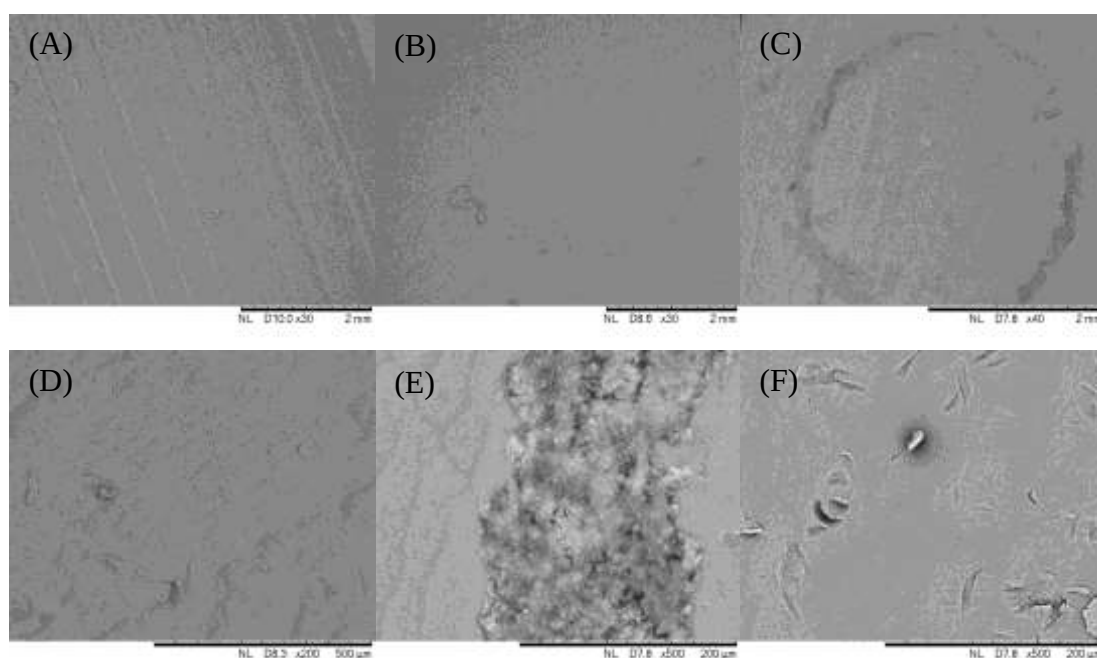


Figure 4-24 - SEM images showing the effect of surfactant application (A) after 1 minute, (B) 10 minutes and (C) 1 hour to a film of 1-D: SA 50: 50 % w/w. (D) Droplet edge after 10 minutes, (E) droplet edge after 60 minutes and (F) droplet centre after 60 minutes.

As Figure 4-24 shows, morphological changes could be induced by the drying surfactants on the surface, consequently leading to coffee-ring effects. However, the exposure must occur over at least 10 minutes in the tested samples to induce observable effects. Similar results have been shown prior on isolated cuticles⁵⁰. For these reasons, it is unlikely that the application of surfactant droplets for 4 minutes will cause extensive morphological changes that could impact spreading and contact angle. This would explain why the contact angle significantly decreased after 1 minute, due to the initial relaxation of the droplet, but then

showed no further significant spreading, as the morphological changes that might induce spreading occurred over a longer period than the testing period.

4.3.4. FT-IR

FT-IR was used, Section 3.3.2.2, to observe spectral changes as a function of composition, to confirm binary waxes formed a eutectic mixture, that no chemical changes had occurred between the waxes and to estimate the crystallinity of the wax powders, Section 3.2.6.

FT-IR of printed waxes was used to confirm the printed wax was not contaminated during printing, the printing process did not induce aberrant chemical interactions and estimate printed wax films crystallinity.

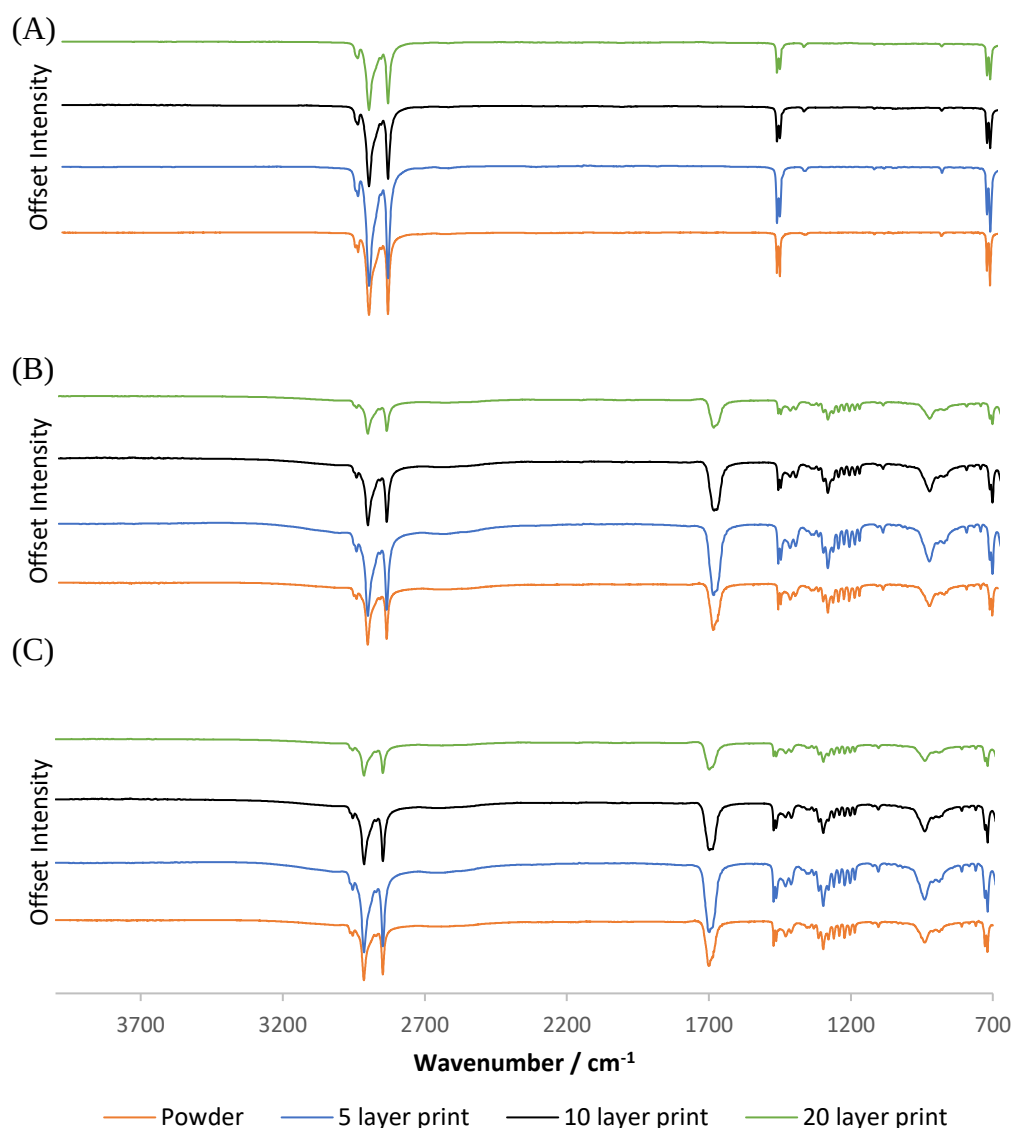


Figure 4-25 - (A) dotriacontane, (B) stearic acid and (C) 1-docosanol. The orange line represents powder wax, the blue line a 5 layer print, the black a 10 layer print and green a 20 layer print.

As Figure 4-25 shows, there was no observable difference between the FT-IR spectra of the powder and the printed samples of either dotriacontane, 1-docosanol or stearic acid. Similar results were found for binary wax mixtures of DOT: 1-D and DOT: SA. This suggests that the printing process has not resulted in new chemical reactions or detectable contamination of the printed waxes. However, some variations could be observed between printed films and powder wax of 1-D: SA, Figure 4-26.

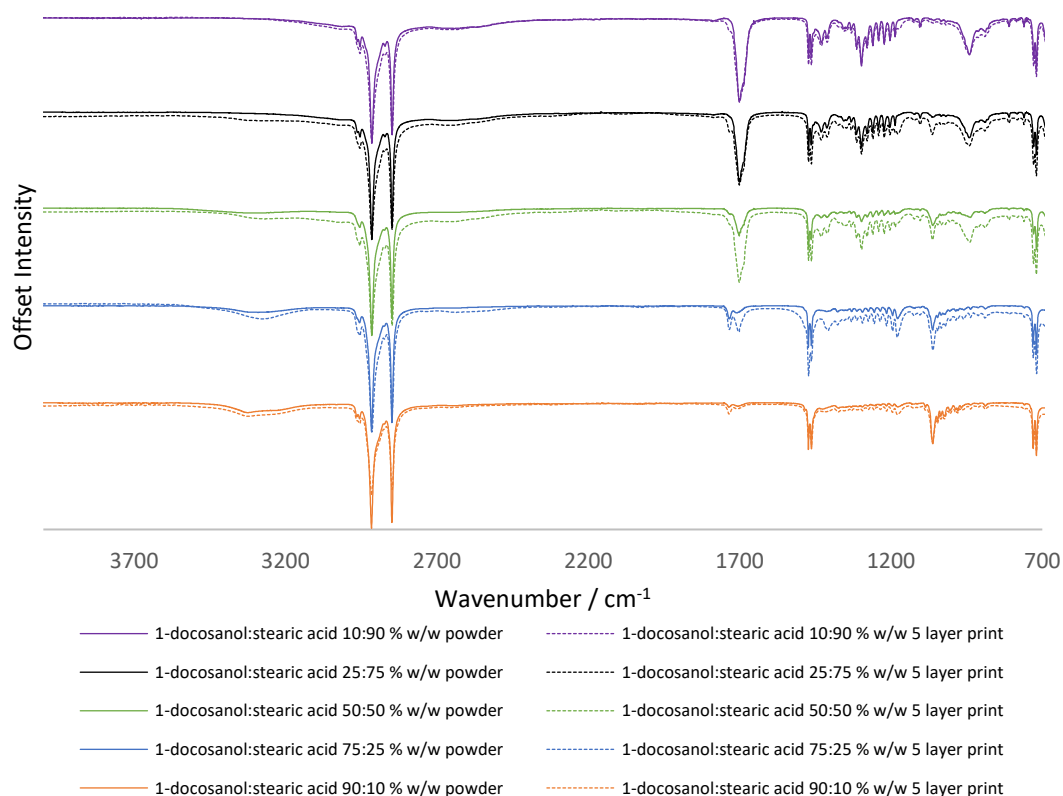


Figure 4-26 - Normalised by the maximum intensity and overlaid FT-IR spectra of binary waxes of 1-D: SA as powder and 5 layer printed films

After normalisation, the diagnostic peaks for stearic acid, an absorbance peaks associated with a C=O bend at 1700 cm^{-1} and the unknown peak at 940 cm^{-1} , within printed samples presented intensity values exceeding the relative intensity in the powder. The C=O bending vibration peak can be seen when the composition of stearic acid is at least 25 % w/w. This peak presents a high-intensity value while diagnostic peaks of 1-docosanol are still dominant, and the second diagnostic stearic acid peak is not visible. This suggests that the printing process has induced the formation of additional C=O bonds, which is unlikely, or there was an accumulation or enrichment of the stearic acid at the surface due to the printing process.

It should be noted that this pattern is also visible in the 50: 50 binary wax. FT-IR spectra of waxes wherein stearic acid is dominant shows the intensity of the C=O bending vibration peak converging with the value found in powder samples. It is thus likely that some aspects of the printing process have increased the intensity of this peak, such as the accumulation of material on the surface interface. Results further exploring this behaviour can be seen in Section 4.3.6.

Table 4-4 FT-IR determined % crystallinity of powder and printed waxes. Sd is the standard deviation.

% w/w composition of 1-docosanol in dotriacontane	Powder wax		5 layer printed films		10 layer printed films		20 layer printed films	
	% crystallinity	Sd	% crystallinity	Sd	% crystallinity	Sd	% crystallinity	Sd
0	73.5	0.3	50.1	2.0	64.3	0.5	59.0	5.2
10	56.0	2.1	52.9	2.2	55.5	1.7	54.7	1.9
25	66.3	4.0	57.6	0.8	60.8	4.7	65.2	1.2
50	84.2	3.6	68.2	0.2	72.0	0.6	66.8	2.5
75	90.0	1.6	77.9	1.3	73.1	1.6	74.0	1.3
90	88.6	2.3	69.6	1.9	70.1	0.8	71.5	0.7
100	86.2	1.3	66.6	5.5	74.8	2.6	79.4	1.6
% w/w composition of stearic acid in dotriacontane	% crystallinity	Sd	% crystallinity	Sd	% crystallinity	Sd	% crystallinity	Sd
0	73.5	0.3	50.1	2.0	64.3	0.5	59.0	5.2
10	57.9	1.4	56.7	2.3	52.8	0.8	49.0	3.0
25	70.2	7.1	51.5	0.2	51.8	0.5	57.3	2.8
50	75.3	1.6	70.1	1.3	62.1	0.1	59.6	2.7
75	84.4	1.5	52.8	0.9	60.9	6.2	66.4	13.4
90	79.9	8.2	54.5	2.5	54.0	1.5	64.7	2.8
100	96.9	0.4	51.6	2.8	50.8	1.6	54.5	9.5
% w/w composition of stearic acid in 1-docosanol	% crystallinity	Sd	% crystallinity	Sd	% crystallinity	Sd	% crystallinity	Sd
0	86.2	1.3	68.4	5.5	74.8	2.6	79.4	1.6
10	87.6	3.9	63.7	9.5	64.7	10.2	71.7	0.4
25	83.4	4.7	68.4	6.2	75.0	0.6	73.5	1.4
50	56.5	5.9	61.0	1.1	-	-	-	-
75	93.1	2.4	77.5	1.3	78.8	1.8	78.5	0.9
90	84.7	4.9	75.3	2.3	-	-	-	-
100	96.9	0.4	51.6	2.8	50.8	1.6	54.5	9.5

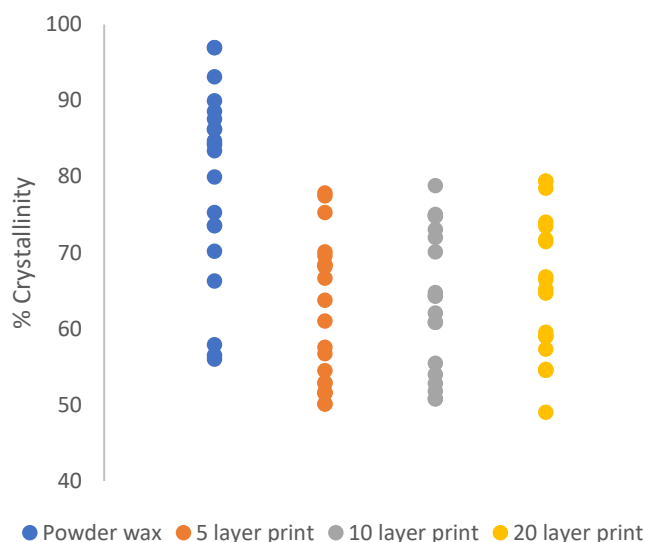


Figure 4-27 - Distribution of % crystallinity as a function of physical form

In addition to evaluating the FT-IR spectra of printed waxes vs powder waxes, the % crystallinity was determined per the CH₂ functionality; Section 3.3.2.2. Figure 4-27 shows the measured % crystallinity of different waxes as powders and the 5, 10 and 20 layered films. All wax compositions experienced a significant decrease in % crystallinity following printing. This is likely a consequence of the more rapid cooling a droplet will experience than a bulk molten wax solution

Figure 4-27 shows the general trend observed wherein printing causes a drop in crystallinity. However, there was no broad trend regarding composition and crystallinity. The waxes did not consistently increase in crystallinity as print thickness increased, suggesting that the change in morphology as observed in Section 4.3.2.3 was not a consequence of changes in crystallinity.

It should be noted that many of the printed and powder waxes present higher % crystallinity values than many cuticle waxes⁵¹⁻⁵³. The low crystallinity of cuticle waxes has been hypothesised to be caused by mixing the many wax classes and chain lengths. This heterogeneity is thought to interfere with crystal formation, increasing the amorphous wax fraction⁵⁴. However, as shown here, binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid did not uniformly present lower % crystallinity values than the component homogeneous waxes, as expected per this hypothesis⁵⁴. However, it should be noted printed waxes might not be comparable to cuticle waxes as the printed waxes are significantly less compositionally complex. In many cases, binary powder and printed waxes often presented a significantly increased crystallinity relative to the single component waxes.

4.3.5. XRD

As seen prior, printing wax in almost all cases leads to a significant decrease in % crystallinity, as per FT-IR measurements. Given this, printing may modify the crystalline arrangement on exposure to raised temperatures for prolonged periods, shear stress at the nozzle and deposition in a patterned structure. The x-ray diffractograms in Figure 4-28, Figure 4-29 and Figure 4-30 show that the powder and printed waxes present several similarities. No printed or powder wax shows amorphous characteristics, as observed by a lack of an amorphous halo⁵⁵. An amorphous halo might be expected in the printed samples considering the reduction in crystallinity following printing. However, waxes with similar % crystallinity, as measured using FT-IR, do not present an amorphous halo. As stated prior, in Section 3.3.2.4, it is not clear if FT-IR-based crystallinity assessments are accurate despite their frequent use in the analysis of Fischer-tropsch waxes⁵⁵.

Additionally, eutectic properties, the simultaneous presenting of peaks from both component materials, can still be observed in binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid. Peaks present in the single component waxes are found in the diffractograms of waxes bulk comprised by these waxes. Finally, the short spacing peaks at $21.4\ 2\theta$ and $24.5\ 2\theta$ degrees, found in almost all wax powder diffractograms, are still observed. This suggests the uniform short spacing peaks, which show the layering of waxes upon each other, has been retained in the printed samples

However, in almost all waxes, the peaks at $21.4\ 2\theta$ and $24\ 2\theta$ are narrower than the powder samples in the printed samples. XRD peak width is inversely proportional to crystal structure size, otherwise known as grain size⁵, suggesting printing resulted in the formation of larger grains. However, it is known that large grains result from slow cooling rates in metals⁵⁷; similar tests have not been conducted on waxes. Considering the droplets that coalesce to form a printed film are small volume and cool individually, or potentially as lines of material, it might be assumed grains would be small due to the rapid cooling involved compared with the powder wax, which was cooled more slowly. However, the printed films were also tempered, which resulted in increased grain size⁵⁸. As stated in Section 4.2.3, printed waxes were allowed to temper for 24 hours on the heated substrate as a printed film to ensure a uniform thermal history due to the slow printing process utilised. Powder wax, Section 3.2.2, was tempered for only approximate 1 hour. Samples showing a narrower $21.4\ 2\theta$ peak also show a shifting of the $21.4\ 2\theta$ and $24.5\ 2\theta$ peaks suggesting a change in unit size, a property typical of tempered materials^{59,60}.

Alternatively, the printed film has undergone extensive shear stress at the nozzle before rapidly cooling. The observed narrow peaks may be a consequence of the preferential orientation of the crystalline structures in the solidified films. Similar semi-crystalline printed material shows extensive preferential orientation of crystalline structures when printed below the glass transition point³⁵.

Additionally, DOT: SA 25: 75 % w/w (Figure 4-29) and 1-D: SA 25: 75 % w/w (Figure 4-30) no longer displayed long spacing peaks, the peaks found at low 2θ values, representing the formation of large scale ordering and stacking of crystal structures, and the intensity of these peaks in stearic acid samples is considerably reduced. It is possible that when waxes cooled more rapidly, relative to powder samples, the arrangement of wax stacking along the c-axis was arrested.

Finally, doublet structures are often observed where singular peaks were found at $21.4\ 2\theta$ and $24.5\ 2\theta$ in printed films. In some cases, these doublet peaks might result from peak narrowing allowing the deconvolution of prior superimposed peaks; DOT: SA 50: 50 % w/w is a possible example (Figure 4-29). However, as shown with 1-docosanol (Figure 4-28), some waxes present novel peaks between $20\ 2\theta$ - $25\ 2\theta$ degrees, including doublets. Similarly, these doublet peaks at $21.4\ 2\theta$ and $24.5\ 2\theta$ might represent the formation of new crystalline arrangements and new spacings between crystalline structures, which the peaks at $21.4\ 2\theta$ and $24.5\ 2\theta$ typically represent in cuticle waxes⁵⁵. Regardless of the origin of doublet and new peaks, it is clear that new crystalline arrangements have formed due to printing. Many waxes show new peaks suggesting a more extensive set of side to side basal spacing between chains have formed. Texture analysis evaluation of the preferential distribution of crystals orientation in a polycrystalline sample⁶¹ may explain the origin of these peaks as prior work has shown a preferential arrangement of printed parts not seen in feedstock material³⁵.

As part of this work, printed wax films underwent permeation analysis. Subsequently, films were heated for prolonged periods to assess the effect of temperature on permeation. XRD was used to assess if heating affects the crystalline arrangement, which is thought to affect permeation¹⁸. Figure 4-31 shows that neither stearic acid nor DOT: SA 25: 75 % w/w film underwent any visible change due to temperature. Similar results have been seen prior on extracted waxes¹¹. Typically the cuticles' crystalline arrangement changes only at temperatures where phase changes. As shown in Section 3.3.2.1, no phase changes occur between $30\ ^\circ\text{C}$ and $60\ ^\circ\text{C}$ in either tested waxes, explaining the lack of change. Given this, crystalline arrangement changes will not occur at the operational temperatures of the

permeation analysis for the materials tested. It should, however, be noted that waxes dominated by dotriacontane may show an Orthorhombic to hexagonal transition at 66 °C,
Section 3.3.2.1

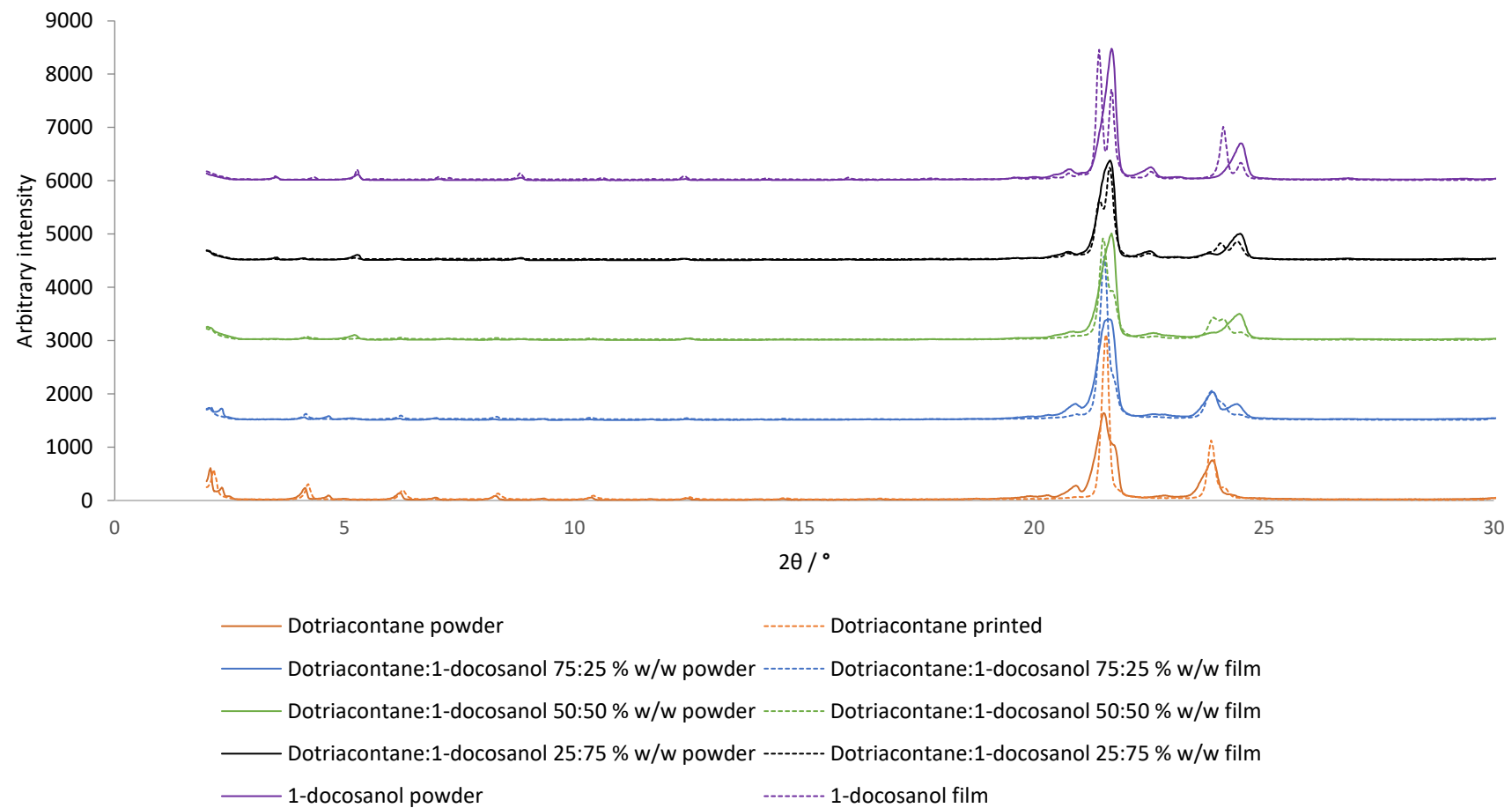


Figure 4-28 - XRD diffractograms of powder and 5 layer printed wax films of dotriacontane, 1-docosanol and associated binary waxes. The printed and powder waxes of each composition are overlaid to allow comparison of each form and composition of wax.

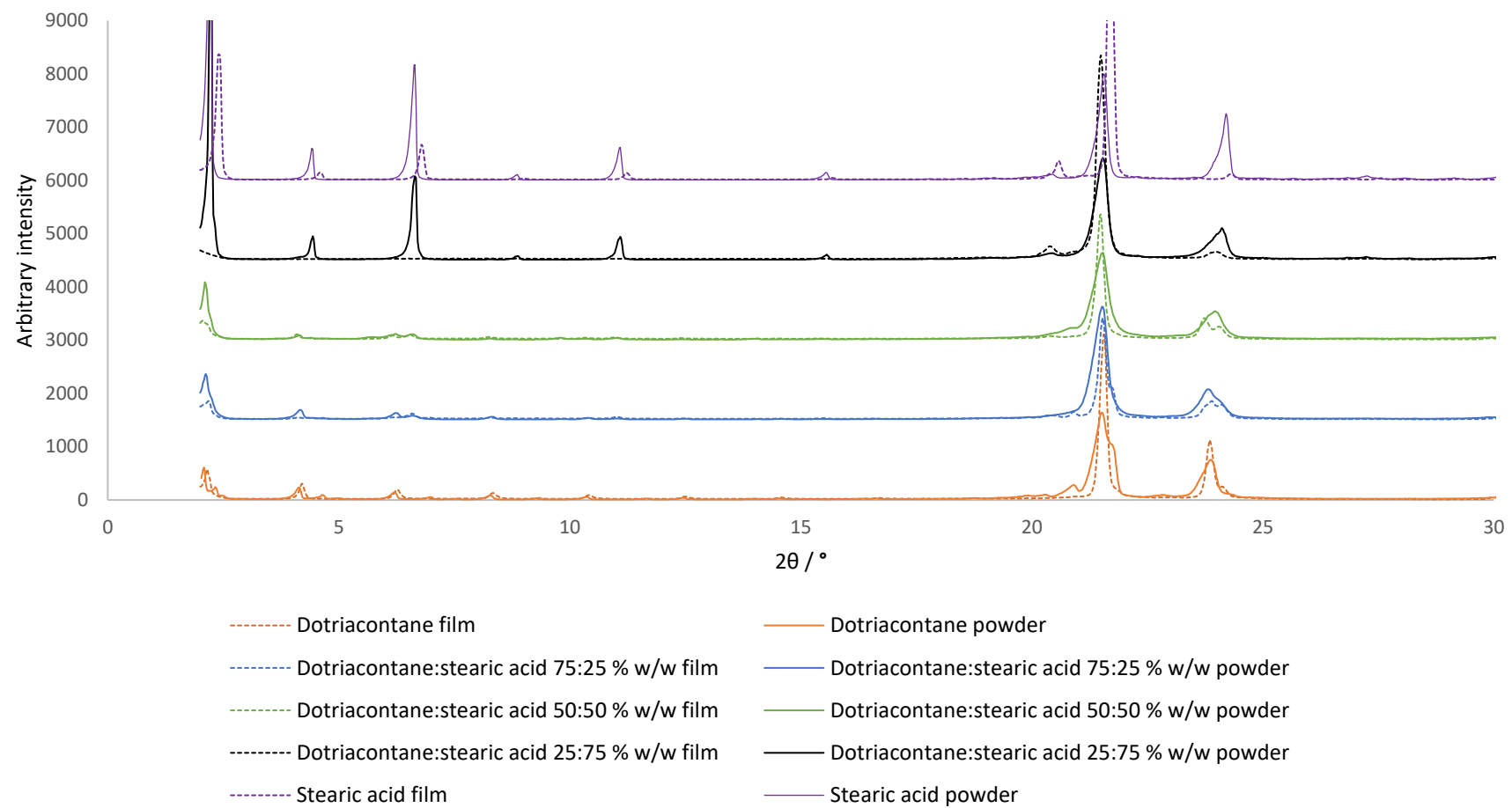


Figure 4-29 - XRD diffractograms of powder and 5 layer printed wax films of dotriacontane, stearic acid and associated binary waxes. The printed and powder waxes of each composition are overlaid to compare each form and composition of wax.

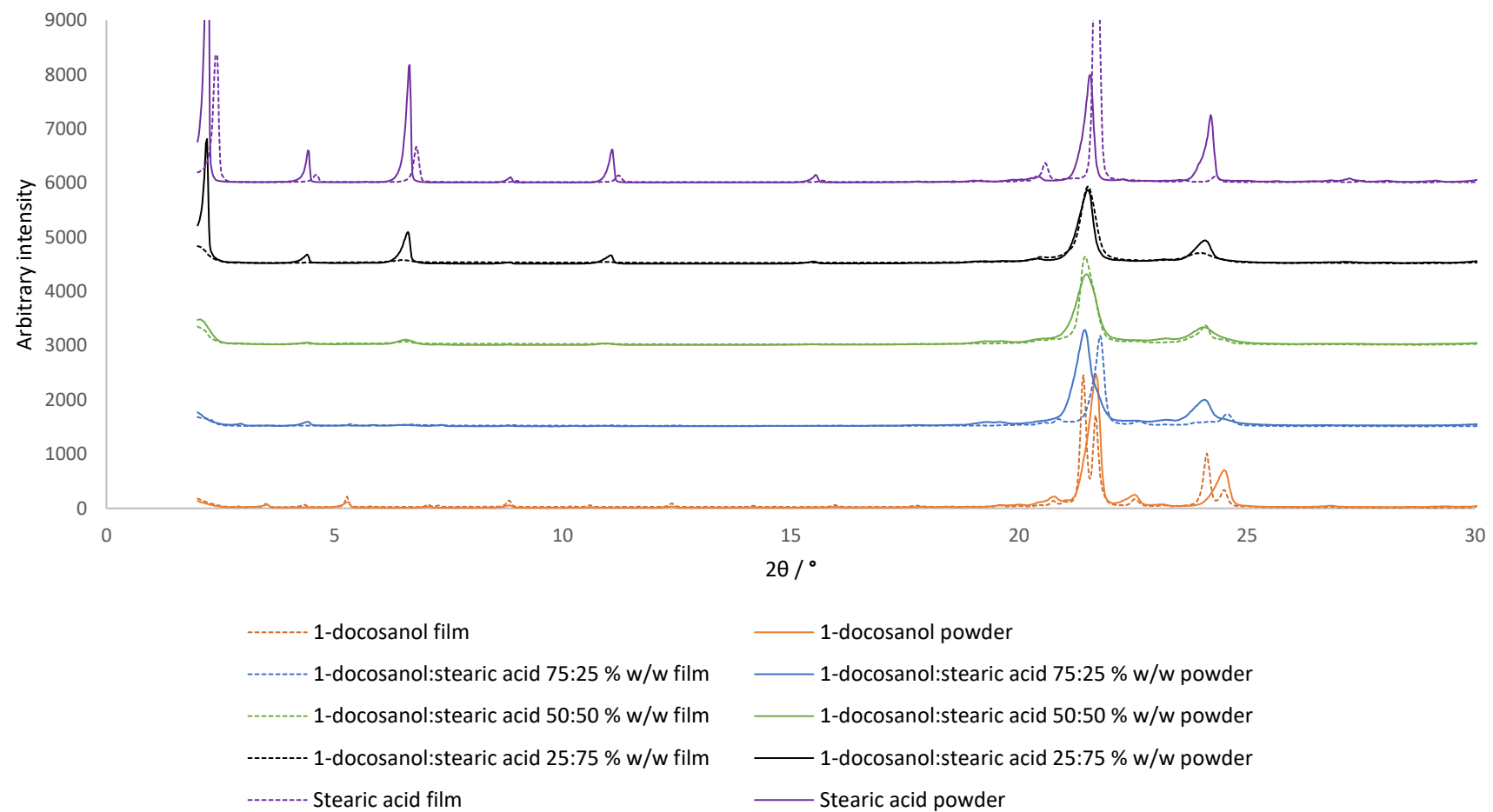


Figure 4-30 - XRD diffractograms of powder and 5 layer printed wax films of 1-docosanol, stearic acid and associated binary waxes. The printed and powder waxes of each composition are overlaid to allow comparison of each form and composition of wax.

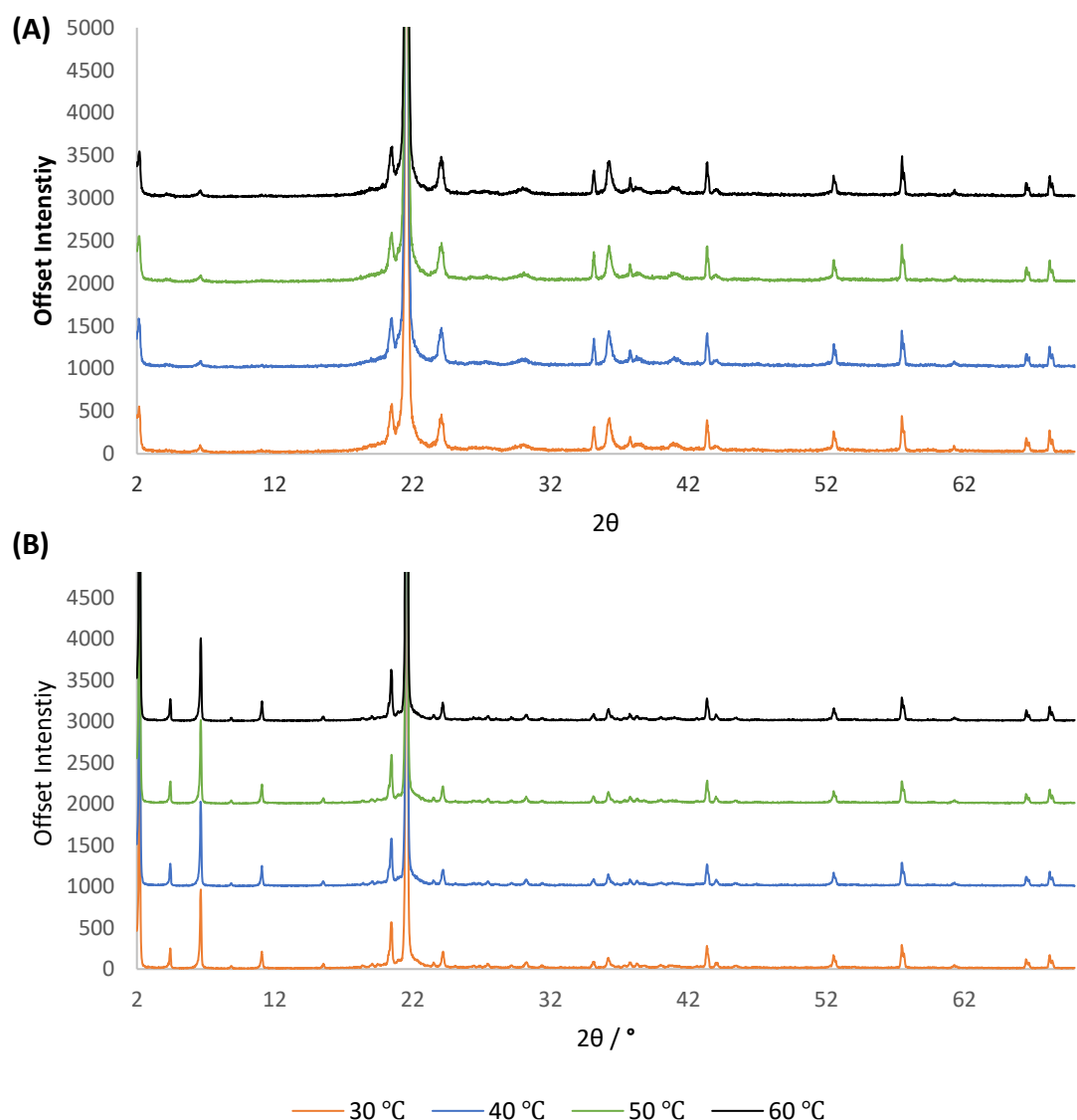


Figure 4-31 - XRD diffractogram of 5 layer printed (A) DOT: SA 25: 75 % w/w and (B) stearic acid between 30 °C and 60 °C.

4.3.6. Raman Spectroscopy

Raman spectroscopy was utilised to define the chemical composition of printed waxes and assess if the printed films displayed a heterogenous wax distribution. As shown in Section 4.3.2.3, such experiments were conducted because multiple wax films, particularly 1-D: SA 25: 75 % w/w, demonstrated extensive crystalloid formation. Similar crystalloids on cuticle surfaces often are comprised of a unique wax composition compared with the bulk wax composition, a consequence of phase separation as wax biosynthesis changes during plant development. Pure

1-docosanol, stearic acid, and 25: 75, 50: 50 and 75: 25 % w/w binary wax films were imaged using Raman Spectroscopy to evaluate the wax distribution.

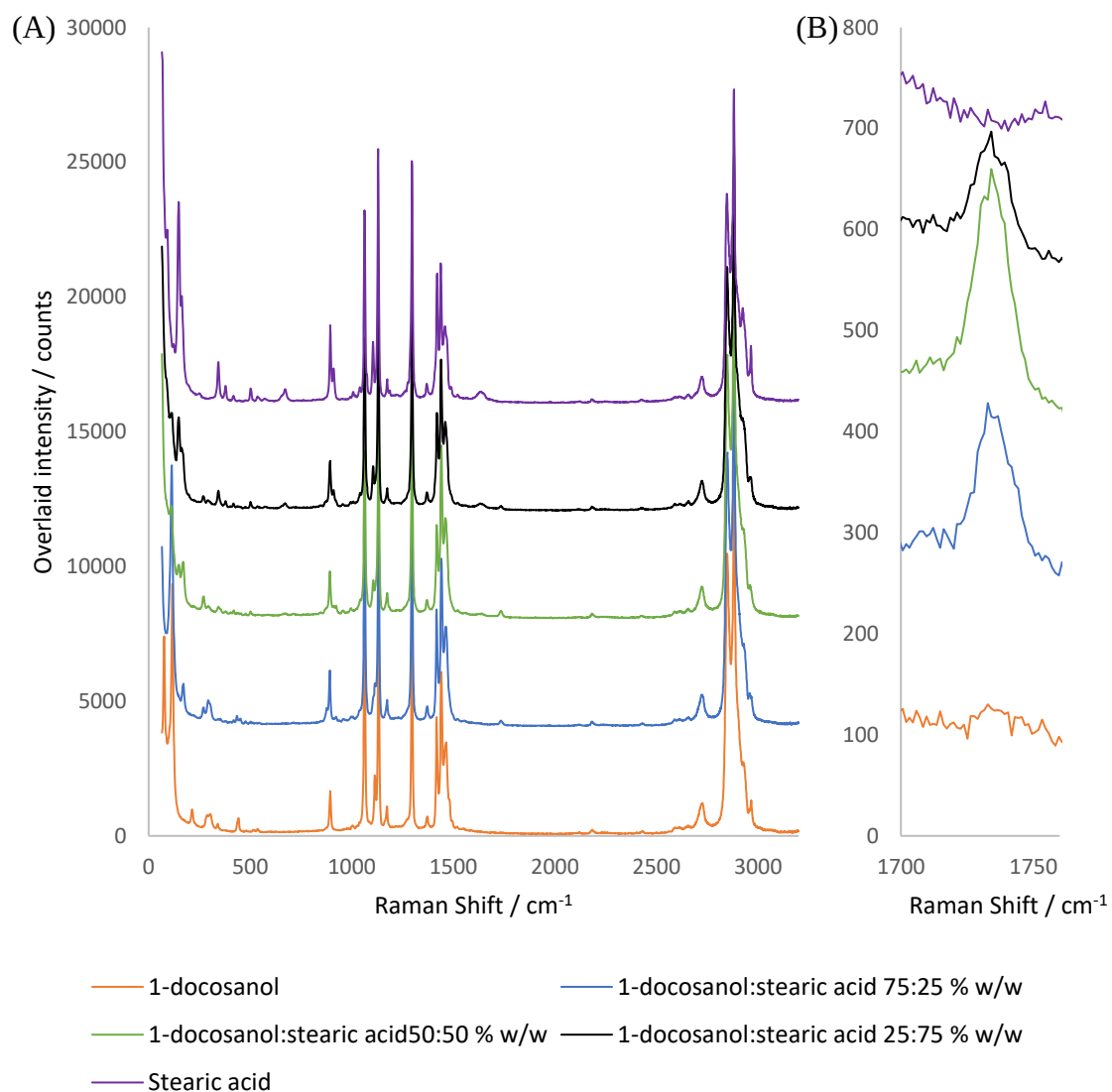


Figure 4-32 - (A) Raman spectra of 1-docosanol, stearic acid and associated 75: 25, 50: 50 and 25: 75 % w/w binary waxes. (B) shows the peak at 1734 cm⁻¹

As shown in Figure 4-32, the two single component waxes showed highly similar spectra, containing a broad series of related bands. Several diagnostics peaks could be identified in the low-frequency regions. The peak at 1635 cm⁻¹ is associated with the C=O stretching vibration and selected as the diagnostic peak representing stearic acid.

Considering point spectra of the tested binary waxes, Figure 4-32 (B), a novel peak could be observed at 1734 cm⁻¹. It is thought this peak is associated with a C=O stretching vibration,

shifted by an intermolecular hydrogen bond between the stearic acid and alcohol. The intensity of this band is greatest in the 50: 50 % w/w composition supporting this theory as both waxes will be evenly distributed near the eutectic point. As shown in Section 4.3.1, 1-D: SA films presented thermostability properties exceeding the component waxes. It is possible that this proposed hydrogen bonding might be responsible for the increased thermostability.

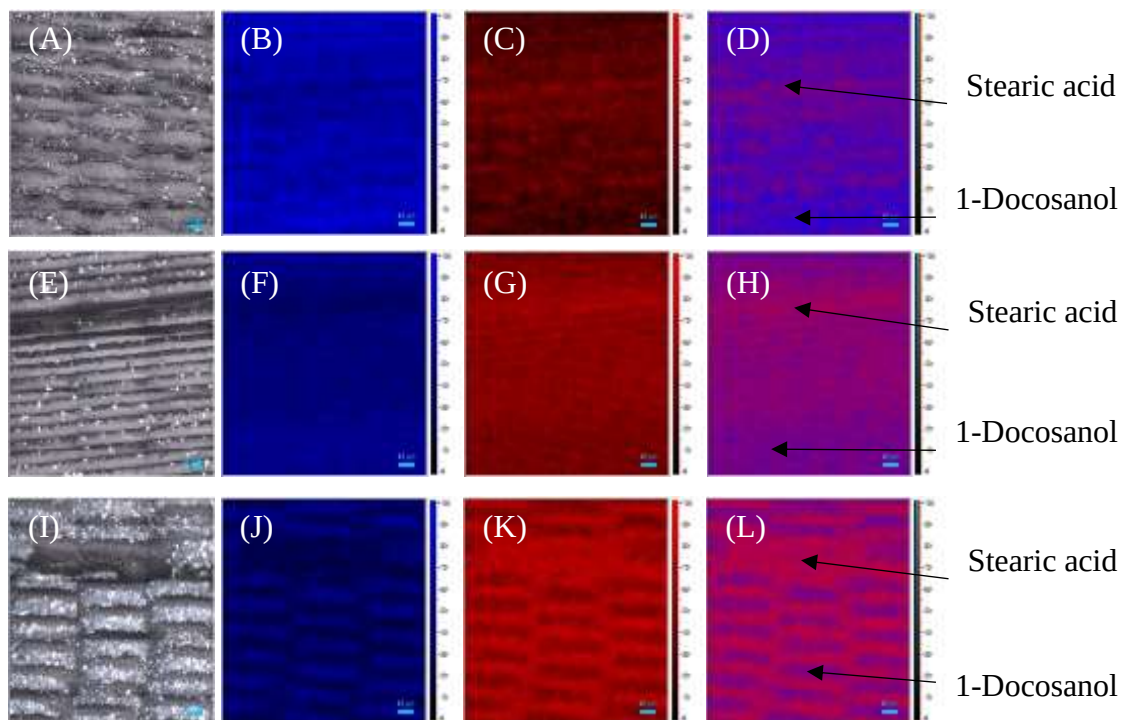


Figure 4-33 - (A) optical image of 1-D: SA 75: 25 % w/w, (B) normalised CLS score map of the CCO stretching vibration, representing 1-docosanol within (A), (C) normalised CLS score map of the C=O stretching vibration, representing stearic acid within (A), (D) overlaid CLS score maps of 1-docosanol and stearic acid within (A). (E) optical image of 1-D: SA 50: 50 % w/w, (F) normalised CLS score map of 1-docosanol within (E), (G) normalised CLS score map of stearic acid within (E), (H) overlaid CLS score maps of 1-docosanol and stearic acid within (E). (I) optical image of 1-D: SA 25: 75 % w/w, (J) normalised CLS score map of 1-docosanol within (I), (K) normalised CLS score map of stearic acid within (I), (L) overlaid CLS score maps of 1-docosanol and stearic acid within (I). Each image is 500 μm x 500 μm and the scale bar is 40 μm.

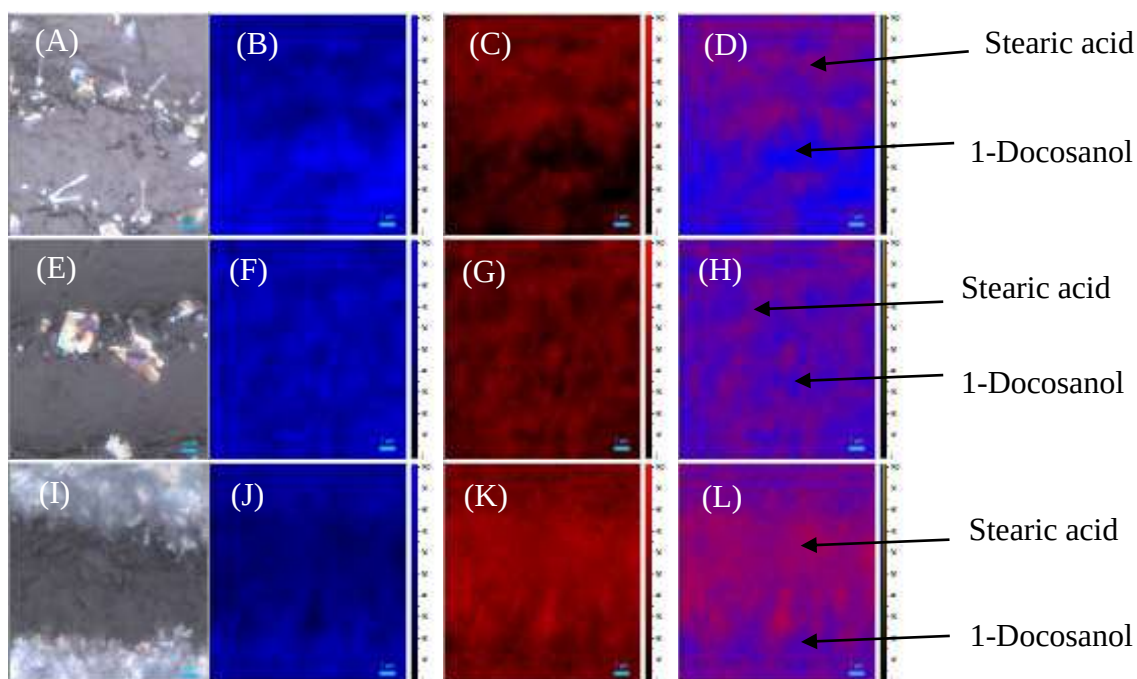


Figure 4-34 - (A) optical image of 1-D: SA 75: 25 % w/w, (B) normalised CLS score map of the CCO stretching vibration representing 1-docosanol within (A), (C) normalised CLS score map of the C=O stretching vibration representing stearic acid within (A), (D) overlaid CLS score maps of 1-docosanol and stearic acid within (A). (E) optical image of 1-D: SA 50: 50 % w/w, (F) normalised CLS score map of 1-docosanol within (E), (G) normalised CLS score map of stearic acid within (E), (H) overlaid CLS score maps of 1-docosanol and stearic acid within (E). (I) optical image of 1-D: SA 25: 75 % w/w, (J) normalised CLS score map of 1-docosanol within (I), (K) normalised CLS score map of stearic acid within (I), (L) overlaid CLS score maps of 1-docosanol and stearic acid within (I). Each image is 50 μm x 50 μm and the scale bar is 4 μm .

Figure 4-33 and Figure 4-34 show Raman maps indicating the distribution of 1-docosanol and stearic acid across the wax films at two different magnifications. Figure 4-33 shows that wax separation and preferential distribution occur with all three waxes in the low magnification images. This is most evident in Figure 4-33 (H and L). Considering Figure 4-33 (I and L), 1-docosanol preferentially distributes into clustered regions similar to the crystalloids shown prior in SEM images of this film (Figure 4-14 E). This is likely a consequence of 1-docosanol cooling and the unique crystalloids that form upon 1-docosanol during cooling (Figure 4-14). It is possible that wax cools more rapidly at the print track interfaces, as already postulated. Subsequently, as per the phase diagram of 1-D: SA binary waxes, Section 3.3.2.1 as the 1-D: SA 25: 75 % w/w wax cools excess stearic acid will precipitate first. If wax at the print track interface does cool more rapidly than the bulk droplet, it is expected that stearic acid would preferentially precipitate at these interfaces where rapid cooling occurs. This is supported in Figure 4-33 (L), where it can be seen that stearic acid forms channels through the film in both the X and Y-axis.

The pattern shown is approximately where it would be expected droplet-droplet interactions would occur. The remaining eutectic material will solidify as the temperature continues to decrease, resulting in the deposition of 1-docosanol crystalloids preferentially in the location comprising the bulk of the droplet.

Table 4-5 - CLS scores describing 1-docosanol distribution across the 500 x 500 μm images (Figure 4-33).

Composition	1-docosanol content / % w/w	1-docosanol CLS score / % (variability / %)
1-docosanol:stearic acid 75:25 % w/w	75	67.1 $\pm$ 8.1 12
1-docosanol:stearic acid 50:50 % w/w	50	48.4 $\pm$ 6.6 13.6
1-docosanol:stearic acid 25:75 % w/w	25	38.1 $\pm$ 8.9 23.5

The theory that stearic acid present in 1-D: SA 25: 75 % w/w wax precipitated preferentially at the print track interface is supported by Table 4-5. 1-docosanol is distributed more extensively than would be expected given its % w/w composition in the 1-D: SA 25: 75 % w/w wax film, Figure 4-33 (I-L). This suggests that some stearic acid is not incorporated within the eutectic mixture, being preferentially distributed into a few locations, leading to the stearic acid distribution being more localised than expected. In this case, it is shown stearic acid preferentially distributed in the print track interface. It is also presumed that stearic acid might preferentially distribute where the droplet interfaces with the substrate. It should be noted that the distribution of 1-docosanol is insignificantly different to the expected value in the 75: 25 and 50: 50 % w/w samples. It is proposed that similar phase separation events are unique to waxes containing large volumes of 1-docosanol.

4.4. Conclusions

The development of a wax cuticle model is desired to study the structure-function relationship of individual waxes within the cuticle, enabling investigation of the physical and chemical properties of the cuticle. However, any model based on replication of chemical composition and distribution of a natural structure must consider factors that could impact the feedstock material or the final product. Additionally, the production methodology must be understood and be capable of generating consistent products. Finally, if it is possible to vary the properties and behaviour of the structures, in this case a film, through modification of factors such as composition and production parameters this must be investigated.

Initial assessment of printing suitability was performed. TGA analysis of the waxes tested showed no wax was found to degraded to an extent at operational temperatures, 90°C, that would render the wax unsuitable for printing. Instead, it was observed every wax tested only experienced 5 % mass after exceeding 200 °C. It was observed that many binary waxes, consisting of two of the component waxes tested, showed degradation patterns where observed peaks that could be attributed to the component waxes and the areas of these peaks corresponded to the w/w % mass of the corresponding wax within the binary wax. This implies the thermal degradation of these binary waxes can be predicted from the degradation patterns of the component waxes. Interestingly one set of the binary wax samples, the 1-D: SA waxes, showed a prolonged degradation compared with its component waxes implying producing the binary wax increased the waxes thermal stability. It is assumed that either the formation of the eutectic material or induced hydrogen bonds between wax molecules increased thermostability relative to the component waxes. In no case did the formation of a binary wax decrease the waxes thermal stability.

Considering printing of the tested waxes, it was shown spherical droplets, absent of satellite droplets or tails, could be successfully ejected, despite the low viscosity of the tested wax. This was achieved by minimising the droplet velocity following droplet ejection through modification of the printing waveform. The printed films produced presented crystalloid surface structures morphologically similar to the crystalloid structures found on native cuticle surfaces. It is predicted that these crystalloids form predominantly in regions where localised purification of the wax component led to local and long spacing ordering subsequent crystalloid structure formation. This is supported by observations that crystalloids predominantly formed in the print

track interfaces, where significant chemical heterogeneity was observed. Here it is predicted localised rapid cooling, relative to the bulk droplet, leading to localised purification of waxes.

Considering the morphology of the printed films control of morphology was achieved by changing substrate temperature and DPI. This could occur while retaining physical properties and general surface morphological features. Modification of DPI enabled heterogeneous morphological features such as aberrant bulges or print induced topography such as print tracks to be controlled. By modifying substrate temperature droplet spreading, and the density of surface crystalloids, following deposition could be controlled allowing the production of thin films with a lower quantity of surface crystalloids through increased substrate temperature.

In addition, morphology and behaviour could be induced via composition modification. The three component waxes and the binary waxes of these components presented unique morphologies depending on the composition. Typically, the morphology of the binary waxes did not share features with its component waxes. This was likely a consequence of two factors. The physical properties of the binary waxes being different to the component waxes, leading to modified droplet formation and spreading. Additionally, as per XRD, the binary waxes did not form a cocrystal and the addition of the second wax affected the formation of large single components waxes. In some samples i.e. DOT: SA 50: 50 % w/w, mixing waxes prevented surface crystalloids forming whereas mixing some waxes i.e. 1-D: SA 75: 25 % w/w, promoted the formation of crystalloids. Furthermore, the morphology of the printed film did not resemble the morphology of larger volume drop cast waxes. This was likely the consequence of printed droplet's smaller volume, relative to the drop cast wax, meaning the cooling rate was increased in the printed droplet which impacted wax crystal formation.

While crystalloid structures can be produced, some of which resemble crystalloids found on cuticles surfaces, no trend relating to composition and morphology was identified. Subsequently, tailoring films to present specific morphologies will be challenging. This is likely a consequence of the simple chemical composition of the printed films, relative to the wax composition of most cuticles, and a consequence of the heating and cooling involved that does not reflect the native biosynthesis and distribution of cuticle wax. To generate morphologies that mimic native cuticles, more complex wax composition will be required. Additionally, the processes involved in the formation of these crystalloids in the native cuticle will need to be

better understood, so a methodology of modelling and replicating this process can be developed.

Considering the physical properties of the printed films some changes were observed relative to the powder waxes. FT-IR identified from the 1-D: SA film suggesting the additional hydrogen bonds had formed due to printing. These results are supported by TGA and Raman spectroscopy. However, the crystallinity of waxes, as measured using FT-IR analysis of the CH₂ bond, was shown to significantly decrease following printing. However, no trend was identified relating crystallinity to composition.

XRD analysis of printed waxes typically showed grain size decreased, relative to the powder wax, possibly caused by tempering. Some printed waxes showed peaks shifting to present more short spacing peaks, representing crystalline structures with short range ordering. In all cases, long spacing peaks found in the powder waxes were either absent or reduced in intensity within the printed waxes. The rapid cooling implicit to the printed droplets likely caused rapid re-solidification and arrested the formation of complex unit cells. To test the effect of heating the wax films two printed waxes, stearic acid and DOT: SA, were heated while undergoing XRD evaluation. Both films showed no change as a function of temperature. This suggests the wax crystalline arrangement is unlikely to change over short periods of time following exposure to temperatures up to 60 °C. Considering permeation experiments will be conducted at temperatures up to 50 °C, and crystalline arrangement is through to affect permeation, identifying that no change occurred upon exposure to this elevated temperature is beneficial. This suggests the wax crystalline structure that acts as a barrier to penetration will not change over the period of the experiment.

Contact angle experiments were performed to assess the effect of composition and film thickness, which visually increases surface roughness, on the spreading of water and surfactants solutions. Changing both composition and the film thickness resulted in significant changes to contact angle. However, while composition significantly changed contact angles it is suggested this was driven by the implied change in topography, not the change in chemical composition. This is because no significant differences were seen in water contact angles on samples formed in conformal contact with the underlying flat substrate, and thus presenting minimal topography. This suggests contact angle and liquid pinning was driven by topography. This would explain why increasing the alcohol content in a film containing bulk alkane increased

the contact angle, as such compositional changes have been shown to increase surface roughness⁴⁷.

Finally, Raman spectroscopy was used to investigate the composition of a subsection of waxes. It was shown that the 1-D: SA films showed clear phase separation of the components waxes regardless of w/w % composition. In particular 25:75 % w/w 1-D: SA wax showed the preferential distribution of stearic acid at the print track interfaces. This phase separation is thought to be driven by the different freezing points of the wax components, resulting in distribution of the freezing 1-docosanol to the edges of the droplets. Subsequently, the localised 1-docosanol might induce local purification of the wax and long-range ordering, resulting in the observed extensive crystalloid structures at these locations.

4.5. References

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4.6. Appendix

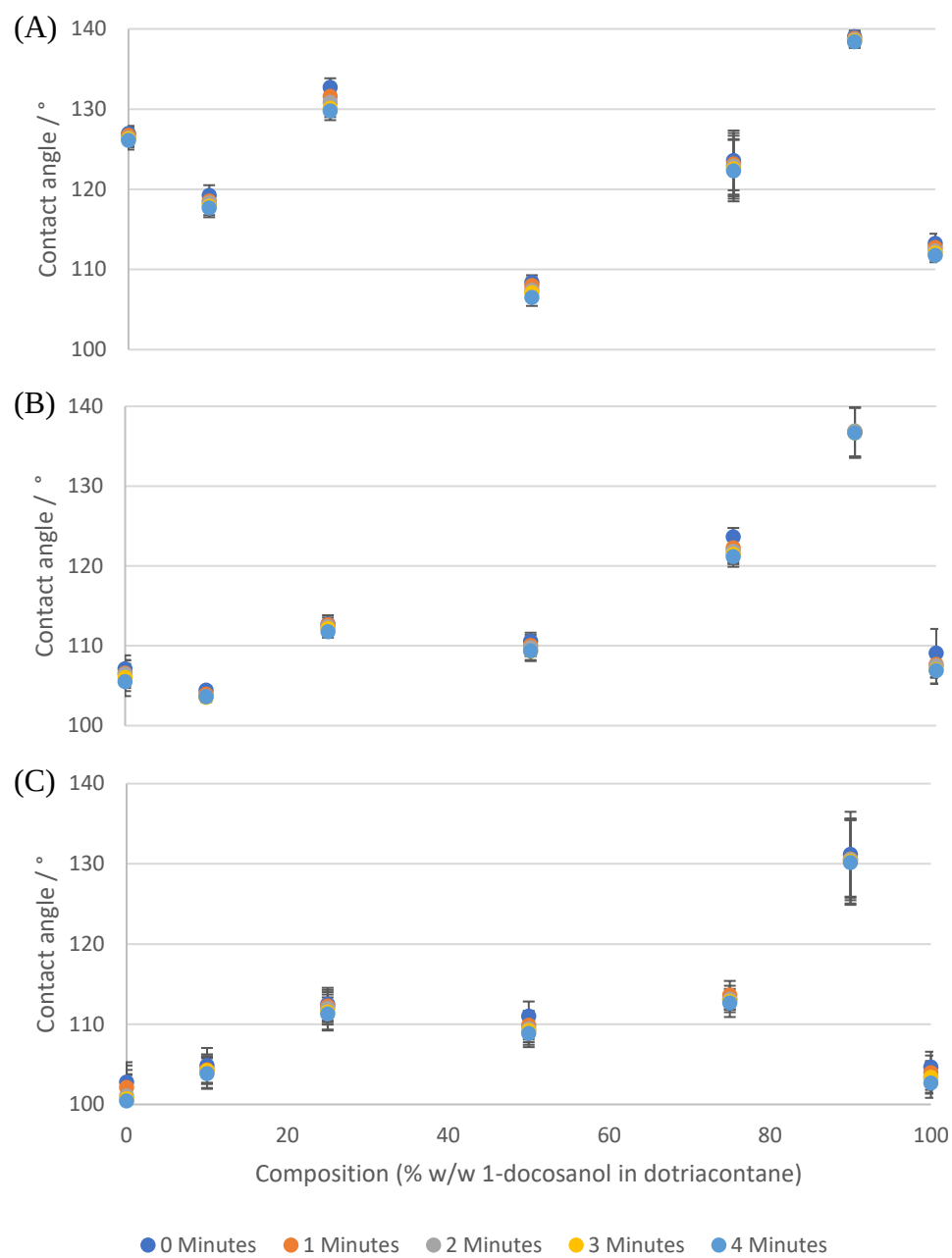


Figure 4-35 Contact angle of water over 4 minutes on (A) 5 layer, (B) 10 layer and (C) 20 layer wax films of DOT: 1-D.

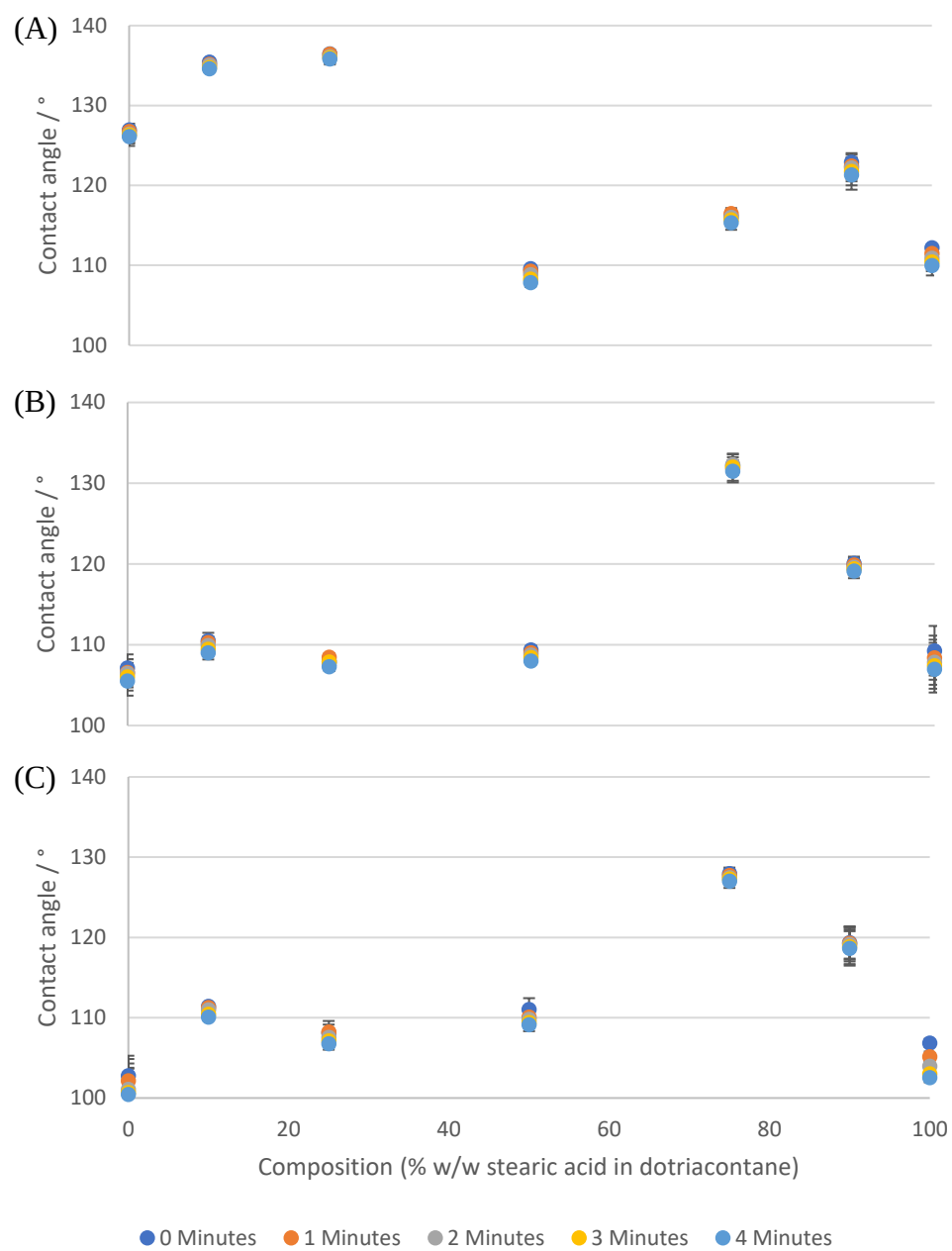


Figure 4-36 Contact angle of water over 4 minutes on (A) 5 layer, (B) 10 layer and (C) 20 layer wax films of DOT: SA.

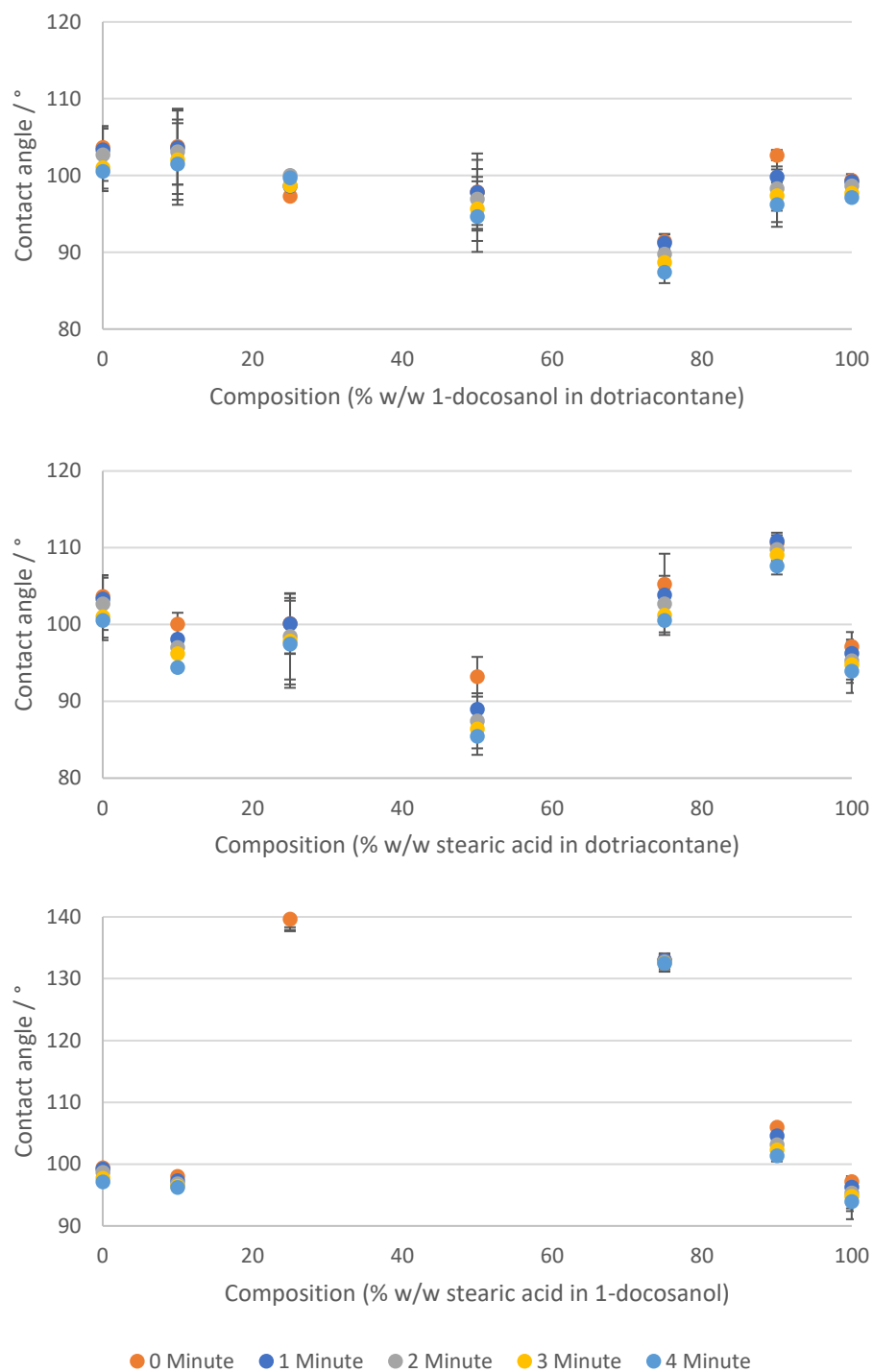


Figure 4-37 Contact angle of water over 4 minutes on the bottom surface of (A) DOT: 1-D, (B) DOT: SA and (C) 1-D: SA.

5. Chapter 5 – Interaction of *Z. tritici* fungi spores and applied azoxystrobin formulation on printed wax films

5.1. Introduction

The function and properties of the plant-environment interface are critical to ensure plant survival in changing environments¹. Primarily, this interface regulates the transport properties between leaf and atmosphere, permitting transpiration² while limiting ingress³. Furthermore, this interface controls and limits interaction with pathogenic microorganisms such as Epiphytic bacteria⁴ and fungal spores⁵. The composition of the cuticle waxes controls the permeation of agrochemicals and fungal attachment. Studies partially or entirely extracting cuticle wax show significant changes regarding these interactions. Typically, wax extraction increases agrochemical permeance^{6,7} compared to cuticles retaining wax, especially if the analyte is lipophilic⁶ and reduces fungal attachment⁸.

Extensive effort has been made to understand the critical processes associated with agrochemical penetration, including sorption⁹, diffusion in the cuticle¹⁰ and desorption¹¹. Furthermore, attempts have been made to identify trends relating permeance to the physical properties of the cuticle¹² and agrochemical¹³. Predominantly these studies investigate the impact of changing species¹¹ and thus modification of the entire wax composition and structure, or extraction methodology, on the individual processes involved with permeance¹¹. Changing species and thus wax composition varies permeation and agrochemical mobility within the cuticle by up to 3 orders of magnitude¹⁴. Understanding composition dictates permeance studies have concluded that modification of singular waxes can modify cuticle function. Some examples include long-chain aliphatic waxes controlling permeation instead of the cyclic triterpenoids waxes¹⁵. Additionally, using mutant enzymes to remove wax components affected wax biosynthesis, generating voids within the cuticle, subsequently increasing permeation¹⁶. Unfortunately, beyond these papers, limited experimentation has evaluated selectively removing waxes on permeance¹⁷. While attempts have been made, conclusive studies evaluating permeation, concerning cuticle wax composition, within a larger group of cuticles have not been performed¹⁸. Instead, research has focused on transpiration, not agrochemical penetration^{19–22}. Hence a structure-function relationship of cuticle waxes concerning permeance has not been developed, and little is understood about the functionality of specific waxes.

It is thought changes in composition change wax crystallinity^{18,23}, and critically the structure and tortuosity of the amorphous regions within the cuticle¹⁴. However, the link between crystallinity and tortuosity is poorly understood. Lipophilic actives²⁴ and water²⁵ must traverse these tortuous, amorphous pathways, which are thought to be nearly chemically identical regardless of species tested, with the only variable being path length^{26,27}. If data regarding the specific function of individual waxes concerning permeance can be collected, it might be possible to model permeance based on cuticle wax composition and understand the impact of composition on tortuosity. The required data to develop this understanding would require measuring the permeance of different agrochemicals through films of waxes presenting known and simple wax compositions. Here, 3D printing wax films could be used to produce the required wax films for permeance testing, offering an attractive avenue to explore this topic.

Beyond evaluating agrochemical penetration, similar films could be used to investigate the contribution of specific waxes to resisting fungal attachment. Many fungal spores attach to cuticles based on the presence of specific receptors²⁸. However, fungal spores also identify kairomones²⁹, signals allowing pathogens to recognise host plant surfaces based on wax composition³⁰.

Generally, it is known that specific types of waxes induce germination and growth of fungal spores^{31–34}, but less is understood about the impact of composition on attachment³⁵. Additionally, much of this work involves simultaneous modification of many waxes inducing physical and chemical changes³⁶, making it difficult to understand the effect of individual waxes.

In addition to wax composition, properties including hydrophobicity^{35,37} and topography^{38–41} impact fungal attachment. For example, the presence of uniform topographical features facilitates hyphae growth⁴². Similarly, hydrophobic fungal spores cannot readily adhere to hydrophilic surfaces^{36,37}. Unfortunately, only limited work has been performed purposefully modifying these properties and assessing the impact on fungal attachment. Furthermore, it is unknown if fungal attachment changes are induced by compositional or associated physical properties changes, i.e. topography and hydrophobicity³⁶.

Given this, the question arises: to what extent can a wax film of controlled composition be used to derive information regarding specific waxes' contribution to agrochemical penetration and fungal attachment? What questions can be asked and answered experimentally with such films?

For example, which waxes, when used alone or in combination with other waxes, enhance permeation? How do induced topographical changes on chemically similar surfaces modify fungal attachment? Are specific crystalline patterns, as per XRD, predictive of permeation?

Prior, these questions could only be answered when considering the entire wax composition using either isolated cuticles or reconstituted waxes. As such, the contribution of specific waxes was challenging to identify. This work aims to assess the nature of surfactant interactions with waxes over time to assess suitability for prolonged exposure. Subsequently, the morphological impact of surface exposure will be evaluated and understood as a function of composition, time and surfactant concentration. Additionally, azoxystrobin sorption and permeance through various waxes will be identified. Finally, the degree of fungal spore attachment to waxes of varied compositions and topography will be measured. To attempts to understand these processes, physical properties prior identified will be correlated against produced permeation and fungal attachment results to identify possible trends warranting further investigation.

5.2. Methods

5.2.1. Materials

Wax materials used were powder dotriacontane (97 %), 1-docosanol (98 %) and stearic acid. (98.5%) (Sigma Aldrich, Dorset, UK). These waxes when used with no further mixing were referred to as single component waxes. Printed films of the three single component waxes: dotriacontane, 1-docosanol and stearic acid and binary waxes: DOT: 1-D, DOT: SA and 1-D: SA were printed as described in Section 4.2.3. DOT: 1-D, DOT: SA and 1-D: SA were printed at % w/w ratios of 10: 90 % w/w, 25:75 % w/w, 50: 50 % w/w, 75: 25 % w/w and 90: 10 % w/w. These waxes were used to print wax films, Section 4.3.2.

Azoxystrobin, ≤ 100 % PESTANAL standard, was purchased from Sigma Aldrich (Dorset, UK). Soybean lecithin (90%) was purchased from VWR international (Leicestershire, UK). Analytical grade acetonitrile and a Hypersil Gold 3UM 150 mm x 2.2 mm column were purchased from Thermo Fisher Scientific (Hemel Hempstead, UK). Milli-Q water was collected from a PURELAB® Ultra reservoir (ELGA, Leicestershire, UK).

Synperonic™ A20 (A20) and Synperonic™ A7 (A7) are alcohol ethoxylate surfactants both with between 13 and 15 carbons in the carbon chain and with 20 and 7 ethylene oxide units, respectively. A20 and A7 was kindly provided by Croda Crop care (Goole, UK). Quadris™, is a highly concentrated commercial agrochemical suspension of azoxystrobin containing 250 g/L. The azoxystrobin is held in an aqueous solution containing 10-20 % of C16 - C18alcohol ethoxylate with no information publicly provided regarding the number of ethylene oxide units. Before use Quadris™ must be diluted by a factor of between 100 and 200. Quadris™ was kindly provided by Syngenta (Jealott's Hill, UK).

5.2.2. Preparation of stock and working solutions

Stock solutions were prepared by dissolving azoxystrobin in three solutions: 2.5 g/L solutions of A20, 50: 50 Milli-Q water: acetonitrile and Milli-Q water at concentrations of 10 mg/L, 5 mg/L and 5 mg/L, respectively. Solutions were vortex mixed, stirred overnight at 600 rpm, covered in aluminium foil, and stored at -20 °C.

Working solutions for sorption studies were prepared by dissolving azoxystrobin in 2.5 g/L A20 stock solutions to produce solutions containing 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0.078 mg/L of azoxystrobin.

Franz cell acceptor solution was prepared by distributing 100 μL of Quadris suspension in 20 ml of Milli-Q water and stirring for 1 hour at 600 rpm to produce a 1.25 g/L azoxystrobin suspension. A20 acceptor solutions were produced by adding 2.5 g/L of A20 to the prepared 1.25 g/L suspension of Quadris before stirring the suspension for 1 hour at 600 rpm.

Receiver solutions were produced by dispersing lecithin in Milli-Q water to produce a 10 g/L suspension before storing the suspension at $-20\text{ }^{\circ}\text{C}$. Lecithin suspension before use would be thawed, sonicated for 20 minutes and then stirred for 1 hour at 1000 rpm to ensure the lecithin was evenly distributed.

5.2.3. SEM

Scanning Electron Microscope imaging of wax films was conducted using a Hitachi TM3030 tabletop SEM (Japan). For additional information, see Section 3.2.9.

SEM was conducted to image surface morphology changes induced by exposing wax films to surfactants solutions for up to 1 week.

5.2.4. Sorption Studies

Film samples of dotriacontane, 1-docosanol, stearic acid and associated 25: 75, 50: 50 and 75: 25 % w/w binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid were freshly produced. See Section 4.2.3 for further details. Approximately 10 mg -15 mg of wax films were weighed into separate 3.5 mL vials sealed with PTFE lined septum caps. The film weight in milligrams was recorded to 2 dp. 3 mL of 5 mg/L, 2.5 mg/L, 1.25 mg/L, 0.63 mg/L, 0.31 mg/L, 0.16 mg/L and 0.078 mg/L azoxystrobin in 2.5 g/L A20 solutions was injected into vials containing waxes. 5 mM CaCl_2 and 200 mg/l NaN_3 was added as a biocide. A blank and control containing 5 mg/L, 2.5 mg/L, 1.25 mg/L, 0.63 mg/L, 0.31 mg/L, 0.16 mg/L and 0.078 mg/L azoxystrobin in 2.5 g/L A20 solutions with no wax powder present was also produced. The blank was prepared to evaluate azoxystrobin loss over the testing period due to photo-oxidation, hydrolysis and sorption into the glass vial and PTFE lid. The control was stored at $-20\text{ }^{\circ}\text{C}$.

To minimise photo-oxidation, vials were covered in aluminium foil and left agitated on a rocking table for 1 week at $25\text{ }^{\circ}\text{C}$.

After five days, 2 ml was removed from each vial, vortexed for 10 seconds and filtered through a 45 μ m filter to remove wax material before HPLC analysis. Blanks would be used to background subtract the effect of photo-oxidation, hydrolysis and sorption into the glass vial and PTFE lid.

Sorption studies were conducted to assess azoxystrobin's movement into the 12 tested waxes, dotriacontane, 1-docosanol, stearic acid and the 25: 75 % w/w, 50: 50 % w/w, and 75: 25 % w/w binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid over five days. Sorption into the cuticle wax is the first process agrochemical actives penetrating the cuticle undergo and was used to determine the partition coefficient between the wax and solution for the sorbing active.

5.2.5. Franz cell penetration studies

Custom Franz cells were produced at the University of Nottingham. Franz cells were made with a 1 ml volume donor chamber and a 1.5 mL volume receiver chamber. The receiver chamber volume was smaller than standard to increase the concentration of penetrating azoxystrobin in the receiver chamber, increasing intensity measured by HPLC. The internal diameter of the receiver/donor chambers was 15 mm in diameter. The donor/acceptor chamber flange joint interface was flame polished to create a smooth surface that was less prone to damaging the brittle wax films than traditionally ground joints. Figure 5-1 shows a representation of the Franz cell used.

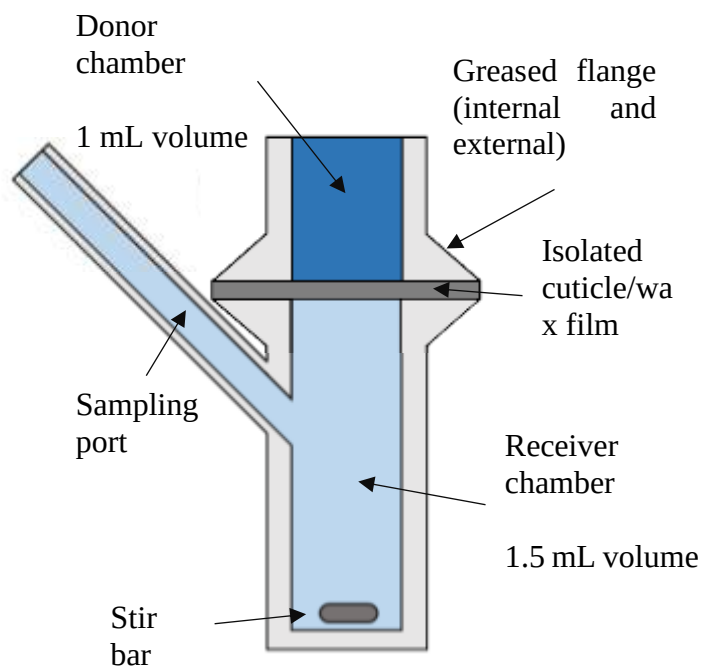


Figure 5-1 - Representation of the Franz cell assembly.

Wax films used for penetration analysis were printed, Section 4.3.2. Before use, all wax films were rinsed with distilled water and dried carefully with a fibre-free cloth. At this point several steps were taken in specific order to ensure the solutions used would not leak, to minimise the incidences of the wax film cracking during preparation and to ensure the correct solution was used in the correct chamber:

1. The flange interface between donor and receiver chambers was coated with a thin layer of paraffin grease and compressed together, in Figure 5-1 this is where "Isolated cuticle/wax film" would later be placed. Excess paraffin grease pushed into the internal tube of the Franz cells was removed. Grease was used as the wax films are inelastic. When the Franz cell was clamped together, without grease, a watertight seal would not be formed. Similar studies using skin or other elastic membranes do not require grease as the membrane will form a seal.
2. The Franz cell was unclamped, and a stir bar was placed in the receiver chamber. Subsequently, a five-layer wax film was placed on the receiver chamber greased flange. The wax film should be placed with the surface to be tested facing away from the

receiver chamber. In this thesis that was always the final printed layer shown in the prior SEM images, Section 4.3.2.2.

3. The receiver and donor chamber were carefully clamped to avoid cracking the brittle wax film. Care should be taken to ensure this is only performed once as repeating this process frequently led to the wax film cracking. A layer of paraffin grease was applied to the outer rim of the flange joint to prevent leaking.
4. 1.5 mL of 10 g/L lecithin suspension was added to the receiver chamber. This solution constituted the receiver solution, into which, the agrochemical would move down its concentration gradient through the wax film from the highly concentrated donor solution in the donor chamber.
5. The Franz cell was inverted for 3 hours to check wax film integrity. If liquid entered the donor chamber, or leaked in any other way, it was assumed the wax film was cracked and the sample was discarded. Transepithelial electrical resistance methods, common to skin studies^{43,44}, were not used as these methods have not been validated for use with these novel wax films.
6. Franz cells that passed the integrity check were placed in a water bath at 25 °C, 40 °C or 45 °C on a submersible stirring plate for 1 hour to equilibrate.
7. 1 mL of Quadris 1.25 g/L suspension or Quadris 1.25 g/L in 2.5 g/L A20 was pipetted into the donor chamber and thus exposed to the printed surface. The acceptor chamber was then sealed using double-sided tape, to minimise any accidental spillage and to minimise evaporation. The water bath was sealed and covered with aluminium foil to minimise photo-oxidisation of azoxystrobin. Because a large concentration of azoxystrobin was applied to the surface of the printed film that could not evaporate, the dose was effectively unlimited, making the Franz cell an unlimited dose system.

At 24 h, 30 h, 48 h, 54 h, 72 h, 78 h, 96 h, 102 h and 162 h, 100 µL of the lecithin suspension was removed from the receiver chamber and placed in a 200 µL HPLC vial insert stored at -20 °C. Franz cells were agitated by repeatedly inverting the Franz cell at each sampling point and after every 12 hours. A rocking table was not used as sustained rocking motion caused damage to the wax films.

Franz cell transport studies were conducted to evaluate the effect of wax membrane composition, temperature, and surfactant presence on azoxystrobin permeation.

5.2.6. HPLC sample preparation

Lecithin samples extracted from the Franz cell were thawed. Lecithin frequently formed sediment during thawing; thus, samples were vortexed for 30 seconds before being sonicated for 30 minutes to redistribute lecithin and azoxystrobin. Because lecithin could damage the HPLC column, the lecithin needed to be precipitated. 60 μ L of lecithin suspension was added to 60 μ L of acetonitrile in a 1.5 ml Eppendorf. The Eppendorf was vortex mixed for 30 seconds and sonicated for 30 minutes to precipitate lecithin and solubilise azoxystrobin in acetonitrile, solubility = 340 g/L. The Eppendorf was centrifuged using a Centrifuge 5425 R (Eppendorf) at 5000 rpm for 10 min at 0 °C, and the supernatant was carefully pipetted into a 200 μ L HPLC vial insert. Due to the low volumes, collected samples could not be filtered, necessitating lecithin precipitation using a centrifuge.

5.2.7. HPLC analysis

Separation of azoxystrobin was conducted on a U3000 HPLC system (Thermo Scientific, USA) equipped with degasser, column thermostat, autosampler, quaternary pump, and UV detector. System control and data acquisition were performed using Chromeleon™ software. The HPLC chromatographic conditions details are as follows: column C18 (150 mm x 2.1 mm) ACE3/ACE-HPLC Hypersil Gold, Thermo Scientific, 3 μ m. The mobile phase composition for analysis of sorption and receptor fluid samples consisted of Milli-Q water: acetonitrile (50: 50). HPLC was operated at a column temperature of 40 °C, injection volume of 40 μ L, a flow rate of 0.2 mL/min and UV detection at λ max = 230 nm.

5.2.8. Fungal spores attachment studies

Fungal attachment analysis was measured using a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) metabolic activity assay. *Zymoseptoria tritici* (strain K4418) was grown on Potato dextrose agar (PDA) for seven days by Catheryn Davies. Spores were harvested using 80% neonate. 40 μ m cell strainers were used to filter hyphae, followed by spinning spores down at 4000 rpm, enabling collection and resuspension in Potato dextrose broth (PDB). A dilution of 2.5×10^6 spore/mL was produced following the counting of the resuspended spore solution using a haemocytometer. Three to five repeats of printed wax circles, coupons, were printed with a diameter of 6.5 mm and were moved into the wells of a 96-well plate using a set of tweezers. Wax coupons were placed in the well with the upper printed surface exposed to any liquid added to the well. Coupons were

printed approximately 0.1 mm smaller than the diameter of the well itself to allow the coupon to slide into the well without cracking. Because the effect of printed topography and chemical composition on fungal attachment was to be tested, any samples placed with the bottom printed surface of the coupon in contact with the liquid was discarded. Additionally, any samples that broke upon insertion to the well were discarded.

100 µl of the spore solution was transferred to each testing well for 5 hours at room temperature enabling initial attachment. Non-adherent spores were removed by three gentle washes of phosphate-buffered saline (PBS) before 100 µl of PDB was added to each test well. The solution was left for 24 hours allowing adherent spores to germinate for 24 hours. Subsequently, wells were rewashed three times with PBS. An XTT reaction was initiated, wherein XTT salt is metabolised within attached fungal spores by menadione to form an orange coloured formazan salt of known colour⁴⁵. To achieve this, XTT and menadione, concentrations of 400 µg/ml and 25 µM respectively, were mixed to a final volume of 200 µl and added to each well. After 6 hours, 100 µl from each well was transferred to a fresh 96-well plate, and absorbance was measured at 490 nm using an EL800 microplate spectrophotometer, Biotek, USA. Fungal attachment is expressed for each wax as metabolic activity, as measured by the intensity of the coloured solution. The intensity of colour signifies the amount of XTT metabolism that occurred and thus the degree of fungal attachment. Non-adherent fungal spores will have been removed prior by washing, and thus, the reaction generating the coloured solution will not occur. Results were background-subtracted by removing the absorbance value for a well possessing a wax coupon but no inoculated fungal spores. Results were displayed as a percentage relative to a control value (100%) wherein inoculated fungal material was exposed to an untreated well plate.

Fungal spore attachment studies were conducted to evaluate if the wax composition and hydrophobicity *Z. tritici* is grown upon can generate variability in attachment to the surface.

5.3. Results and discussion

5.3.1. Sample degradation

To observe permeation, the agrochemical must likely be exposed to the printed wax film for at least one day⁴⁶. Such active solutions often contain surfactants used to accelerate permeance, which solubilises components of wax surfaces¹⁰. Surfactants induce wax solubilisation and plasticisation through accumulation within amorphous wax, with minimal effect on the

crystalline domains^{27,47,48}. Surfactant exposure is thus likely to modify the printed wax surface morphology and possibly the wax film integrity⁴⁹. Unfortunately, the morphology of waxes exposed to surfactants is poorly reported, and it is thus unclear what the morphological impact of surfactant exposure is. Subsequently, it is unclear if surfactant exposure is likely to cause film failure.

As such, it is critical to assess the morphology of the wax films following surfactant exposure before beginning these prolonged transport studies to guide surfactant selection. As seen in Section 4.3.3, surfactant drying on wax films induced morphological changes in a coffee ring pattern. Similar interactions may occur over a sufficient period without the solution drying. To achieve this, 5-layer printed wax films were exposed to Milli-Q water, 2.5 g/L and 10 g/L solutions of A20 and A7 for a week at 40 °C. SEM images were taken on days one and seven.

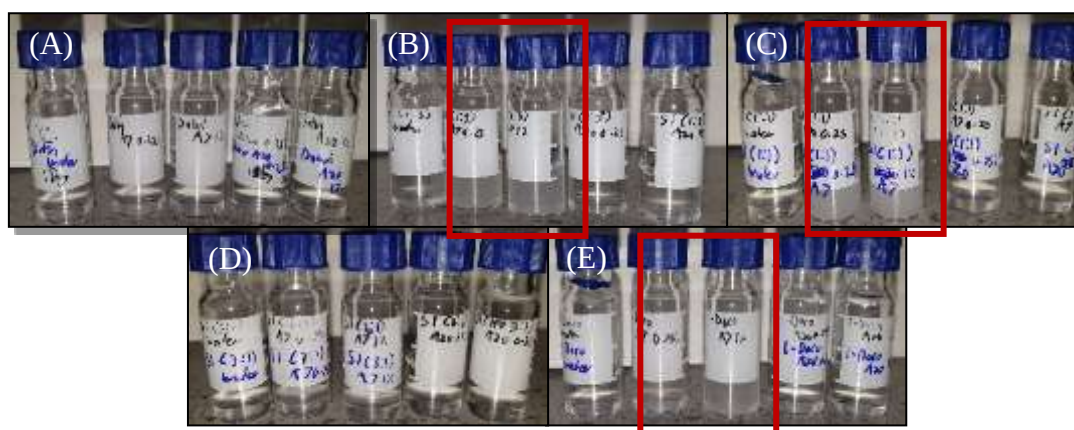


Figure 5-2 - Photographs of (A) dotriacontane, (B) DOT: 1-D 25: 75 % w/w, (C) DOT: 1-D 50: 50 % w/w, (D) DOT: 1-D 75: 25 % w/w and (E) 1-docosanol. Vials were filled with Milli-Q water, 2.5 g/L A7, 10 g/L A7, 2.5 g/L A20 and, 10 g/L A20, from left to right. Red boxes highlight solutions that became cloudy after one day.

Following one day of exposure, several samples had begun visibly to degrade, as the applied clear surfactants solution became cloudy (Figure 5-2). Cloudiness formed only with samples containing 1-docosanol when exposed to 2.5 g/L or 10 g/L A7 and resulted in samples physically breaking apart upon removal for imaging. This physical degradation of the film is likely the origin of the cloudy solutions. Over time, this effect progressed; many samples containing 1-docosanol could not be retrieved and imaged after seven days when exposed to A7. A qualitative visual inspection shows the degree solutions are cloudy does not correlate with 1-docosanol % w/w mass nor with % crystallinity, Section 4.3.5. For example, the DOT: 1-D 50: 50 % w/w presented an insignificant change in crystallinity compared with 1-docosanol but more visible cloudiness.

With neither A20 solutions nor water was any solution cloudy following seven days of exposure to wax films.

5.3.2. Wax films following water exposure

SEM imaging of wax films was conducted to identify the morphological changes induced by surfactants exposure. As a control following exposure to water, no sample showed observable signs of degradation. However, some morphological changes could be observed. These changes were apparent on samples containing 1-docosanol and or dotriacontane (Figure 5-3 A-F). Samples containing 1-docosanol and or dotriacontane became smoother, the print tracks became less prominent, and there was a decreased density of platelet structures on the 1-docosanol films relative to untreated films. Stearic acid films presented a greater density of pitted structures. Considering these waxes are hydrophobic, Section 4.2.7, the observed morphological changes are likely mainly due to exposure to high temperatures, 45 °C. However, it is possible that sorption and swelling have also occurred due to exposure to water⁵⁰. Morphological changes progressed as a function of time. Stearic acid, for example, showed greater pitting structures following seven days than after one day. Critically films showed no visible defects that could modify penetration, such as cracks between print tracks. This indicates that exposure to water at temperatures elevated to 45 °C is unlikely to cause film failure over one week.

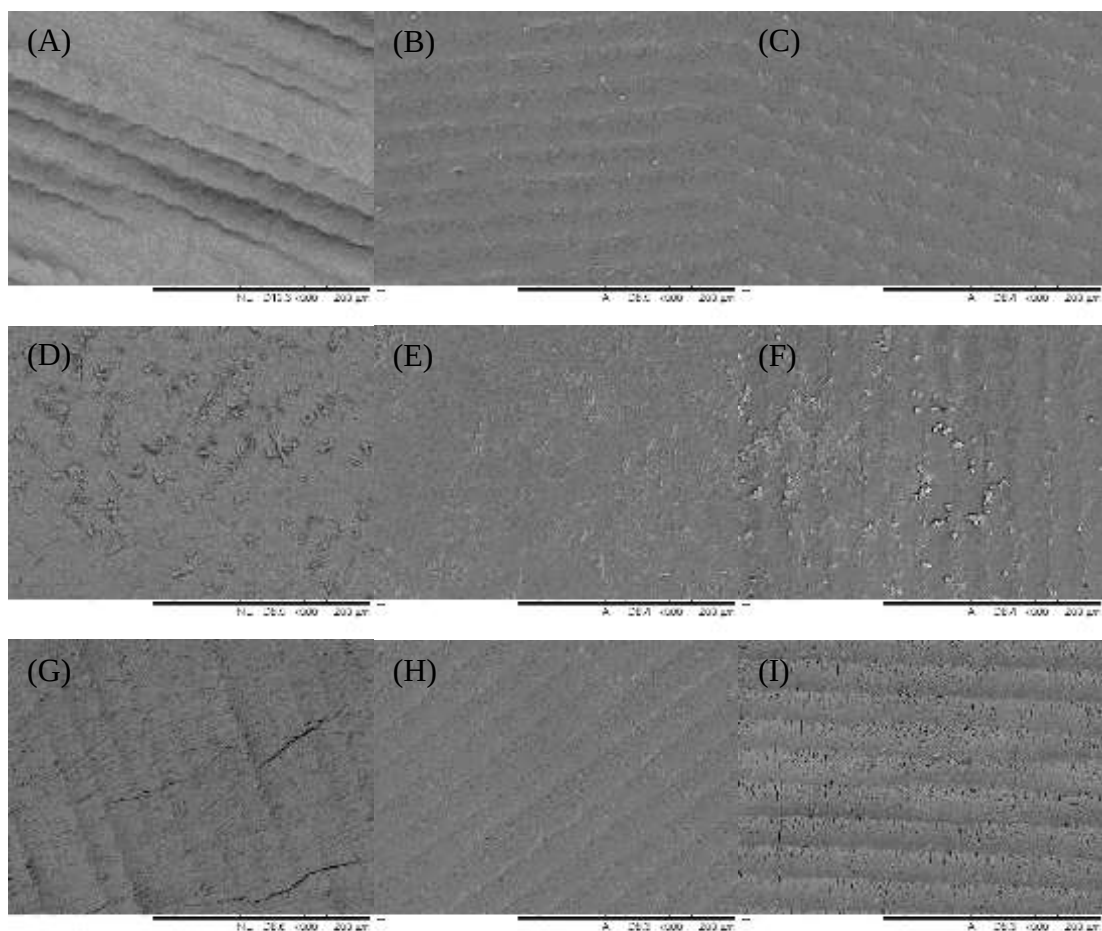


Figure 5-3 - (A) untreated dotriacontane, (B) dotriacontane exposed to water for one day, (C) dotriacontane exposed to water for seven days, (D) untreated 1-docosanol, (E) 1-docosanol exposed to water for one day, (F) 1-docosanol exposed to water for seven days, (G) untreated stearic acid, (H) stearic acid exposed to water for one day and (I) stearic acid exposed to water for seven days.

Considering morphological changes induced upon binary waxes, the effect of the water was visibly more extensive than the changes induced on single component wax films (Figure 5-3). Binary waxes of DOT: 1-D (Figure 5-4) showed minimal degradation in their surface morphology; however, the surface did become visibly smoother and presented a greater amount of crystal structures in the print track interface. The density of crystalline structures increased over time. This is possibly caused by removing amorphous wax at these locations that have been previously shown to show preferentially distributed waxes, Section 4.3.6.

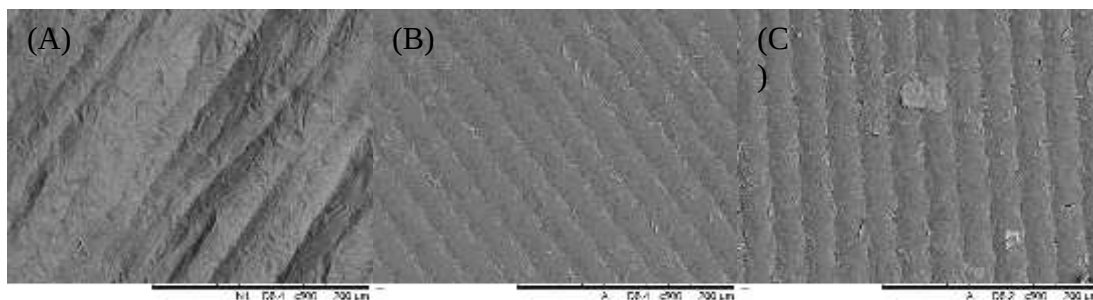


Figure 5-4 - (A) untreated DOT: 1-D 75: 25 % w/w, (B) DOT: 1-D 75: 25 % w/w exposed to water for one day and (C) DOT: 1-D 75: 25 % w/w exposed to water for seven days.

Considering binary waxes of DOT: SA (Figure 5-5), the seven-day sample was not suitable for imaging due to extensive degradation of the printed film, making retrieval unfeasible and it is thus not shown. However, it was clear that the sample's morphology had undergone extensive change over one day. Print track interfaces were no longer readily visible, and the surface showed a heterogeneous covering of platelet structures. The structures observed in Figure 5-5 are similar to those in Figure 5-4, suggesting they result from dotriacontane.

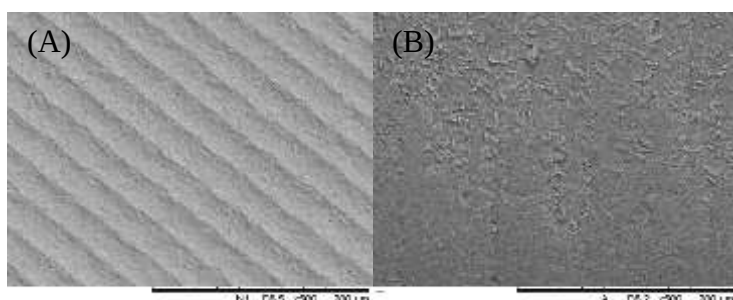


Figure 5-5 - (A) untreated DOT: SA 50: 50 % w/w and (B) DOT: SA 50: 50 w/w exposed to water for one day. The seven day sample was contaminated and thus not shown.

The 1-D: SA binary wax (Figure 5-6) showed minimal visible degradation of the surface nor the revealing of crystal structures after one day. However, crust crystal structures coated the surface homogenously after seven days, and the print track interfaces could no longer be observed. The crystalloids observed do not match those seen in 1-docosanol or stearic acid, nor are they present in the film prior to water exposure, Section 4.3.2.3.

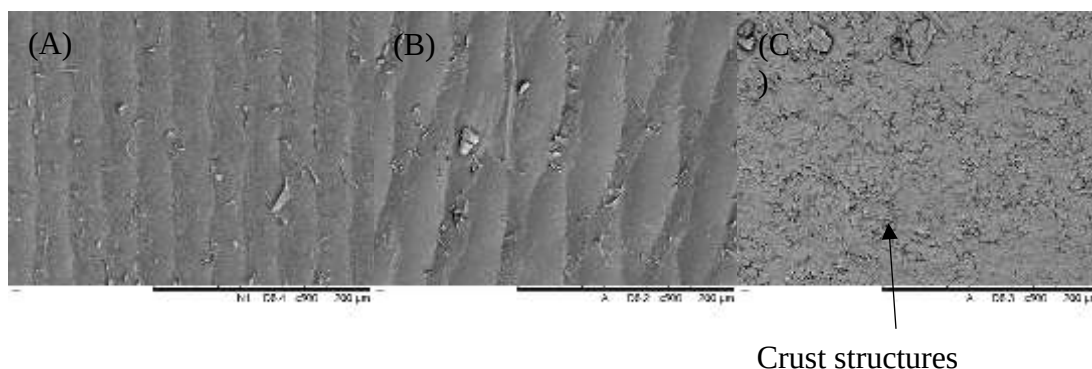


Figure 5-6 - (A) untreated 1-D: SA 75: 25 % w/w, (B) 1-D: SA 75: 25 % w/w exposed to water for one day and (C) 1-D: SA 75: 25 % w/w exposed to water for seven days.

Overall, exposure to 40 °C water did not cause significant surface degradation on single component waxes. However, binary waxes showed the formation of crystalloids following water exposure. This impact progressed as a function of time.

In many cases, the crystalloids found on binary waxes samples are not seen in their component waxes (Figure 5-3). While it is difficult to understand why this occurs, it suggests that binary waxes do not necessarily simply show a summation of physical properties comparable with component waxes. Instead, binary waxes present new properties not seen in the component dotriacontane, 1-docosanol or stearic acid.

5.3.3. Wax films following A20 exposure

Samples exposed to 2.5 and 10 g/L A20 solutions showed substantial surface morphology modifications (Figure 5-7, Figure 5-8, Figure 5-9, Figure 5-10, Figure 5-11 and Figure 5-12) within one day that progressed after seven days.

Considering single component waxes, the changes to stearic acid (Figure 5-7) are the most striking. Extensive degradation of the original morphology after seven days and pitting can be seen. The effect of surfactant concentration is also evident. Exposure to 2.5 g/L A20 resulted in a surface free of striated structures, frequently seen in stearic acid films, and a surface approximating a 'cracked fissure' cuticle structure. These images suggest that the surfactant has interacted with the wax as voids are present. Similar structures were not seen following water exposure suggesting solubilisation of wax occurred with the surfactant. Stearic acid exposed to 10 g/l showed greater solubilisation. Visually, extensive amounts of material have been liberated, leaving pitting. These pitted structures are seen homogenously; no localisation of

interaction is seen, for example, at the print tracks interface. While it cannot be confirmed, it is likely that the remaining structures are predominantly the crystalline domains, whereas the material liberated was amorphous stearic acid. This conclusion is based on observation of surfactants only acting on amorphous wax^{27,47,48} and, as seen in Section 4.3.4, stearic acid 5 layer films present 51.64 % crystallisation.

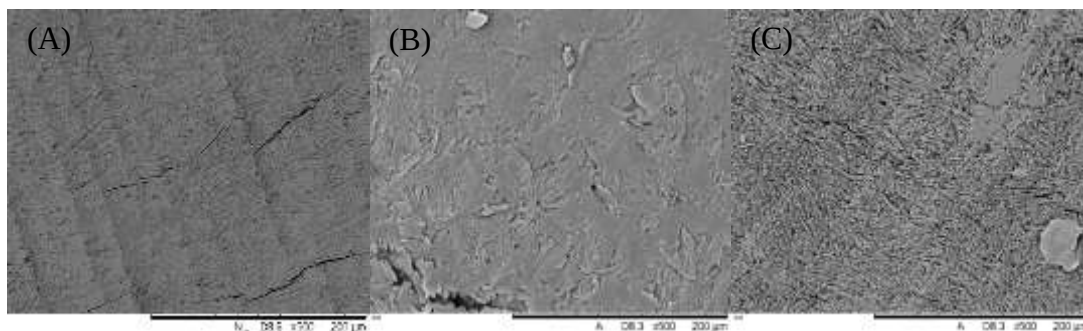


Figure 5-7 - (A) untreated stearic acid, (B) stearic acid exposed to 2.5 g/L A20 for seven days and (C) stearic acid exposed to 10g/L A20 for seven days.

1-docosanol and dotriacontane exposed to A20 show less extensive changes. Dotriacontane showed a visual reduction in print induced topography (Figure 5-8). 1-docosanol showed the removal of surface crystalloids (Figure 5-9). On both dotriacontane and 1-docosanol, A20 effects were localised to the print track interface. Dotriacontane presented plate structures, not dependent on exposure time, whereas 1-docosanol showed clearly defined print tracks and a time-dependent reduction in surface structures. After seven days, films exposed to either 2.5 g/L or 10 g/L showed no visible crystalloids. Print tracks are more defined, surface topography reduced, and surface crystals absent as the exposure increased suggest the wax has solubilised surface structures.

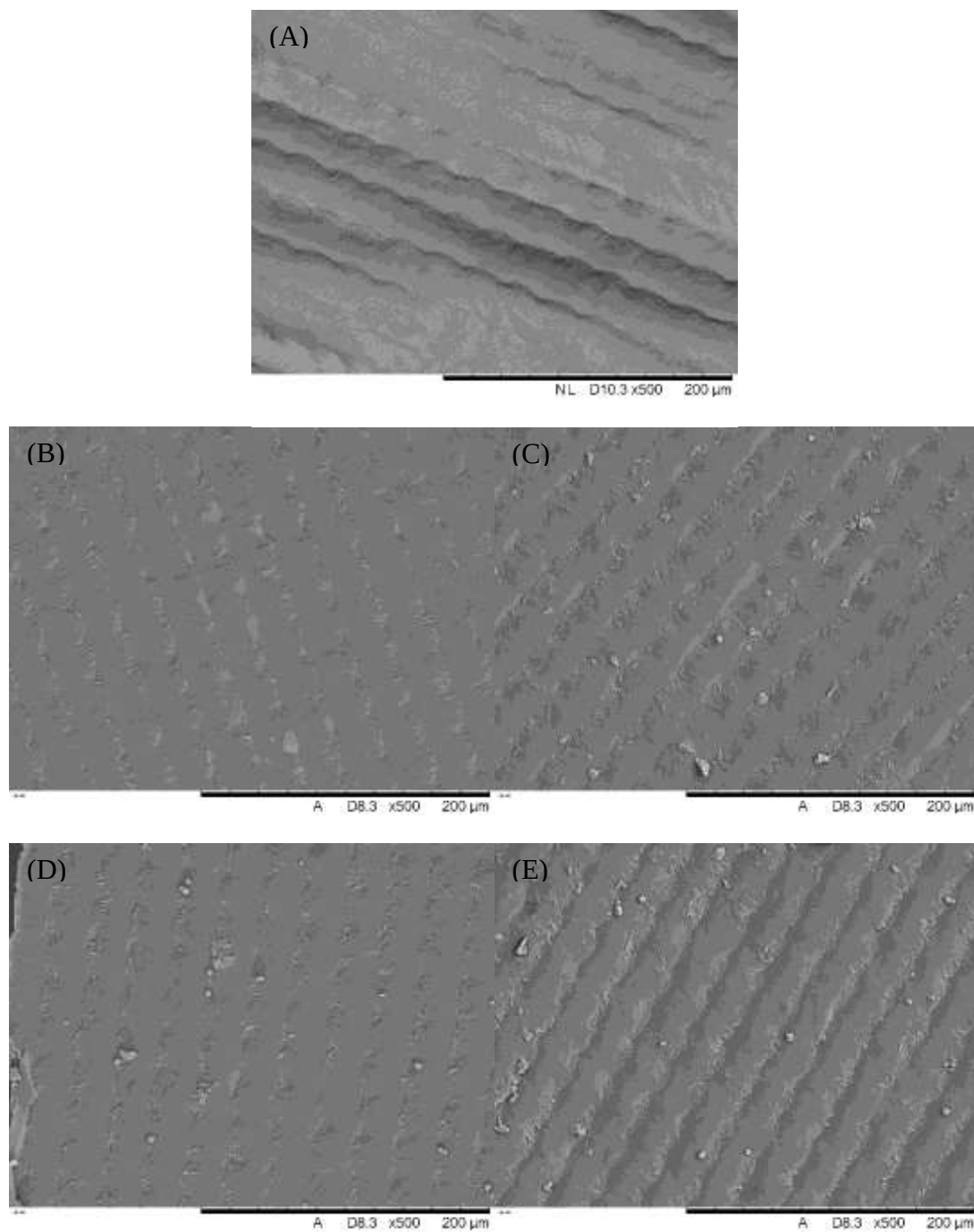


Figure 5-8 - (A) untreated dotriacontane, (B) dotriacontane exposed to 2.5 g/L A20 for one day, (C) dotriacontane exposed to 2.5 g/L A20 for seven days, (D) dotriacontane exposed to 10 g/L A20 for one day, (E) dotriacontane exposed to 10 g/L A20 for seven days.

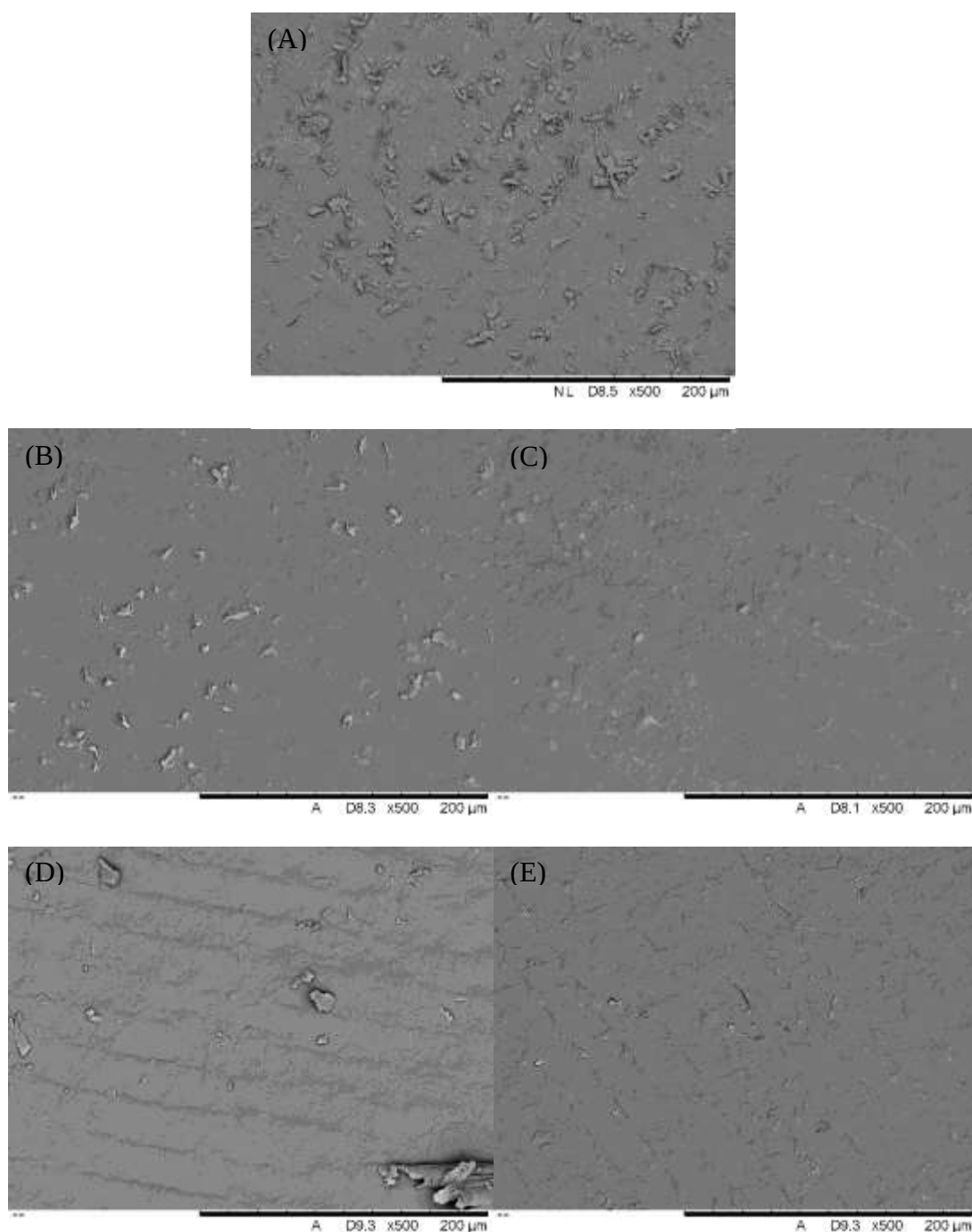


Figure 5-9 - (A) untreated 1-docosanol, (B) 1-docosanol exposed to 2.5 g/L A20 for one day, (C) 1-docosanol exposed to 2.5 g/L A20 for seven days, (D) 1-docosanol exposed to 10 g/L A20 for one day, (E) 1-docosanol exposed to 10g/L A20 for seven days.

Binary waxes exposed to A20 show morphology changes similar to the component waxes. DOT: 1-D binary waxes (Figure 5-10) showed plate crystalloid structures in the print track interface and reduced print-induced topography. Similar behaviour was seen following dotriacontane

exposure to A20 (Figure 5-10); however, this binary wax's morphology change appears to be more extensive.

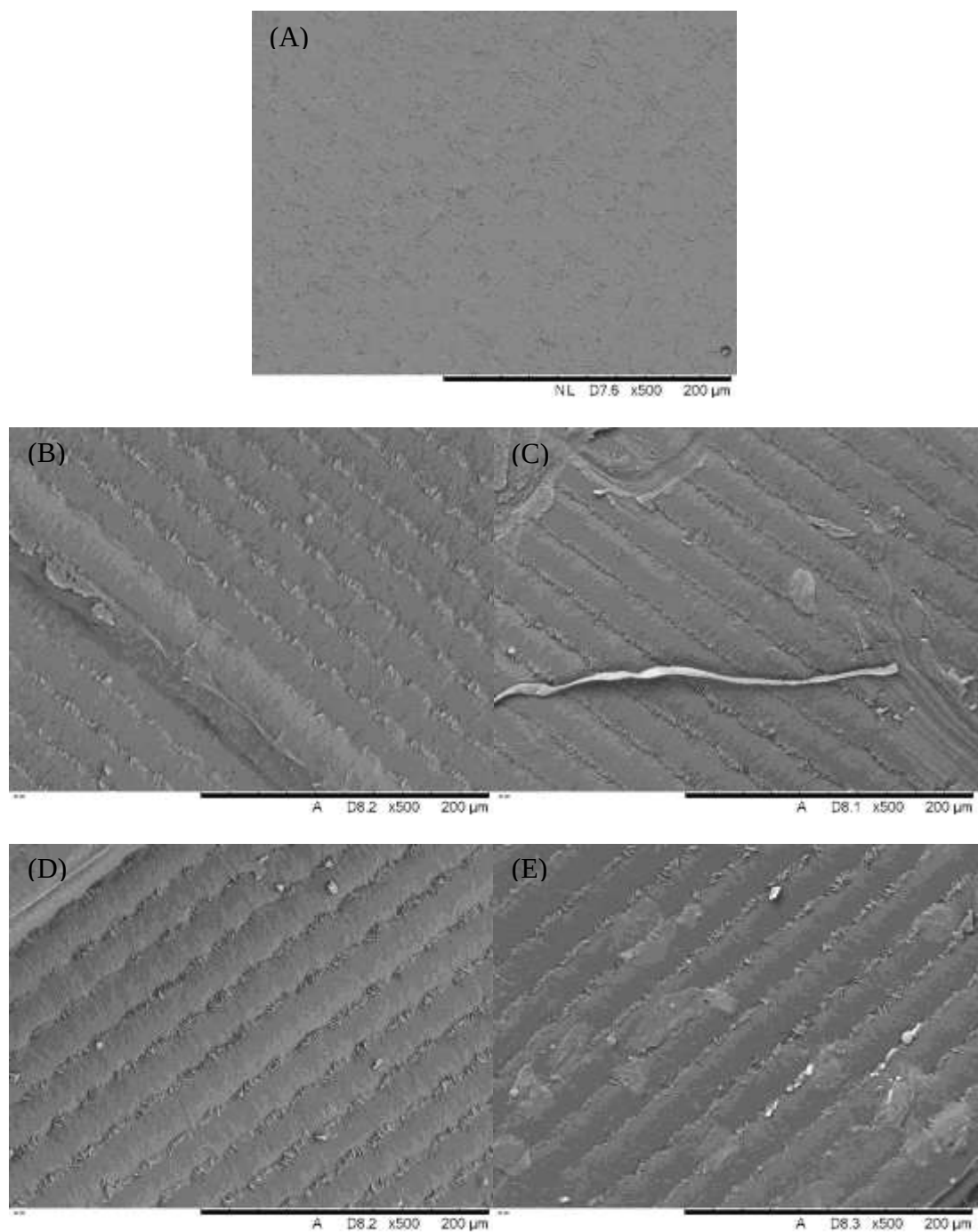


Figure 5-10 - (A) untreated DOT: 1-D 50: 50 % w/w, (B) DOT: 1-D 50: 50 % w/w exposed to 2.5 g/L A20 for one day, (C) DOT: 1-D 50: 50 % w/w exposed to 2.5 g/L A20 for seven days, (D) DOT: 1-D 50: 50 % w/w exposed to 10 g/L A20 for one day, (E) DOT: 1-D 50: 50 % w/w exposed to 10 g/L A20 for seven days.

DOT: SA films (Figure 5-11) showed extensive local degradation at the print track interface, particularly after one day, this is particularly shown in Figure 5-11D where it can be seen the crystalline structures observed cluster and form a heterogeneous distribution of structures. After seven days, the morphological changes are distributed uniformly over the wax film surface, Figure 5-11E compared with Figure 5-11D and Figure 5-11C compared with Figure 5-11B. This suggests the surfactant preferentially interacts with the print track interfaces initially, but over time is capable of interacting with the bulk wax film, similar to what was seen following surfactant exposure to stearic acid, Figure 5-7. Crystal structures seen do not reflect structures seen in stearic acid (Figure 5-7). It is thus postulated that mixing dotriacontane with stearic acid might have resulted in the excluded crystalline structures that restrict the permeation of lipophilic agrochemicals, Section 1.5.1 presenting a new structure. For this reason, it is possible that the structures seen at the print track interfaces of Figure 5-10 are the proposed novel crystalline structure formed when dotriacontane and 1-docosanol are mixed.

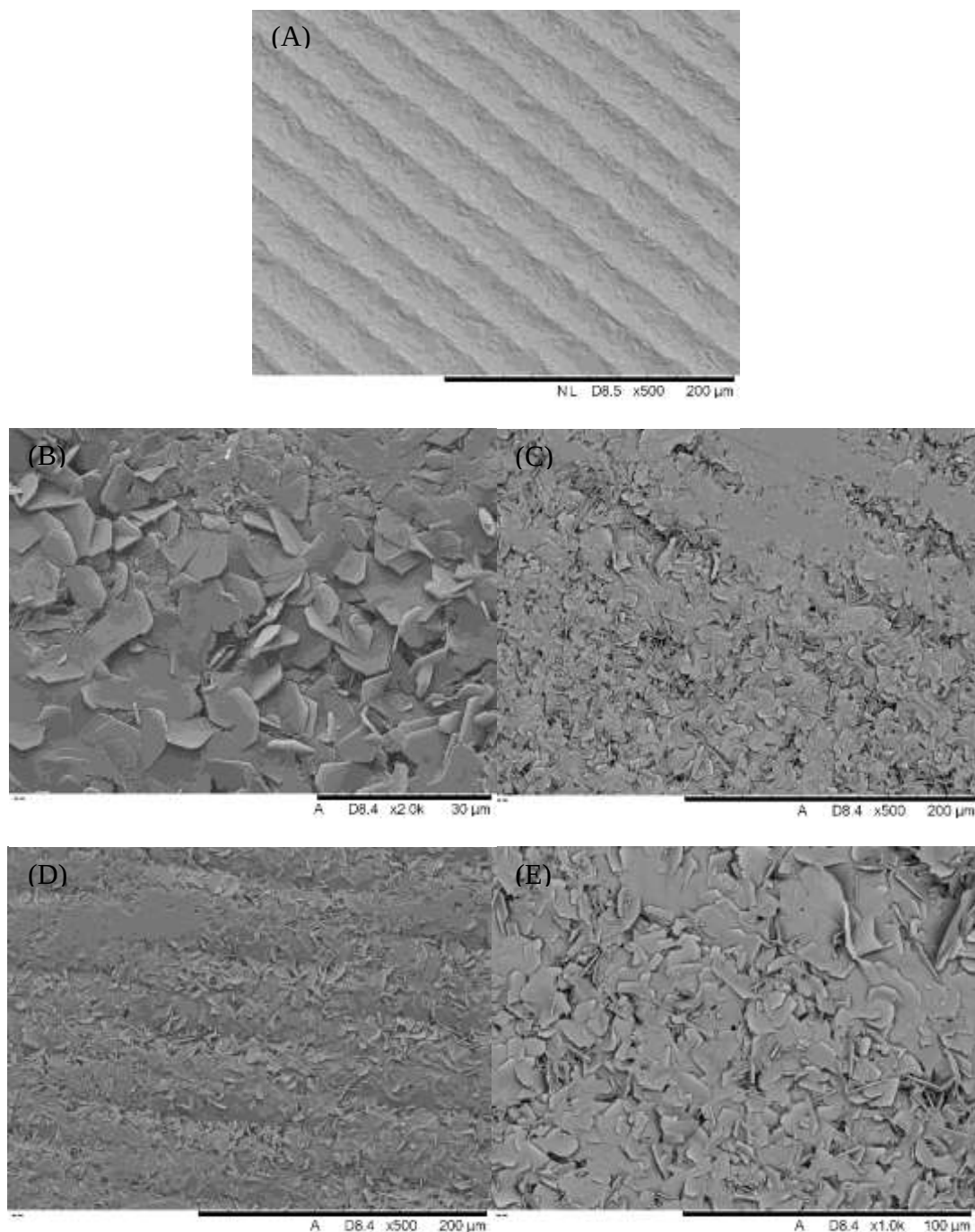


Figure 5-11 - (A) untreated DOT: SA 50: 50 % w/w, (B) DOT: SA 50: 50 % w/w exposed to 2.5 g/L A20 for one day, (C) DOT: SA 50: 50 % w/w exposed to 2.5 g/L A20 for seven days, (D) DOT: SA 50: 50 % w/w exposed to 10 g/L A20 for one day, (E) DOT: SA 50: 50 % w/w exposed to 10 g/L A20 for seven days.

1-D: SA (Figure 5-12) showed considerable development of crystal structures. The crystal structures relieved following surfactant exposure are predominantly found at the print track interface; however, a lower density of these structures can be found uniformly across the film.

The observed structures are very similar in size and shape to the 1-docosanol crystals seen at the interface of printed 1-docosanol droplets Section 4.3.2.2. This is unexpected as the film contains by weight 50 % stearic acid, and no features of the stearic acid film Section 4.3.2.3 are seen showing that the relationship between composition and morphology is highly complex and not simply linear as a function of composition. Interestingly, the use of either A20 2.5 g/L or ten g/L had a limited effect on the morphology changes to the wax film.

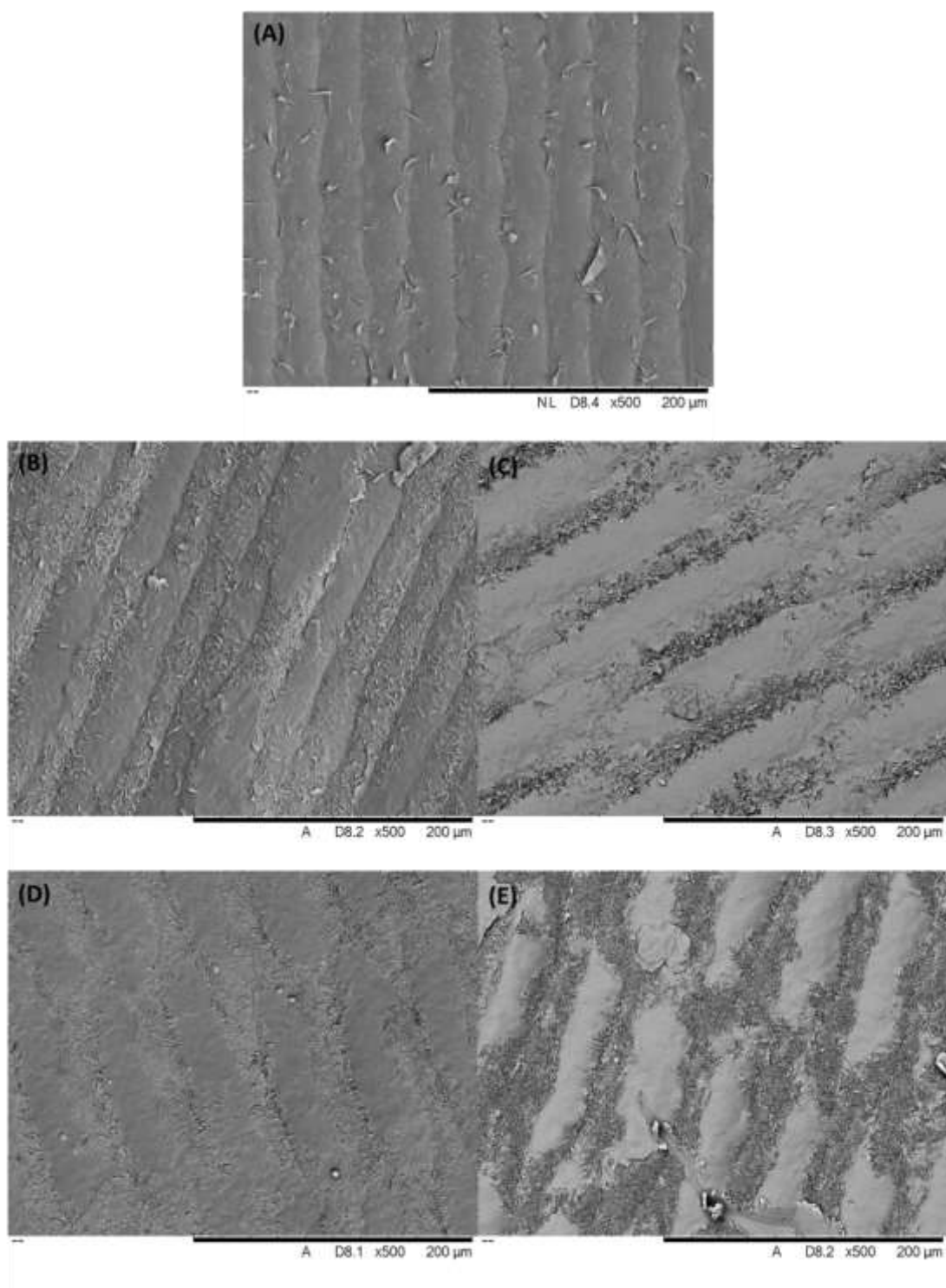


Figure 5-12 - (A) untreated 1-D: SA 25: 75 % w/w, (B) 1-D: SA 25: 75 % w/w exposed to 2.5 g/L A20 for one day, (C) 1-D: SA 25: 75 % w/w exposed to 2.5 g/L A20 for seven days, (D) 1-D: SA 25: 75 % w/w exposed to 10 g/L A20 for one day and (E) 1-D: SA 25: 75 % w/w exposed to 10 g/L A20 for seven days.

Generally, films exposed to A20 underwent morphological changes almost entirely localised at the print track interface with the pure stearic acid and DOT: SA 50: 50 % w/w film being exceptions. These morphological changes did not cause films to be visually structurally compromised. No cracks were observed in any films, and on only one film type, stearic acid exposed to A20 10 g/L for seven days showed voids that might directly permit permeation of the active solution. The similarity of the images in Figure 5-12 (C-E) and the 1-docosanol crystals found at the interface of droplets, Section 4.3.2.3, is of particular interest. It is proposed that the localisation of the surfactant effect on the film is a consequence of preferential solubilisation at this location, revealing the crystal structures already present at this interface.

5.3.4. Wax films following A7 exposure

Samples exposed to A7 solutions (Figure 5-13, Figure 5-14, Figure 5-15, Figure 5-16, Figure 5-17 and Figure 5-18) show localised morphological changes uniformly distributed across the film. Many films could not be retrieved from the surfactant solution as they were too delicate after seven days limiting the images taken. Dotriacontane films (Figure 5-13) showed extensive morphological changes and crystals localised at print tract interfaces after one day. After seven days, localised morphological changes were uniformly distributed across the film, and voids were present between print tracks following exposure to 10 g/L A7.

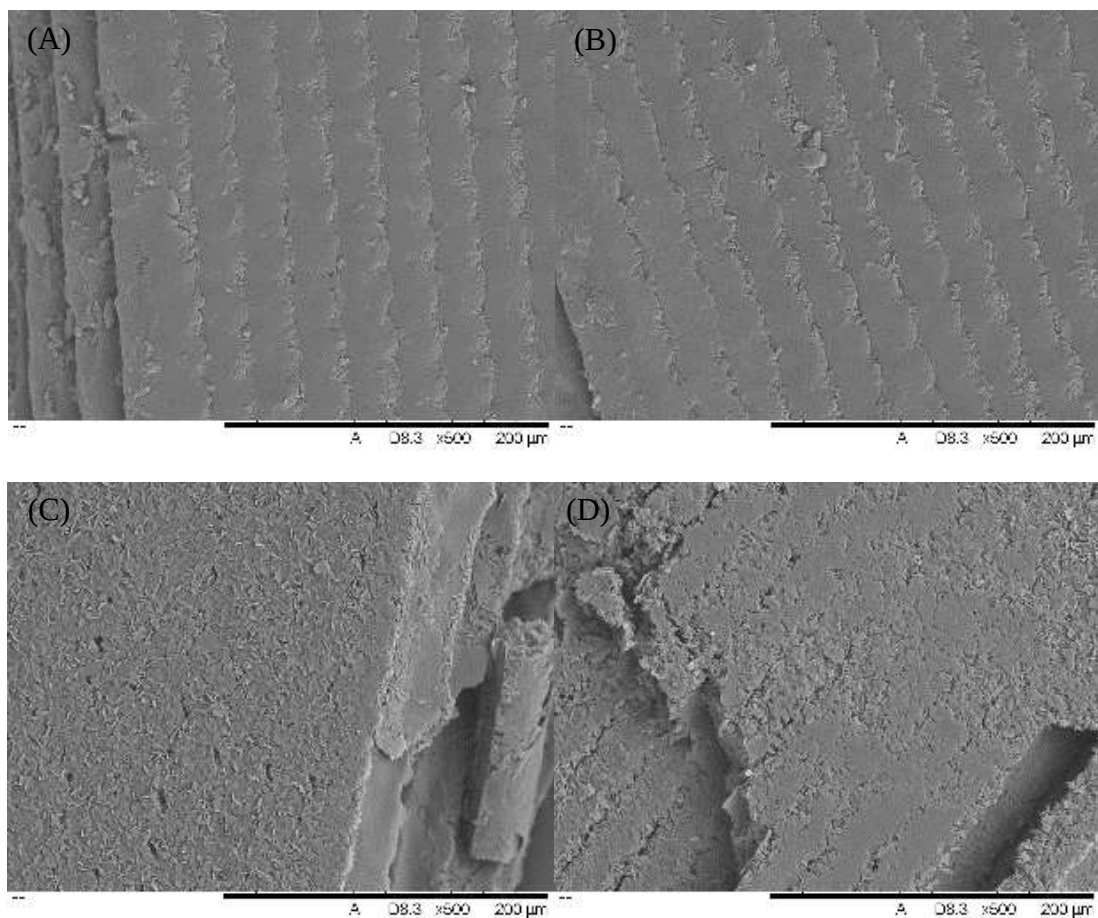


Figure 5-13 - (A) dotriacontane exposed to 2.5g/L A7 for one day, (B) dotriacontane exposed to 10 g/L A7 for one day, (C) dotriacontane exposed to 2.5g/L A7 for seven days, (D) dotriacontane exposed to 10 g/L A7 for seven days.

1-docosanol films after one day (Figure 5-14) showed regions, likely at the print track interface, where the wax had been removed by the A7 surfactant, leaving voids following 2.5 g/L A7 exposure. Following ten g/L A7 exposure, print tracks are misaligned, suggesting fragments of the film broke away from the bulk structure before these fragments adhered to the surface during removal from solution. After seven days (Figure 5-13 G-H), surfactant exposure caused the film to crumble following removal from the solution.

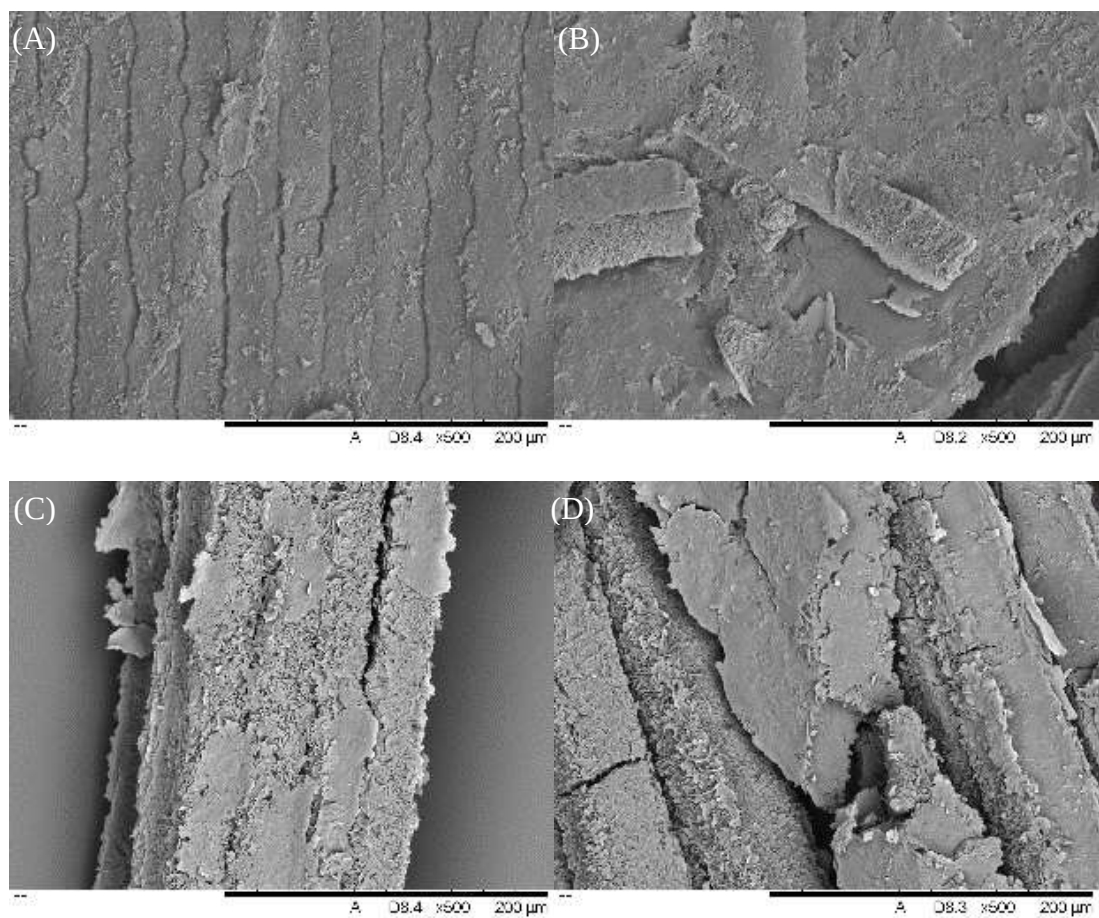


Figure 5-14 - (E) 1-docosanol exposed to 2.5g/L A7 for one day, (F) 1-docosanol exposed to 10 g/L A7 for one day, (G) 1-docosanol exposed to 2.5g/L A7 for seven days, (H) 1-docosanol exposed to 10 g/L A7 for seven days.

Stearic acid (Figure 5-15) presented a long plate-like crystal structure on the surface of the wax. The origin of these is unknown. The size and number of these structures is greater following ten g/l solution exposure. After seven days, typical stearic acid striations were visible. Solubilisation occurred heterogeneously like stearic acid exposure to A20; however, pitting was less extensive following A7 exposure relative to A20.

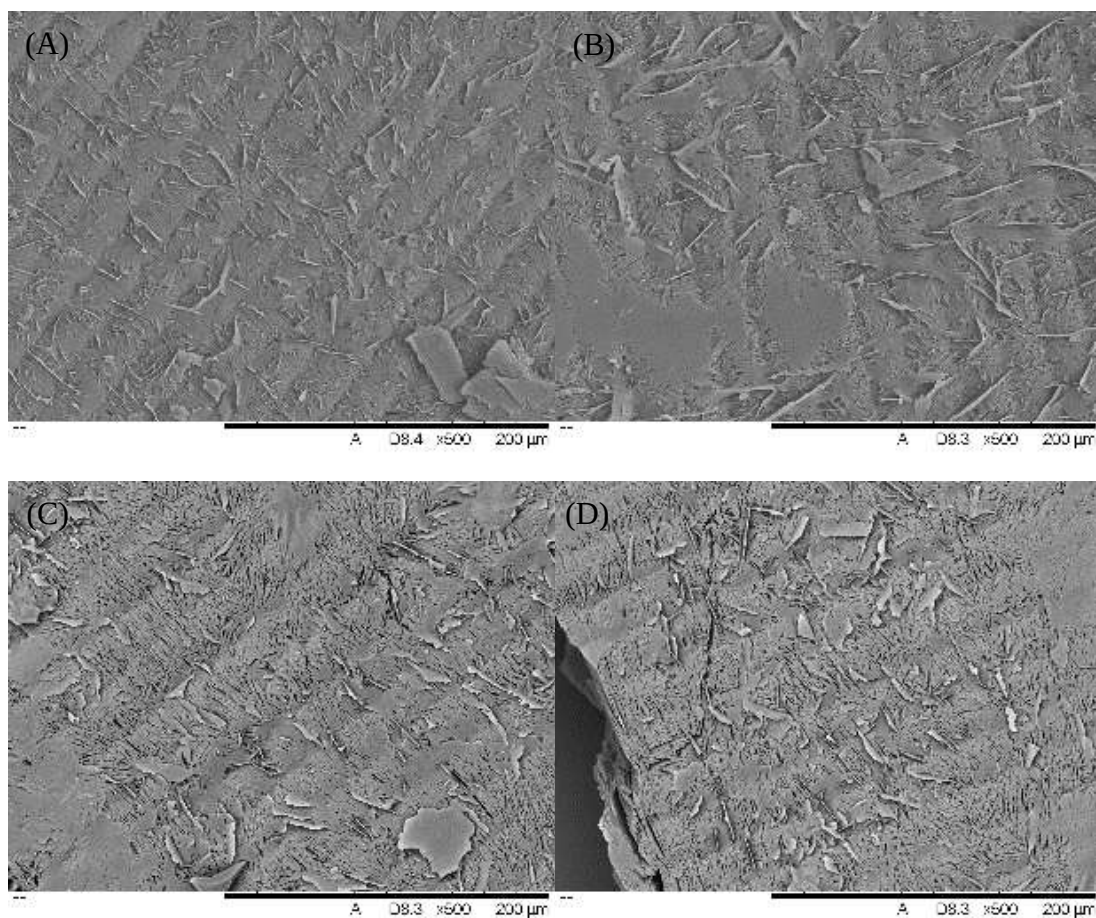


Figure 5-15 - (A) stearic acid exposed to 2.5g/L A7 for one day, (B) stearic acid exposed to 10 g/L A7 for one day, (C) stearic acid exposed to 2.5 g/L A7 for seven days, (D) stearic acid exposed to 10 g/L A7 for seven days.

Considering the binary waxes, all samples containing 1-docosanol were fragmented along print tracks and displayed considerable morphological modification after one day (Figure 5-16 and Figure 5-18). All samples containing 50 % w/w 1-docosanol or more, apart from pure 1-docosanol, were not possible to retrieve due to sample degradation.

Binary waxes of DOT: 1-D (Figure 5-16) show general degradation that progresses over time. After one-day print, track interfaces clearly show small voids, particularly following exposure to the 10 g/L A7 solutions. After seven days, the original print morphology was no longer visible, and exposure to 10 g/L A7 degraded the film almost entirely. Crystal structures are shown in Figure 5-16(D) correspond with crystals seen on 1-docosanol exposed to 10 g/L A7 (Figure 5-13 G-H).

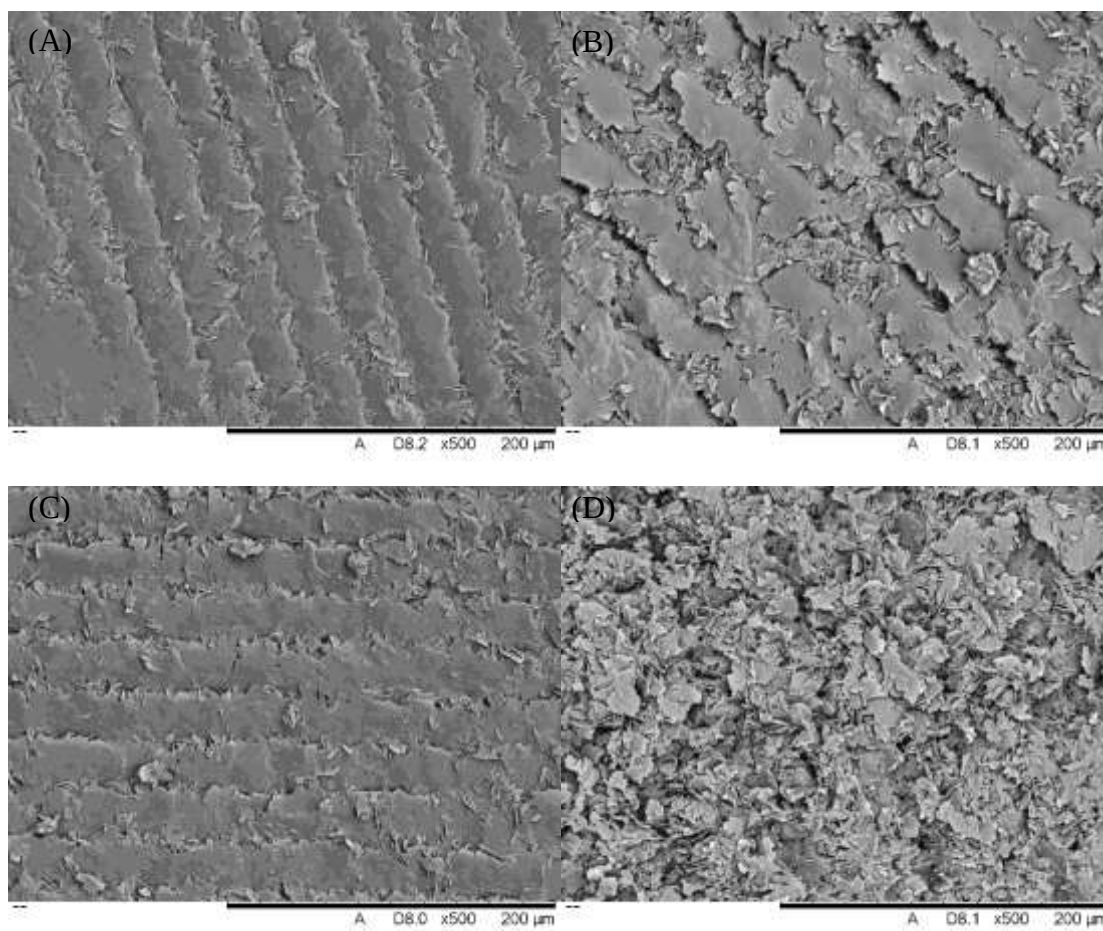


Figure 5-16 - (A) DOT: 1-D 75: 25 % w/w exposed to 2.5 g/L A7 for one day, (B) DOT: 1-D 75: 25 % w/w exposed to 2.5 g/L A7 for seven days, (C) DOT: 1-D 75: 25 % w/w exposed to 10 g/L A7 for one day and (D) DOT: 1-D 75: 25 % w/w exposed to 10 g/L A7 for seven days.

DOT: SA binary waxes exposed to A7 (Figure 5-17) show homogenous platelet structures. After one day, films exposed to 2.5 g/L A7 showed a lower density of crystals relative to the film exposed to 10 g/L A7, however, print tracks were not visible. Films exposed to 10 g/L showed a higher density of crystals; however, print tracks were still visible. After seven days, both films showed a high density of crystals. Print tracks are still tentatively visible on samples exposed to 10 g/L.

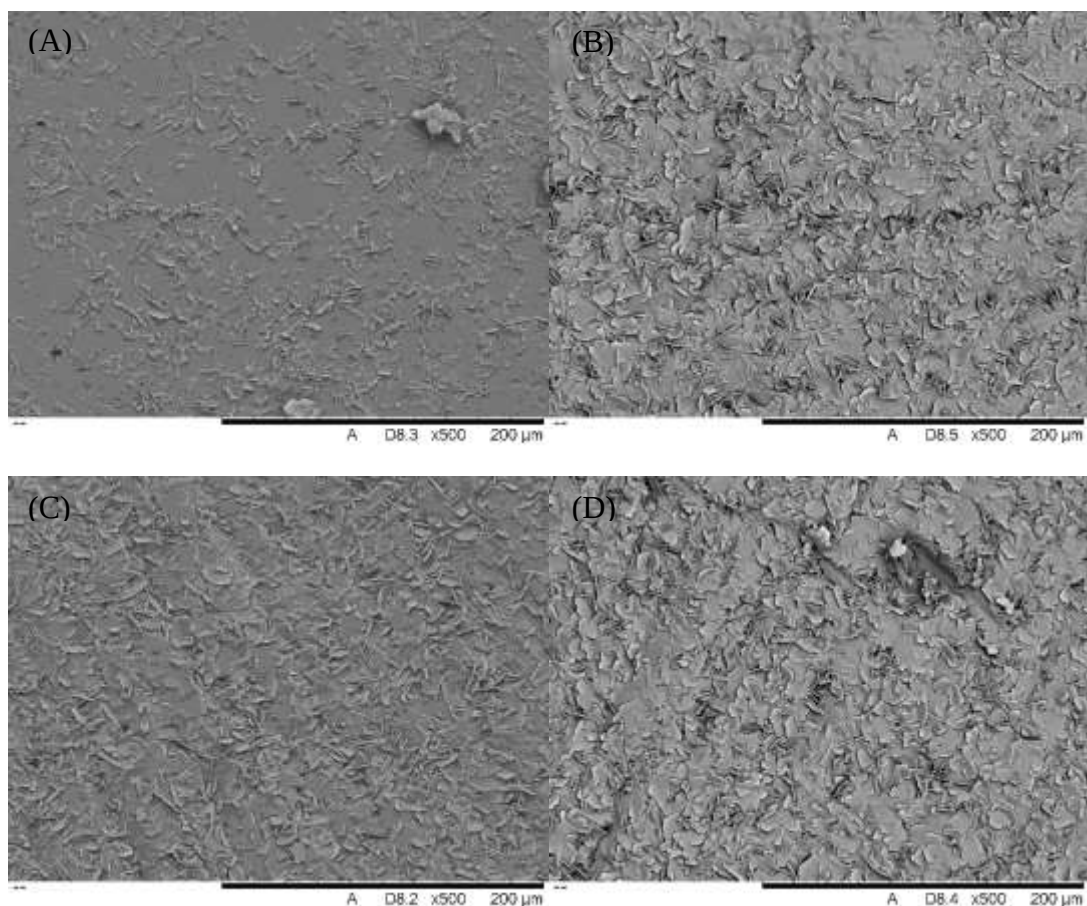


Figure 5-17 - (A) DOT: SA 25: 75 % w/w exposed to 2.5 g/L A7 for one day, (B) DOT: SA 25: 75 % w/w exposed to 2.5 g/L A7 for seven days, (C) DOT: SA 25: 75 % w/w exposed to 10 g/L A7 for one day and (D) DOT: SA 25: 75 % w/w exposed to 10 g/L A7 for seven days.

1-D: SA binary waxes (Figure 5-18) degraded rapidly after A7 exposure, regardless of surfactant concentration or time. Wax films retrieved broke apart, leaving only powder, particularly when exposed to 10 g/L A7. Films retrieved showed extensive uniform crystal coverage. Because of film fragmentation, identification of print tracks was not possible. Considering crystals were present uniformly across the surface, this suggests that the surfactant wax interactions were not limited to the print tracks. Such behaviour is common following exposure to A7, whereas only DOT: SA 50: 50 % w/w showed this behaviour following A20 exposure.

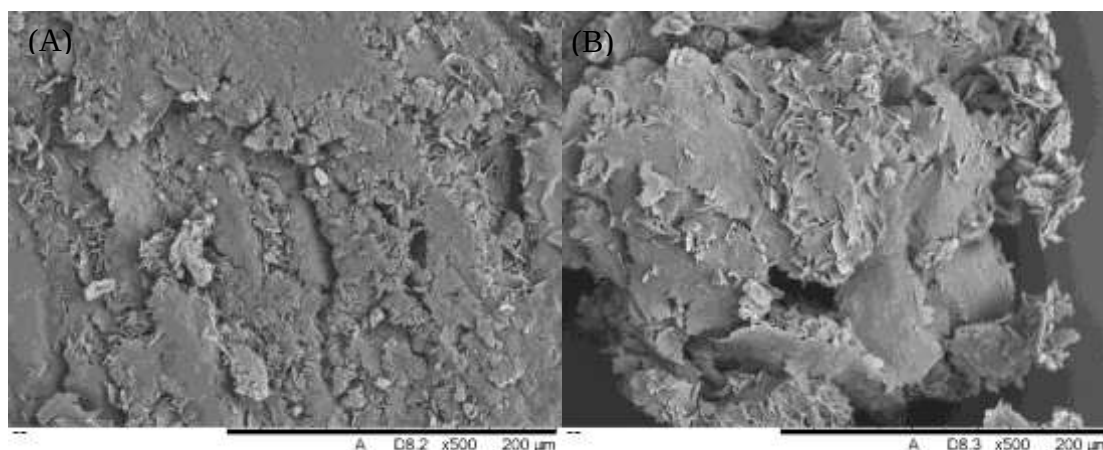


Figure 5-18 - (A) 1-D: SA 25: 75 % w/w exposed to 2.5 g/L A7 for one day and (B) 1-D: SA 25: 75 % w/w exposed to 10 g/L A7 for one day.

Considering this data, several trends can be observed. Following exposure to A20, surfaces presented a range of morphological changes, primarily determined by wax composition. With dotriacontane, 1-docosanol, and associated binary wax showing minimal changes such as the loss of crystalline structures and the generation of plate-like crystals at print track interfaces. A20 induced considerable changes to the surface topography of stearic acid films and stearic acid containing binary waxes with extensive pitting across the entire surface. Considering binary waxes, A20 caused deterioration of the film. This was localised mainly to the print track interfaces and was associated with the formation of crystals at these locations and hence likely loss of amorphous material.

Regarding A7 surfactants, it is challenging to conclude as many samples were degraded and images could not be taken. After seven days, the three single component wax films underwent homogenous morphological changes with no localisation of these interactions at the print track interface. Further, A7 surfactant exposure resulted in different morphological changes compared with A20. For example, following A20 exposure, stearic acid films (Figure 5-7C) showed extensive pitting. However, following A7 exposure, stearic acid films (Figure 5-15) presented long plate crystals and limited pitting leading to the generation of a striated surface. Considering binary waxes, all waxes show progressive uniform degradation as a function of time and concentration. Waxes containing 1-docosanol were degraded to a greater extent. Many wax films were found as a powder following 7 days of A7 exposure. Films of 1-D: SA 25:75 % w/w 1-docosanol following exposure to A7 (Figure 5-16) fragmented rapidly compared with component

waxes. It has been suggested that small quantities of secondary waxes result in extensive disorder and physical property changes induced by mixing waxes are not linear with composition¹⁸. As shown in Section 4.3.6, many waxes with 1-docosanol show preferential deposition of 1-docosanol. It is suggested that similar phase separation and localisation of waxes resulted in localised degradation at these locations and deterioration of the physical structure of the film.

It is thought that ethoxylate chain length determines the quantity of wax solubilisation⁵¹. Such behaviour can be seen; however, surfactants induced different morphological changes, varied by ethoxylate chain length. This suggests there is no simple linear trend relating ethoxylate chain length and solubilisation. If additional Synperonic surfactants were tested, a range of morphological changes would likely be observed determined by chain length.

In all cases, the concentration used was above the surfactant CMC⁵². Subsequently, any surface morphology changes induced by increasing surfactant concentration are not a consequence of exceeding the CMC and are a consequence of increasing the concentration. Many surfactant-cuticle/wax interactions follow a trend wherein, below the CMC, minimal interaction occurs as micelles do not form and thus cannot remove wax from the cuticle and above the CMC, the degree of interaction increases with concentration⁵². These SEM images suggest similar results are seen on printed films. As shown, the degree of surface modification increases as a function of surfactant concentration in all cases, suggesting greater removal of waxes as a function of increased surfactant concentration.

From this imaging, it was concluded that A7 surfactants were not suitable for further penetration testing. Many wax films would likely degrade during testing. As such, penetration would not be possible to attribute only to permeance through the tortuous amorphous domain. Instead, transport might also occur through surfactant induced voids. Only 2.5 g/L A20 showed no void formation in any imaged wax. Subsequently, only aqueous and 2.5 g/L A20 solutions would be used in sorption and penetration studies to remove the risk of samples degradation induced by surfactants.

5.3.5. Sorption studies

Sorption is when an analyte diffuses into a material, a sorbent, from a solution. This process is passive and measured to calculate the partition coefficient of an analyte between the solution

and the sorbent⁹. It is essential to understand which films an analyte, azoxystrobin, is capable of sorbing into to understand the penetration of an active across a wax film.

This work measures sorption and the partition coefficient in 12 waxes powders: dotriacontane, 1-docosanol, stearic acid, and binary waxes comprised of 25: 75 % w/w, 50: 50 % w/w and 75: 25 % w/w of two of dotriacontane, 1-docosanol or stearic acid. The wax was suspended in solutions containing a range azoxystrobin concentrations. If azoxystrobin was capable of sorbing into the material the concentration of azoxystrobin in solution would decrease as it sorbs into the wax. This change in concentration would then be measured, background-subtracted against a blank, and used to identify the amount of azoxystrobin sorbed into the wax. In the relevant literature, sorption is often measured using Liquid scintillation counting^{11,51,53-55}, but this method was not available.

Figure 5-19, Figure 5-20, Figure 5-21 and Figure 5-22 show the sorption isotherms of all 12 tested waxes at 25 °C in a solution of 2.5 g/L A20. Linear trends with reasonable R^2 values and positive gradients indicate analyte sorption into the wax film. The gradient reflects the rate of sorption with respect to the available analyte. Isotherms with shallow or no gradient indicate limited, or no sorption, such as Figure 5-19 C. Negative and anomalous points are likely associated with measurement errors, perhaps caused by poor wax filtration or fouling of the HPLC column. Sorption was tested at 25 °C instead of 40 °C or 45 °C; the temperature penetration was tested because sorption occurs more rapidly at lower temperatures^{56,57}, meaning differences in sorption would be more visible as a function of composition.

It was observed that only binary waxes containing stearic acid permitted sorption, 50: 50 % w/w DOT: 1-D wax being an exception. In each case, these waxes showed a linear relationship between analyte sorbed as a function of analyte concentration. Isotherms showing linear trends are considered “C” or constant partitioning type isotherms⁵⁸. These isotherms are observed when the availability of sorption sites remains constant, irrespective of analyte concentrations⁵⁹. Subsequently, as sorption occurs, more sorption sites must be generated. For this to occur, the sorbent must display microporous structures with variable crystallinity⁵⁹.

Additionally, the analyte must have a greater affinity for the sorbent than the solvent. This is required for the analyte to disrupt intermolecular interactions within the sorbent, resulting in additional sorption sites opening proportionally with the quantity of analyte sorbing⁵⁷.

The analyte, azoxystrobin, is a lipophilic compound and thus can be assumed to tend to partition into lipophilic materials such as wax. Many printed waxes showed a significant difference regarding % crystalline measurements, Section 4.2.5, with evidence of heterogeneous distribution of waxes across binary wax films, Section 4.3.6. This suggests that % crystallinity variability is a function of location and composition. It is reasonable to assume that these isotherms are C type and that the partition coefficient does not vary as a function of analyte concentration.

It should however be understood that the results shown below despite showing strong correlations, as indicated by the large R^2 values, also show large errors bars suggesting poor precision in the methodology. It might be a consequence of limited azoxystrobin sorption occurring per mg of wax powder used and the small mass of powder used. This would mean only a small amount of azoxystrobin would be removed from solution, resulting in difficulty accurately measuring the change in peak area, as identified by HPLC. Alternatively, it could be associated with the use of power wax compared with wax films or with the selection of analysis techniques, HPLC vs Liquid scintillation. It is possible the filtration methodology was not rigorous enough to completely filter out the wax, especially considering it was filtering wax from a surfactant solution above the surfactants CMC, resulting in fouling of the column.

Alternatively, it is possible that the use of a surfactant solution, rather than an aqueous solution, resulted in poor sorption of the azoxystrobin due to the azoxystrobin preferentially partitioning into the surfactants micelles, and thus be retained in the surfactant solution.

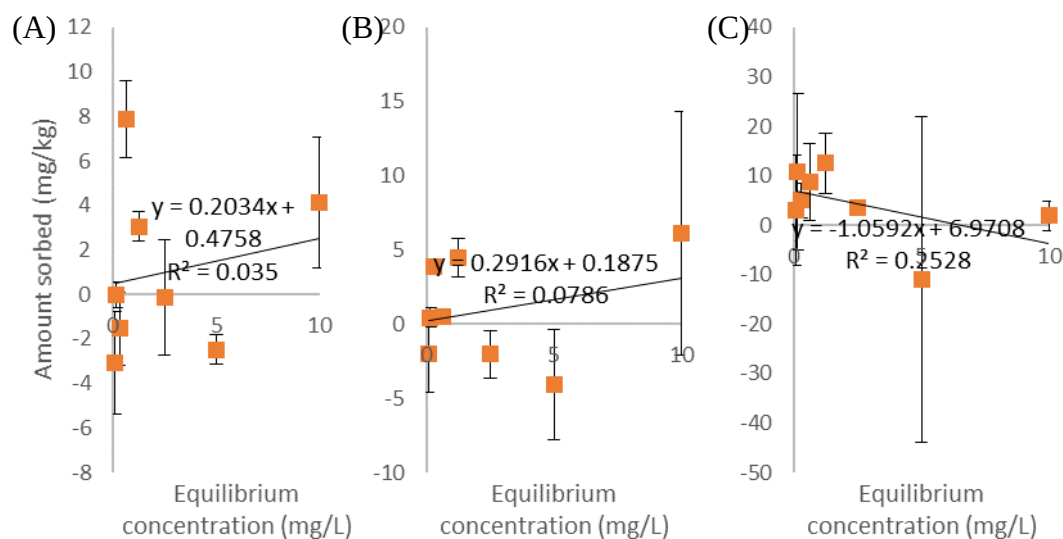


Figure 5-19 - Sorption isotherm of (A) dotriacontane, (B) 1-docosanol and (C) stearic acid at 25 °C after five days exposed to 2.5g/L A20 solutions containing a range of azoxystrobin concentrations.

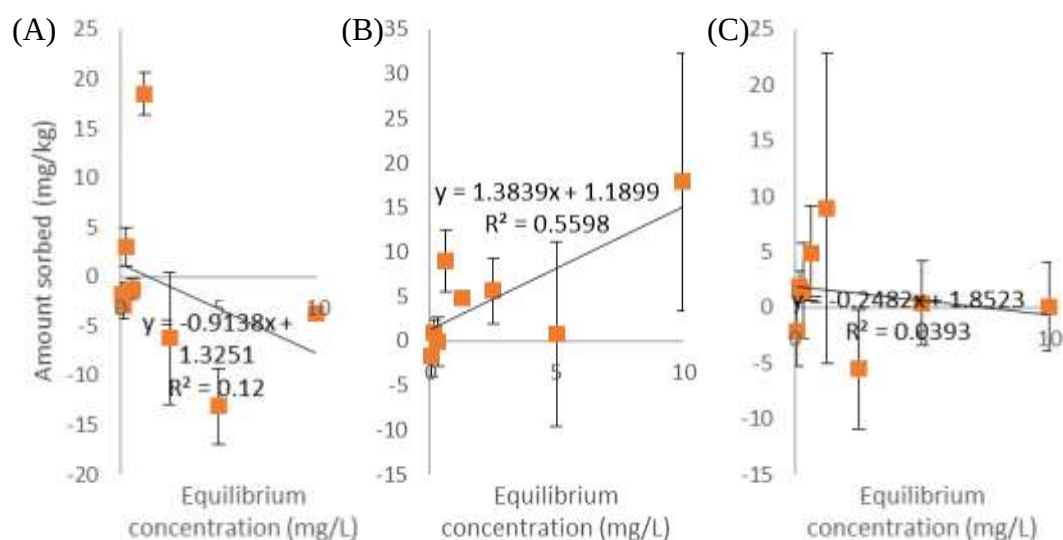


Figure 5-20 - Sorption isotherm of DOT: 1-D (A) 75: 25 % w/w, (B) 50: 50 % w/w and (C) 25: 75 % w/w at 25 °C after five days exposed to 2.5g/L A20 solutions containing a range of azoxystrobin concentrations.

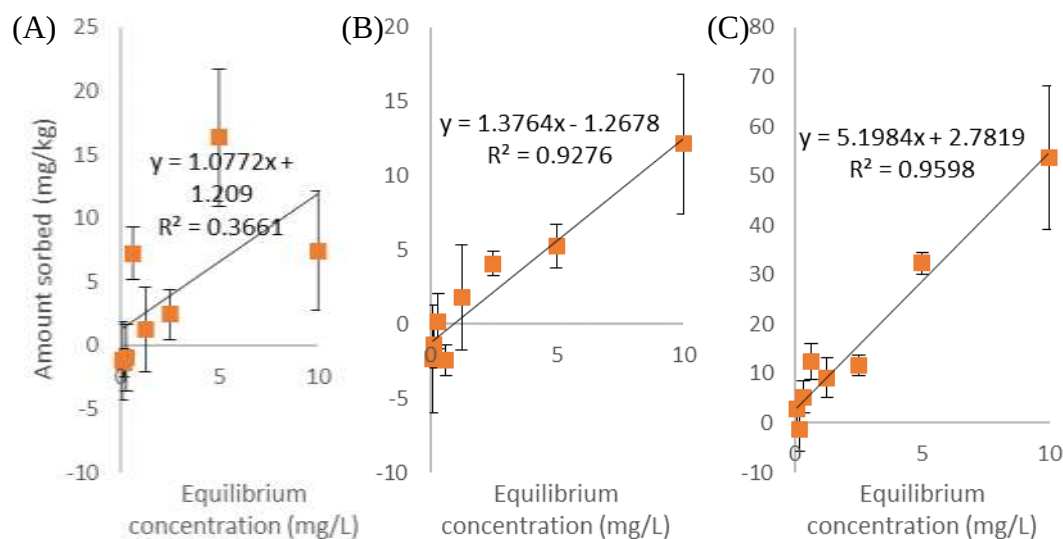


Figure 5-21 - Sorption isotherm of DOT: SA (A) 75: 25 % w/w, (B) 50: 50 % w/w and (C) 25: 75 % w/w at 25 °C after five days exposed to 2.5g/L A20 solutions containing a range of azoxystrobin concentrations.

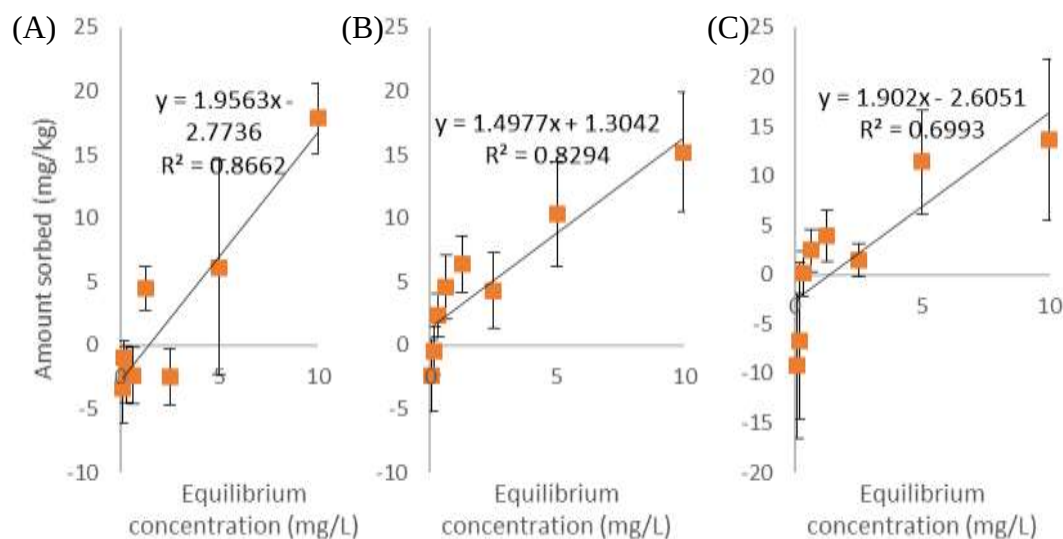


Figure 5-22 - Sorption isotherm of 1-D: SA (A) 75: 25 % w/w, (B) 50: 50 % w/w and (C) 25: 75 % w/w at 25 °C after five days exposed to 2.5g/L A20 solutions containing a range of azoxystrobin concentrations.

To determine the effect of wax composition on sorption, understanding the partition coefficient is critical. The equation for measuring partition coefficient between wax and surfactant solution, K_{ws} , was modelled after equations for determining the cuticle/water partition coefficient, Equation 5-1⁶⁰:

Equation 5-1

$$K_{ws} = (M_w/m_w)/(M_s/m_s)$$

Where M is the concentration (mg/L) of active in wax (M_w) or solution (M_s) and m is the known mass (mg) of the wax (m_w) or solution (m_s). Table 5-1 shows the calculated partition coefficient for each wax.

Table 5-1 - Partition Coefficients of azoxystrobin between wax powders and A20 solution

	% w/w composition of 1-docosanol in dotriacontane				
	0	25	50	75	100
Log K_{ws}	-0.95	-0.05	0.15	-0.51	0.08
Standard deviation	0.57	0.21	0.32	0.35	0.09
	% w/w composition of stearic acid in dotriacontane				
	0	25	50	75	100
Log K_{ws}	-0.95	-0.23	0.11	0.74	-0.42
Standard deviation	0.57	0.43	0.15	0.03	0.2
	% w/w composition of stearic acid in 1-docosanol				
	0	25	50	75	100
Log K_{ws}	0.08	0.3	0.17	0.24	-0.42
Standard deviation	0.09	0.05	0.11	0.22	0.2

As Table 5-1 shows, the three single component waxes show negative, or approximately zero, Log K_{ws} suggesting azoxystrobin preferentially partitions into solution rather than the single component wax films. Dotriacontane, in particular, showed the lowest observed partition coefficient of -0.95. The binary waxes of DOT: 1-D present partition coefficients that are negative or only just positive. 50: 50 % w/w DOT: 1-D was the only binary waxes of DOT: 1-D that showed a positive partition coefficient.

When mixed with a secondary wax, wax films containing stearic acid present positive partition coefficients, except DOT: SA 75: 25 % w/w. Similarly, DOT: SA 75: 25 % was the only binary wax containing stearic acid not to show a significant increase in partition coefficient relative to the component wax comprising the bulk of the film.

Waxes containing multiple components showed greater azoxystrobin sorption than single wax systems, particularly following the addition of stearic acid. The general increase in partition

coefficient within binary waxes might result from an increasing presence of suitable sorption sites through the introduction of disorder within the crystalline matrix. As shown in Section 4.2.6, the addition of secondary waxes to stearic acid resulted in the loss of XRD long spacing peaks found only in materials showing head to head alignment of molecules along the C-axis. This suggests that these waxes have lost much of their long spacing order, enabling the formation of more significant numbers of sorption sites. Prior authors have similarly shown that the addition of small molecules, including waxes, to waxes, frequently introduces considerable changes to the crystalline arrangement of excluded crystalline domains and their arrangement, which is similar to what is seen in this printed waxes⁴⁸. Such changes might be associated with increasing disorder within the crystalline arrangement, i.e. the loss of long spacing peaks being associated with the generation of new sorption sites.

Further, it has been hypothesised that the number of sorption sites in waxes is negatively correlated with crystallinity¹⁸. As crystallinity decreases, more amorphous regions are available wherein sorption sites can present. Such behaviour was suggested to explain why cuticles with differing wax compositions show different partition coefficients. Using crystallinity data from Section 3.2.6. Figure 5-23 shows the correlation between measured partition coefficients and the degree of crystallinity. The lack of a meaningful R^2 value suggests no such linear relationship for the systems studied here¹⁸.

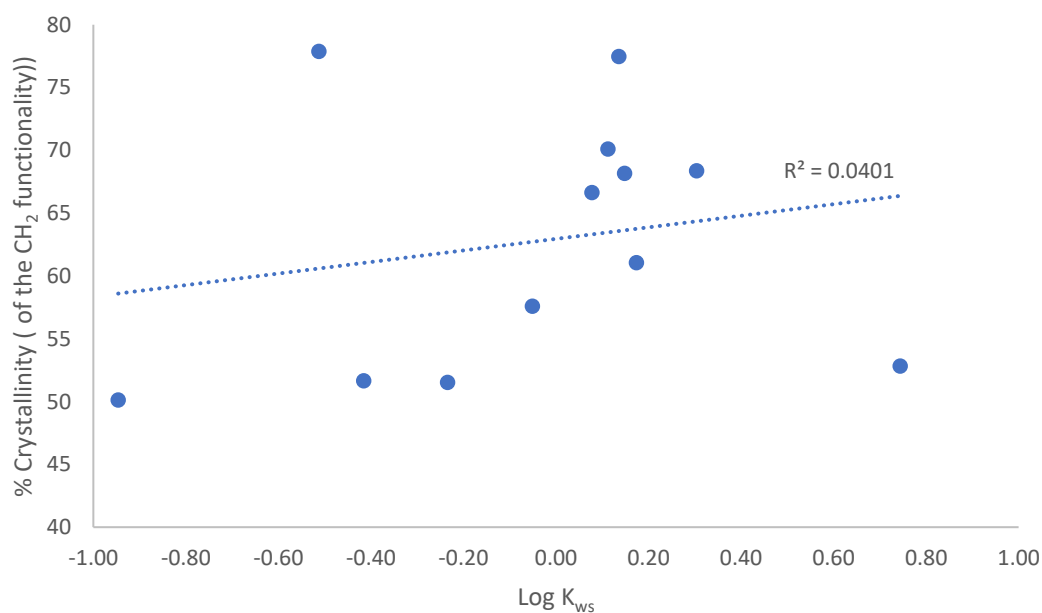


Figure 5-23 – Graph showing the measured relationship of each waxes crystallinity, as measured using FT-IR, and the waxes wax partition coefficient.

Rather than crystallinity determining the K_{ws} it is postulated that there is some unknown factor associated with chemical composition and the structure of the crystalline component that controls sorption as a function of composition. In support of this conclusion, the three single component waxes present significantly different partition coefficients, and the addition of a secondary wax in almost all cases resulted in a significant increase. Further, C-type sorption isotherms (Figure 5-21 and Figure 5-22) present when increasing analyte concentration causes the number of sorption sites to increase. The addition of secondary material, particularly stearic acid, often is associated with a significant increase in the isotherm gradient showing increased sorption. It is possible that the addition of a secondary wax results in increased sorption by decreasing intermolecular bond strength rather than through reduction of % crystallinity. It is known that long-chain aliphatic materials added to wax integrate into the crystalline wax structure, as shown by the low mobility of these integrated compounds reconstituted into the wax compared with the significantly higher mobility of these same compounds when they are exposed to the original film^{48,61,62}. It should also be noted that it has also been postulated that composition may affect sorption. Cuticles presenting a homogenous composition permit lower analyte sorption compared with more compositional complex cuticles. This result is similar to what is seen in the presented data taken on wax films suggesting the printed wax films present similar sorption properties to reconstituted waxes and may be suitable models of cuticle waxes¹⁸.

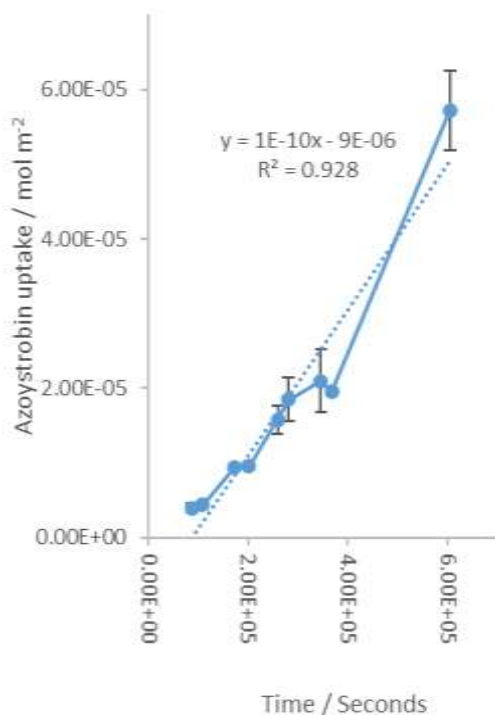
Additionally, it should be understood that the sorption experiments were conducted using azoxystrobin solubilised in 2.5 g/L A20, not water. Surfactant solution effects on sorption are variable; studies have shown significant decreases and increases in sorption. It is understood that surfactant influence on sorption is based on their chemical composition, the concentration of the surfactants and physical properties of sorbent and analyte. Why surfactants decrease, the partition coefficient is thought to be associated with increased agrochemical partitioning into the surfactant micelles⁵⁷. Surfactants at a concentration below their CMC do not modify the partition coefficient, supporting this conclusion^{57,63,6}. At concentration above the CMC, sorption increases temporarily⁶⁵ or steadily fall⁵⁷ as sorption into the micelles increases. When surfactants increase partition coefficient, the plasticisation of the wax film is the cause, enabling more significant ingress of analyte into the wax and mobility within the cuticle^{65,66}.

5.3.6. Permeation

Understanding that the A7 solution was unsuitable for use, Section 5.3.4, azoxystrobin penetration was tested with 1.25 g/L azoxystrobin suspension and a 1.25 g/L azoxystrobin suspension modified with 2.5 g/L A20.

5 layer wax films comprised of dotriacontane, 1-docosanol, stearic acid, and 25: 75 % w/w, 50: 50 % w/w and 75: 25 % w/w binary wax films comprised of two of dotriacontane, 1-docosanol and stearic acid were used to measure penetration. Films were exposed to an aqueous suspension of azoxystrobin with and without 2.5 g/L A20 surfactants using a Franz cell at heated to 25 °C, 40 °C or 45 °C for seven days as has been described in Section 5.2.5 To reiterate this process the wax film would be exposed to two solution in separate sealed chambers on opposite faces of the wax film surfaces. On one surface a solution containing no azoxystrobin would be found and on the opposite surface a suspension of highly concentrated azoxystrobin was found. Azoxystrobin thus will move down its concentration gradient, through the wax film, which would act as a barrier to penetration. Depending on if the agrochemical could sorb into the film, and the rate of Azoxystrobin permeation through the film, Azoxystrobin concentration in the receive solution would over time increase and be measured to calculate rate of penetration.

(A)



(B)

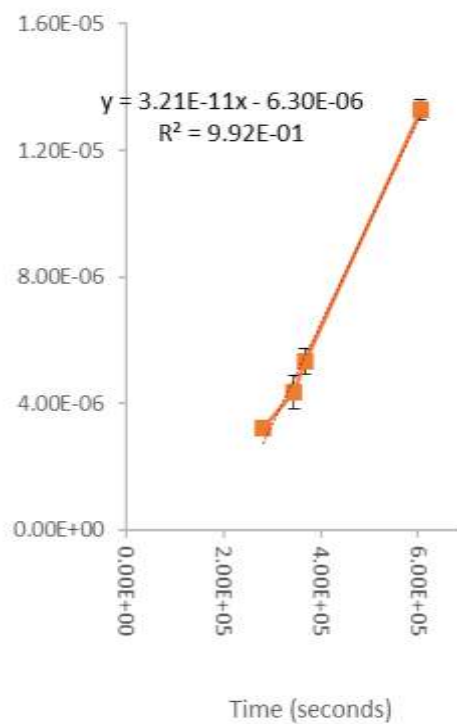


Figure 5-24 - Permeation of Quadris, azoxystrobin 1.25 g/L, through 5 layer printed films of (A) DOT: SA 25: 75 % w/w and (B) stearic acid, at 40 °C over one week. Note that the scale of the Y-axis on (A) and (B) are different.

72 sets of Franz cell tests were conducted, see Table 5-4. Only two demonstrated penetration of the azoxystrobin through the wax film (Figure 5-24). These involved wax films comprised entirely or mainly of stearic acid where permeation only occurred at relatively high temperatures. Furthermore, azoxystrobin was maintained at a high concentration wherein sorption and subsequent permeation is principally affected by the relative partition coefficient between active in solution and wax⁶. For lipophilic actives such as azoxystrobin, Log P = 2.5, sorption and penetration occur immediately⁶⁷, while the use of an unlimited dose system ensures the maintenance of a steep concentration gradient between donor and receiver solutions⁶⁸. Given this, it is proposed that the absorption of azoxystrobin into wax and permeation into the receiver chamber will not significantly diminish azoxystrobin concentration within the acceptor chamber. All these factors have been shown to increase the permeation rate of active through the cuticle.

To measure permeance, a value measuring the rate of permeation unaffected by film thickness mg/L was converted to mol/m², where the area measured is derived from the internal diameter of the circular Franz cells, 15 mm.

Figure 5-24 shows azoxystrobin permeations show a linear regression line. As such, flux (F, mol s⁻¹ m⁻²) equals the gradient of this curve vs time. Subsequently, permeance (P, m s⁻¹) through the wax films can be calculated using the below Equation 5-2¹¹.

Equation 5-2

$$P = \frac{F}{\Delta c}$$

Azoxystrobin was permeating from an acceptor suspension held in excess, with no surfactant, into a solution where azoxystrobin would rapidly solubilise in lecithin micelles. Azoxystrobin was rapidly removed from the free volume in the receiver solution, and the concentration gradient was maintained. Subsequently, change in concentration (Δc) was calculated as the difference between azoxystrobin concentrations, in water: 6.7 mg/L, within the acceptor chamber at t_0 , and the receiver solution, wherein c is nearly zero. The concentration of azoxystrobin in water was used, as azoxystrobin is confined to the suspension apart from the small volume soluble in water. As such, the bulk of the azoxystrobin will not interact with the film and is not available for sorption.

Table 5-2 - The Flux and Permeance of all tested waxes exposed to Quadris 1.25 g/L suspension. Only stearic acid and DOT: SA 25: 75 % w/w showed any permeation. All other waxes are shown as blanks.

	% composition of 1-docosanol in dotriacontane				
	0	25	50	75	100
Flux ($\text{mol s}^{-1} \text{ m}^{-2}$)	-	-	-	-	-
Permeance (m s^{-1})	-	-	-	-	-
	% composition of stearic acid in dotriacontane				
	0	25	50	75	100
Flux ($\text{mol s}^{-1} \text{ m}^{-2}$)	-	-	-	9.78E-11	3.21E-11
Permeance (m s^{-1})	-	-	-	5.89E-06	1.93E-06
	% composition of stearic acid in 1-docosanol				
	0	25	50	75	100
Flux ($\text{mol s}^{-1} \text{ m}^{-2}$)	-	-	-	-	3.21E-11
Permeance (m s^{-1})	-	-	-	-	1.93E-06

Permeation values of $5.89 \times 10^{-6} \text{ m s}^{-1}$ and $1.93 \times 10^{-6} \text{ m s}^{-1}$ were found for DOT: SA 25: 75 % w/w and stearic acid, respectively. Only waxes containing a large component of stearic acid permitted penetration of the azoxystrobin. All other waxes presented no visible penetration after seven days. It is clear permeation only occurred through wax films of specific composition, and thus composition affected permeation rates.

Interestingly, permeation was significantly increased following the addition of dotriacontane to stearic acid, relative to permeation through a film of pure stearic acid. However, mixing the same quantity of 1-docosanol into stearic acid resulted in the cessation of penetration. This highlights the importance of wax-wax interactions on permeation, a topic that could not be readily explored prior.

In both cases, the Y-intercept was negative, consistent with similar studies suggesting initial time-dependent sorption and permeation within the wax film¹⁶. Positive Y-intercepts are associated with permeation through a pathway that leads to a reduced onset time, i.e. permeation through stomata, followed by bulk permeation through the cuticle¹⁶. That the Y-

intercept is negative and follows a linear regression line suggests no defects are present that might allow the rapid onset of penetration. This agrees with the images shown in Section 5.3.2.

Why only stearic acid and DOT: SA 25: 75 % w/w allowed permeation, and what feature of DOT: SA 25: 75 % w/w wax increased permeance is unknown. Literature and prior results, however, allows some predictions to be made.

It is understood that permeation is limited by the cuticle wax film^{47,6}, with permeance being possible to predict using the octanol/water partition coefficient, diffusion coefficient and molar volume^{24,70}. Despite this, permeance can vary by up to 4 orders of magnitude¹⁵ depending on wax composition. It is suggested that wax composition controls the tortuosity of the barrier to agrochemical penetration¹⁴, which cannot be easily accounted for in the prior models. Subsequently, studies have shown that permeation is controlled by long-chain aliphatic waxes that form impermeable regions of the wax barrier, through which agrochemicals cannot penetrate¹⁵. Cyclic waxes, however, allow permeation through the permeable amorphous cuticle waxes¹⁵. This might explain the variability as a function of composition observed here. Unfortunately, this study did not attempt to determine if the aliphatic waxes chemical functionality influenced permeation. Additionally, it is currently unknown which waxes specifically increase or decrease tortuosity nor if wax-wax interactions can change tortuosity.

The presented results show that stearic acid, a carboxylic acid, permits penetration, whereas both pure dotriacontane and 1-docosanol, an alkane and alcohol, respectively, showed no permeation. It is suggested that different pure wax films form unique quantities and distributions of excluded crystalline domains. Agrochemical penetration cannot occur within these regions, and the wax film's associated tortuosity thus changes within each wax. Adding a secondary wax, dotriacontane, to bulk stearic acid resulted in increased permeance but adding the same mass of 1-docosanol resulted in cessation of penetration. It is possible that stearic acid and DOT: SA 25: 75 % w/w films are the only films presenting a sufficiently low tortuosity allowing azoxystrobin to penetrate. Additionally, the mixing dotriacontane into stearic acid reduced tortuosity further, increasing permeance, whereas the addition of 1-docosanol increased tortuosity, causing a cessation of penetration.

Similar results showed that adding a C₂₈ acid to a mixture of alkanes resulted in increased permeation. It was concluded that low amounts of secondary wax disrupted the waxes physical

structure, causing a decrease in tortuosity. However, as the quantity of secondary wax increased, this secondary wax would form unique excluded crystallite regions increasing tortuosity. This agrees with results in Section 3.3.2.1, wherein adding further dotriacontane to stearic acid increases the eutectic component, a unique crystalline structure. This theory agrees with Section 4.2.6, wherein 50: 50 % w/w mixtures of all three binary waxes form a set of unique crystal structures comprised of both component waxes.

It has been theorised that correlating crystallinity with permeation might explain the variance in permeation as a function of composition¹⁸. Unfortunately, plotting the crystallinity of the printed film, Section 4.2.5 against permeance, does not positively correlate with the data presented. The films that permitted penetration did present the lowest crystallinity measured: 51.64 ± 2.76 and 52.82 ± 0.931 , stearic acid and DOT: SA 75: 25 % w/w respectively. However, they were insignificantly different to many waxes, wherein penetration was not observed, suggesting a clear correlation is not present. This would further suggest that the relationship is not as simplistic as prior suggested¹⁸.

Because there is unlikely a simple relationship between % crystallinity and permeance, the arrangement of the crystallinity may be critical. XRD diffractograms of reconstituted waxes are often similar to the isolated cuticle, except for reducing the amorphous halo associated with the cutin polymer^{71,7}. Additionally, as a self-organising structure¹¹, wax presents the same mobility⁵¹ and desorption¹¹ properties regardless of the physical form, i.e. a reconstituted cuticle wax or an isolated cuticle. Considering this, as per Section 3.3.2.4, it was initially predicted that only waxes with long spacing peaks should permit permeation. Similar peaks are found in a small number of extracted cuticle waxes⁷¹, suggesting that waxes showing long spacing order did indeed permit more rapid penetration.

Unfortunately, XRD diffractograms of wax films, Section 4.2.6, showed no long spacing peaks in DOT: SA 25: 75 % w/w wax film. Stearic acid printed films showed, relative to powder wax, less intense long spacing peaks. Long spacing peaks thus cannot be considered an indication of potential permeation. Unfortunately, no physical property measured supports a hypothesis explaining why only stearic acid and DOT: SA 25: 75 % w/w permitted azoxystrobin penetration.

One theory, however, relates to the nature of Fischer-Tropsch waxes. Cuticle amorphous waxes typically are comprised of cyclic waxes with the tails of waxes integrated into excluded

crystalline domains⁶¹. Stearic acids present a relatively short chain length, compared with dotriacontane and 1-docosanol, and the trend of secondary waxes integrating into waxes⁶¹ may result in dotriacontane forming large tails expanding the amorphous regions. It was shown in Section 3.2.7 that adding dotriacontane to printed stearic acid results in a loss of the long spacing peaks found in stearic acid, suggesting that the addition of dotriacontane increased the disorder with the film, supporting this theory.

Considering temperature and surfactant use, as already stated, all tests at 45 or 25 °C showed no penetration. Similarly, all tests containing surfactants showed no penetration.

Considering temperature, penetration not occurring at lower temperatures is not surprising. Data showing azoxystrobin penetration through cuticles at 40-45 °C is not available in the literature. However, increasing penetration temperature by 10 °C can increase permeance by an order of magnitude⁷. Considering the measured permeated azoxystrobin concentration (Figure 5-24) was within two orders of magnitude of the HPLCs limit, the concentration of any azoxystrobin that permeated the film at 25 °C was possibly below or near the HPLCs limit of detection, limiting the HPLCs ability to discriminate a peak.

Permeation was not observed in any Franz cell where a surfactant was used. This might be explained as surfactant enhanced permeation only occurs at specific concentrations where the cuticle, agrochemical and surfactants appropriately interact⁵⁷. It is seen that agrochemical penetration is greatest at an optimum surfactant concentration. If surfactant concentration increases, the agrochemical may preferentially partition into surfactant micelles⁵⁷. As shown in Section 5.3.5, the A20 surfactant likely induced preferential partitioning of azoxystrobin into surfactant micelles at the tested concentration. This traps the azoxystrobin, thus preventing penetration.

Additionally, when agrochemicals are solubilised within surfactant micelles, release occurs predominantly when the surfactant solubilises the wax⁷. As shown in Section 5.3.1, the 2.5 g/L surfactant solution did not induce considerable morphological changes to many wax films, suggesting minimal solubilisation had occurred. The active will remain solubilised within the micelle, explaining the lack of observed permeation. In this experiment, it is thus postulated that 2.5 g/L A20 and A7 was at sufficient concentration wherein permeation had decreased because

of the surfactant presence and that azoxystrobin remained solubilised within the micelle, explaining the lack of observed permeation

5.3.7. Fungal attachment to wax films

In addition to investigating agrochemicals and surfactants interactions with wax, the printed films provide a surface to test the effect of composition on fungal attachment. Different cuticle surfaces are known to show different degrees of fungal attachment⁷. This is self-evident, considering a specific fungal spore can only attach to a small number of plant surfaces. This is related to specific receptors or chemical composition detected by the fungal spore triggering *adhesion* and further colonisation²⁹. However, physical and topographical features are also associated with fungal attachment, such as roughness⁷⁶ and hydrophobicity⁷⁷. Hydrophilic fungal spores, such as *Z. tritici*, are expected to adhere more readily to rough, hydrophilic surfaces.

Further, following infection hyphae, a branching filament of the adhered spore that forms the infection structure is not random. This enables the hyphae to grow towards stomata and begin colonising and infection of the plant. Often the growth of such hyphae shows a clear directionality suggesting some topographical^{76,78} or chemical⁷⁹ tropism and growth towards the stomata.

As already shown, wax surface topography can be modified by changing print thickness and wax composition, Section 4.3.2.3. Additionally, the print thickness and composition affect the hydrophobicity of the surface, Section 4.3.3. Given this, it was hoped it would be possible to assess if these factors affect fungal attachment. Figure 5-25 show the % attachment of *Z. tritici* spores to 17 of the 18 printed wax films compared with a PET control. There is a clear trend concerning composition vs attachment. As the % weight of stearic acid increased in films containing dotriacontane or 1-docosanol fungal attachment increased (Figure 5-25 B and C, respectively). A similar pattern is seen when 1-docosanol is added to dotriacontane. While many of the DOT: 1-D wax films show significant differences from the two constituent single component wax films, only the 5-layer films show a significant trend of increasing attachment as a function of increasing 1-docosanol content.

However, as per a two-tailed t-test, only the 5- layer and 10-layer samples are significantly different to attachment compared with 1-docosanol. The 20 layer films were insignificant different (Figure 5-25 A).

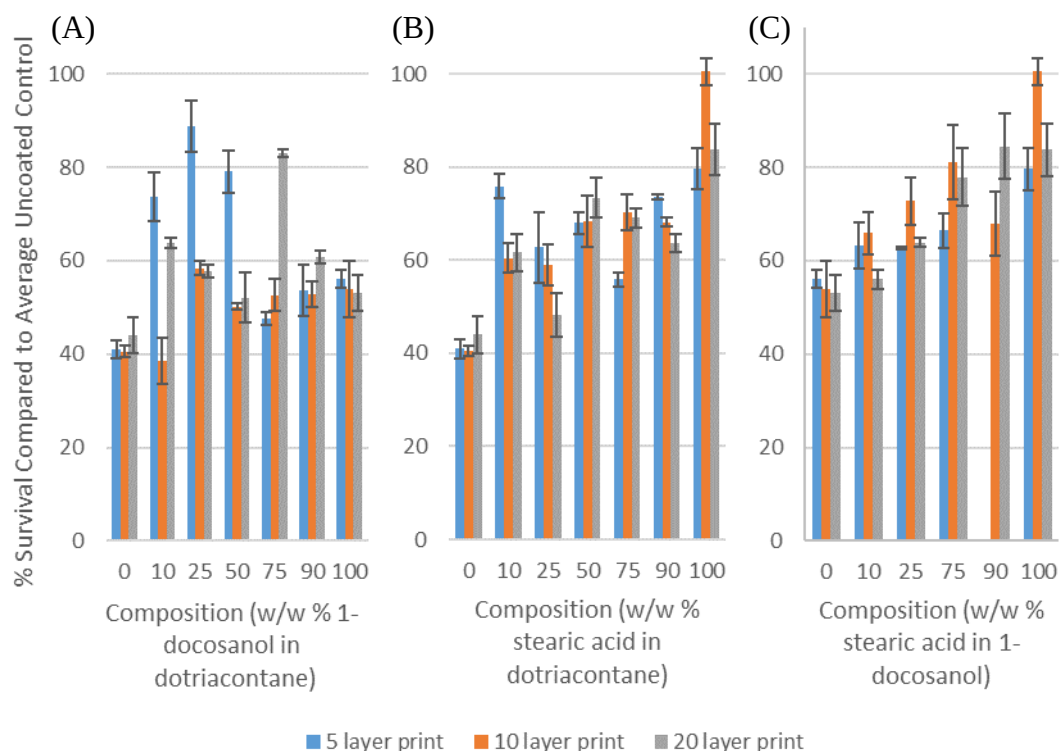


Figure 5-25 - Fungal attachment, compared with a PET control (attachment = 100%), of *Z. tritici* onto 5, 10 and 20 layers thick printed wax coupons. 5, 10 and 20 layers 1-D: SA 50: 50 % w/w and 5 layer 1-D: SA 10:90 % w/w repeatedly broke during testing and was excluded from the analysis. (1-D: SA 10:90 % w/w, N=2)

Interestingly, adding 10 % w/w stearic acid to dotriacontane caused a significant increase in the fungal attachment, which was not seen when stearic acid was added to 1-docosanol. Trends between single component waxes are more clearly seen in Figure 5-26. Stearic acid shows a significantly increased attachment of *Z. tritici* compared to dotriacontane and 1-docosanol films, regardless of film thickness. However, a significant difference between dotriacontane and 1-docosanol attachment only occurred in 5 layer films. The impact of film thickness, seen in Figure 5-25 and Table 5-3, shows layer thickness largely had an insignificant effect, except for the 5 layer films of 90: 10, 75: 25 and 50: 50 DOT: 1-D. The cause of this is unknown, and this may be an outlier result that further work is required to explore.

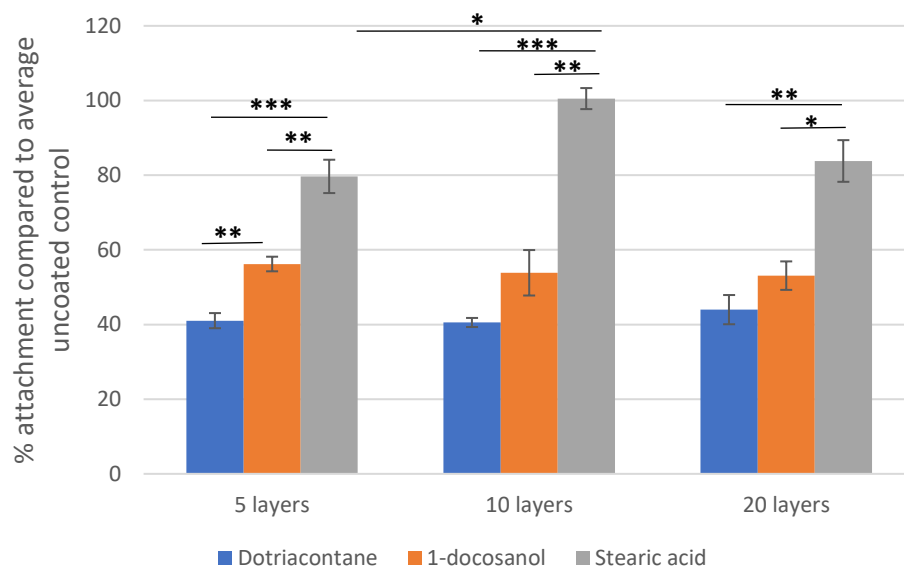


Figure 5-26 - A pruned version of Figure 5.25 showing only the fungal attachment to the single component wax films printed with 5, 10 and 20 layer thicknesses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5-3 - % Attachment of *Z. tritici* onto wax films compared with a PET control. Means connected by the same letter within a row or column, first and second letter respectively, are not significantly different according to a paired two-tail students t-test $\alpha = 0.05$.

% w/w composition of 1-docosanol dotriacontane in	Attachment: <i>Z. tritici</i> level after 24 hours (XTT signal, % of control)					
	5 layer % attachment	SEM	10 layer % attachment	SEM	20 layer % attachment	SEM
0	41.03 a, a	2.03	40.55 a, a	1.21	43.99 a, a	3.91
10	73.64 a, b	5.21	38.56 b, a	4.95	63.77 c, b	1.12
25	88.86 a, b	5.44	58.43 b, b	1.44	57.74 b, c	1.48
50	79.15 a, b	4.53	50.22 b, c	0.75	52.12 b, c	5.28
75	47.58 a, c	1.33	52.69 a, c	3.38	83.08 b, d	0.87
90	53.74 a, c	5.53	52.77 a, c	2.72	60.78 a, b	1.44
100	56.21 a, d	1.96	53.86 a, c	6.09	53.09 a, c	3.82

% w/w composition of stearic acid in dotriacontane	5 layer % attachment	SEM	10 layer % attachment	SEM	20 layer % attachment	SEM
0	41.03 a, a	2.03	40.55 a, a	1.21	43.99 a, a	3.91
10	75.91 a, b	2.72	60.46 b, b	3.15	61.63 b, b	4.08
25	62.80 a, b	7.59	58.92 a, b	4.37	48.23 b, a	4.62
50	68.02 a, b	2.37	68.27 a, c	5.51	73.44 a, c	4.23
75	55.84 a, b	1.54	70.25 b, c	3.79	69.07 b, c	2.09
90	73.65 a, b	0.58	68.18 b, c	1.06	63.61 c, b	1.87
100	79.68 a, b	4.47	100.52 b, d	2.82	83.80 a, d	5.58
% w/w composition of stearic acid in 1-docosanol	5 layer % attachment	SEM	10 layer % attachment	SEM	20 layer % attachment	SEM
0	56.21 a, a	1.96	53.86 a, a	6.09	53.09 a, a	3.82
10	63.29 a, a	5.05	65.96 a, b	4.56	56.08 a, a	2.07
25	62.64 a, a	0.32	72.76 b, c	5.01	63.89 a, b	0.92
50	- -	-	- -	-	- -	-
75	66.44 a, a	3.67	81.09 b, c	8.00	77.86 b, c	6.18
90	- -	-	67.98 a, b	6.93	84.49 a, c	7.04
100	79.68 a, b	4.47	100.52 b, d	2.82	83.80 a, c	5.58

While it is clear that specific waxes, stearic acid, in particular, promote attachment, why this occurs is unknown. Based on the literature, it was hypothesised that hydrophobicity could be a causative factor⁷.

Hydrophilic surfaces minimise the attachment of hydrophobic fungal spores³⁷. Mutant *Medicago truncatula* demonstrates this, showing a decrease in surface hydrophobicity alongside decreased attachment of three different rust fungal spores³⁶. It is postulated that a decreased concentration of C₃₀ primary alcohols, increased the relative concentration of C₂₉ and C₃₀ alkane, and loss of epicuticle surface crystalline structures caused the increased attachment³⁶. However, whether this change was induced by physical (hydrophobicity and topography) or chemical (primary alcohol) cues is unknown.

In this thesis, evaluating *Z. tritici* fungal attachment to different composition wax films vs the film water contact angle on 5, 10, and 20 layer films showed no direct link between hydrophobicity and fungal attachment (Figure 5-27 A-C). Also, film thickness, and thus visible topography, had a minimal effect on fungal attachment (Figure 5-25 and Figure 5-26). It was thus theorised that the different topographical features of the different composition films, Section 4.3.3, affected the wettability of the surface, such that any correlation between hydrophobicity and fungal attachment was masked. To assess this attachment was correlated against contact angle on the bottom surface of the printed film. This surface largely eliminates the topography, and composition dictates hydrophobicity, Section 4.3.3.

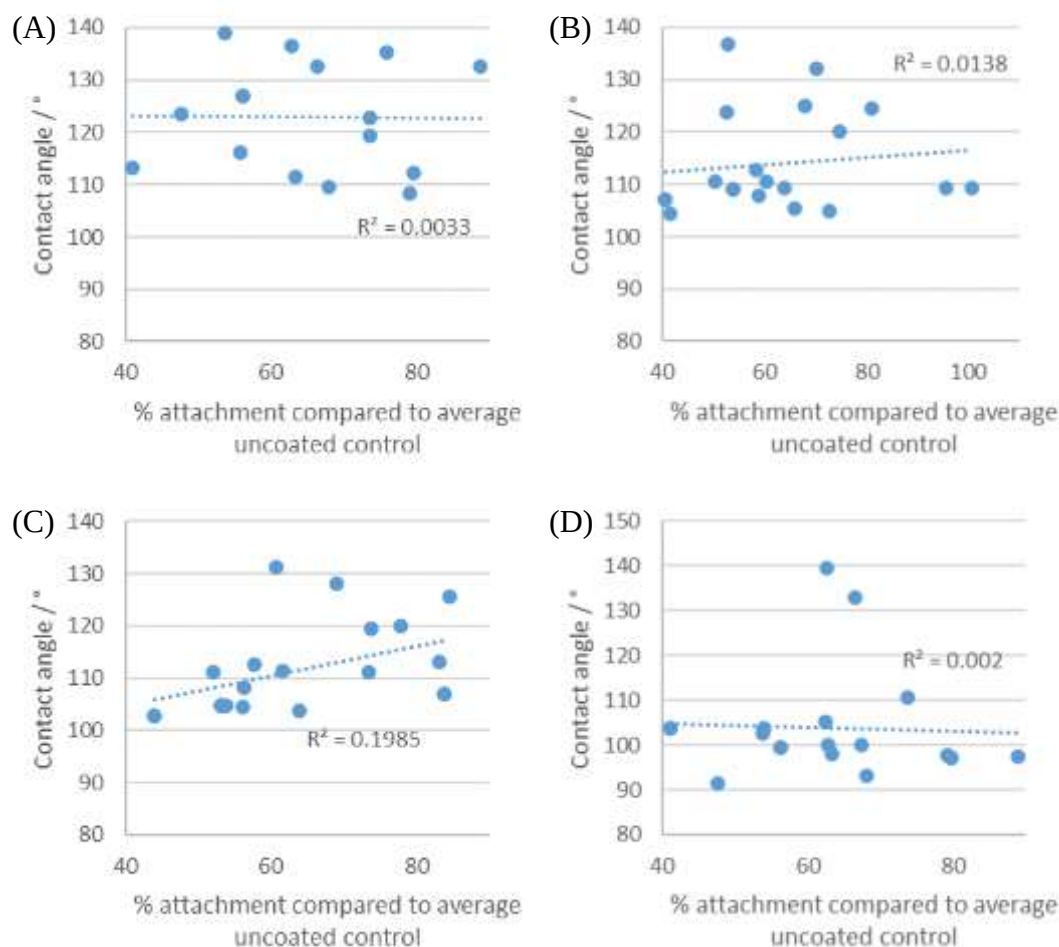


Figure 5-27 – Fungal attachment of *Z. tritici* onto wax films of different compositions vs the water contact angle of these wax films. (A) Shows attachment vs contact angle on a 5 layer film, (B) 10 layer film, (C) 20 layer films and (D) attachment to a 5 layer film vs contact angle on the bottom surface of a printed 5 layer film.

This should not be considered a direct comparison as fungal spores were not exposed to this surface. However, as shown in Figure 5-26, the effect of topography between films of the same composition is often insignificant.

Figure 5-27 (D) shows that no correlation could be drawn with the hydrophobicity of the flat bottom surface of a printed film. This seems to contradict prior literature drawing correlations between hydrophobicity and fungal attachment. However, the study by Uppalapati et al.³⁶ on *Medicago truncatula* found a correlation between loss of alcohol and gain of alkane surface groups with a decreased in hydrophobicity and increased fungal attachment. As shown in Section 4.3.3, hydrophobicity on printed waxes was primarily driven by topography, not composition, as on the bottom surface, many films showed insignificant differences in contact

angle. This would suggest that the change in attachment, in these results, was principally driven by composition, not the wax films physical properties such as hydrophobicity and topography.

It should be noted that the wild type *Medicago truncatula*³⁶ abaxial surface tested showed a wax composition similar to the dotriocntane:1-docosanol 10:90 % w/w printed film. Similarly, mutant type *Medicago truncatula* showed a wax composition similar to the dotriocntane:1-docosanol 50: 50 % w/w. If the 50: 50 % w/w and 10:90 % w/w DOT: 1-D waxes are considered crude models of these cuticles, the fungal attachment and hydrophobicity results for these films match literature results

5.4. Conclusions

Printed wax films have considerable scope for investigating the fundamental interactions between waxes and a range of environmental conditions or endogenous compounds and pathogen infections. Depending on the composition of the wax exposure to surfactants, it has been shown that surfactants can induce considerable morphological changes. The extent and induced morphology change depending on the time and concentration of the surfactant used. Furthermore, it has been shown that depending on the surfactants used. It is possible to modify the morphology or deteriorate the integrity of a printed film with different wax compositions. Synperonic A7 surfactant caused extensive modification of 1-docosanol waxes and waxes containing 1-docosanol, whereas Synperonic A20 caused modification principally to stearic acid waxes and waxes containing stearic acid.

Interestingly single component waxes visibly underwent less visual surface modification than binary mixed waxes. Additionally, the induced morphological changes were different depending on the surfactant used. It has been suggested that wax solubilisation follows a linear trend wherein surfactants with smaller ethoxylate chains induced greater solubilisation. The presented data further suggests that the physical effect of solubilisation may also change due to ethoxylate chain length.

Considering the permeation of the active azoxystrobin through these wax films, it has been shown that the composition of the film modifies permeation. Only two of the 12 films tested permitted any permeation, with no permeation observed when surfactant was present. Both films showing permeation contained either pure stearic acid or a significant component of stearic acid. There is no clear link between crystallinity and the related XRD pattern with these

two waxes, so it is likely not currently possible to predict permeation from this perspective at present. It is proposed that some aspects of stearic acid and the stearic acid: dotriacontane wax induced lower tortuosity values relative to the other waxes such as dotriacontane or 1-docosanol. Transmission electron microscopy imaging of these waxes films could be conducted¹⁶ to investigate this in the future.

The sorption experimentation can explain the lack of permeation following the application of agrochemicals within a formulation containing surfactants. These experiments inferred that azoxystrobin would preferentially partition into the surfactant micelle relative to the wax surface in almost all cases.

Fungal attachment studies showed a variance in % attachment as a function of the composition of wax tested with stearic acid in almost all cases showing a more significant attachment of *Z. tritici* than dotriacontane or 1-docosanol. Analysis of binary mixtures of wax showed a relatively linear trend in attachment in waxes containing stearic acid, whereas waxes lacking stearic acid showed no trend in terms of attachment. This is one of the few experiments that showed a linear trend between composition and behaviour. It is postulated that this linear trend was caused by the observed phase separation of stearic acid in the 1-D: SA films. In many eutectic waxes, it would be expected that the material will form a uniform structure of repeating units comprising the component materials. Stearic acid showed distinct phase separation. Perhaps through inducing phase separation, extensive stearic acid regions are presented, allowing fungal spores to preferentially attach to these locations resulting in the generally linear increase in fungal attachment as stearic acid concentration was increased.

No trend could be observed between wax surface hydrophobicity and fungal attachment. Comparing results against literature showed that several of the printed wax films showed similar fungal attachment and hydrophobicity results as shown in this work, which is promising in terms of the printed wax films acting as a model for native cuticles.

5.5. References

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5.6. Appendix

Table 5-4 Table of all tested Franz cell parameters. Green boxes signify testes wherein azoxystrobin was detected in the receiver solution within one week after initiating the transport study, whereas crosses signify tests wherein no azoxystrobin was detected in the receiver solution at any time point

Test parameters	Waxes											
	Pure waxes			% composition of 1-docosanol in dotriacontane			% composition of stearic acid in dotriacontane			% composition of stearic acid in 1-docosanol		
	Dotriacontane	1-docosanol	Stearic acid	25	50	75	25	50	75	25	50	75
25 c, no A20												
25 c, 2.5 g/L A20												
40 c, no A20												
40 c, 2.5 g/L A20												
45 c, no A20												
45 c, 2.5 g/L A20												

6. Chapter 6 – Conclusions

This thesis's original aim was to develop a scalable 3D printed wax film on which experimentation of wax-environment and wax-agrochemical interactions could be conducted and related to the same interaction as they occur on the cuticle. The prevailing wisdom relating to cuticle composition and agrochemical interaction is that the extent of the interaction is driven by wax composition. This results in the variance of agrochemical penetration rates, UV reflection and fungal attachment, as examples, as a function of the reconstituted cuticle wax or isolated cuticle's wax composition.

This thesis has shown that applying a combined analytical process investigating 3D printed wax films generates broadly similar results as would be expected when assessing similar interactions on reconstituted cuticle waxes or isolated cuticles that possess a broadly similar wax composition as printed model cuticles. This suggests printed wax films could be utilized as models of cuticle waxes and be used to investigate the contributions of different waxes to these processes in native cuticles. Native cuticles possess a highly complex wax component, so understanding the contribution of specific waxes in cuticle-environment interactions will aid the current understanding of how to tailor formulations to the plant of interest, based on the known wax composition of this plant.

This was achieved in three parts. The first part of this thesis shows that it is possible to utilize ToF-SIMS and associated MVA analysis to identify wax composition as a function of location on the cuticle. This information and prior literature allowed the selection of three cuticle waxes analogues: dotriacontane, 1-docosanol and stearic acid, based on the composition of the tested *Solanum Lycopersicum* fruit cuticle. It was subsequently found that these three single component waxes were printable and presented a high degree of crystallinity like reconstituted cuticles waxes. These waxes were mixed, and FT-IR, DSC and XRD were used to determine if these binary waxes comprised of two of dotriacontane, 1-docosanol and stearic acid were printable, crystalline, and eutectic, like many cuticle waxes in native cuticles. Subsequently, 18 waxes were identified for further printing and analysis.

The second part of this thesis showed that a commercial hot-melt inkjet printer and printhead could be utilized to eject droplets as predicted in the prior chapter successfully. Furthermore, surface structures similar to cuticle crystalloids were observed, and their formation could be

controlled through modification of printing parameters such as film thickness, substrate temperature and DPI and wax composition. Understanding this, it was possible to replicate some native cuticles' morphological features and crystalloids, allowing us to mimic, to a limited degree, the surface of native cuticles. Similar crystalloid structures were not observed when using a more traditional method of preparing reconstituted cuticle wax, drop-casting. The ability of 3D printing wax to modify the surface feature was further explored through the generation of a multi-layered-multi-material structure.

The impact of printing on surface features was evident; however, it was unclear what caused this. Raman Spectroscopy showed that crystalloid distribution was controlled by the phase separation of wax components within droplets. It was theorized that given that the waxes are eutectic, the two different wax components would cool at different times leading to phase separation. Additionally, it was found that the following printing, while the film's chemical composition had not changed, as per FT-IR analysis, the crystalline arrangement and crystallinity had changed as shown through both XRD and FT-IR analysis. It was theorized that this is a consequence of the printing and cooling process resulting in a reduction in % crystallinity for all printed waxes compared with their powder forms. Finally, it was found that through modification of the printing parameters and wax composition, the functional properties of the wax could be readily modified. The most pertinent example identified was through modification of wax composition and printing parameters, wettability of the 3D printed wax surface, in response to exposure to water and two surfactant solutions with different HLB values, could be controlled. This was best shown with 1-docosanol containing waxes where increasing the w/w % of 1-docosanol resulted in a significant increase in contact angle. This is thought to be associated with the spontaneous formation of heterogeneously distributed crystalline surface structures that reduce the interfacial energy between substrate and liquid.

In the final part of the thesis, it was found that prolonged exposure to a range of different solutions, water and two surfactants for a week resulted in the degradation of a large majority of the wax films when exposed to surfactants solutions with no clear trend relating crystallinity, as measured using FT-IR and XRD or the wax composition. Based on this, it was determined that any agrochemical permeation experiment would need to be conducted over a prolonged period. Only low concentration surfactants would be utilized to avoid causing degradation of the wax film and subsequent failure of the experiment or inappropriately high permeation caused by

gradual degradation of the film. When the penetration of azoxystrobin was measured across the printed films, it was readily found that azoxystrobin had only managed to penetrate through a small selection of waxes containing either pure 1-docosanol or stearic acid:1-docosanol over one week. No trend could be identified relating to azoxystrobin penetration through these waxes with either sorption or crystallinity, which have been previously considered as cuticle functionalities correlated with the rate of permeation. However, it was identified that prior literature assessing penetration of agrochemical through reconstituted waxes had identified 1-docosanol as a wax that permitted a high penetration rate. It is thus possible that a similar trend is identified here and that the printing process has not disrupted this functionality of 1-docosanol. If this is true, considering reconstituted waxes are known to retain much of the cuticle's waxes functionality and are used as a model cuticle for testing permeation and sorption, evidence showing 3D printed films show similar trends and properties as reconstituted waxes these films might be good models of the cuticle. Finally, the relationship between surface morphology, wax composition, and fungal attachment of the *Z. tritici* fungal spore was measured. Here, the use of high % w/w stearic acid in dotriacontane and 1-docosanol films increased attachment, which agreed with literature that had previously shown *Z. tritici* preferentially grows upon cuticles presenting a significant carboxylic acid component.

All this suggests printed films produced in this thesis could be utilized to understand the fundamental relationship between wax composition and cuticle function and understand the variance in agrochemical permeation and fungal attachment as a function of plants species. Such understanding could lead to developing novel agrochemical formulations accurately targeted to the specific plant of interest based on the known wax composition of that species' cuticle surfaces.

6.1. Future work

6.1.1. Short term goals

The below suggestions focus on the key immediate gaps in this thesis that upon resolution would improve the quality of the data presented in this thesis.

6.1.1.1. Further exploration of wax film properties - contact angle and topography

Within Section 4.2.7 the contact angle of water, A7 and A20 was measured providing information regarding the interaction of these liquids and the printed film and the relationship between wax composition, topography, and contract angle. However, no information regarding roll-off angle, advancing or receding contract angle, contact angle hysteresis or surface free energy was collected.

Understanding roll of angle would enable investigation of pinning on these surfaces and the impact of composition on the retention of surfactant solutions on the printed surface. Agrochemicals must remain in contact with the cuticle so understanding the contribution of specific waxes to roll off angle and relating this to the wax composition of cuticles of interest, would be helpful when developing agrochemical formulations. Additionally, contact angle hysteresis and, advancing and receding contact angle could be used to investigate the effect of print parameters and wax composition on the. The contact angle hysteresis data would be complementary to roll off angle data.

Free energy would be another contact angle measurements that could be collected. This would be useful for generating qualitative measurements of the effect of changing wax composition and print parameters on the free energy of the film. In addition, it might be an interesting measurement to use to directly compare the interactions of the wax films with liquids against these interactions on native cuticles.

6.1.1.2. Further exploration of wax film properties – topography

In this thesis topographical information was derived from microscopy images of the surfaces of interest. No quantitative measurements of topography were presented in this thesis. In part, this was because of measuring the topography of a translucent, white surface with most optical surface measurement is challenging. However, quantitative topography data would enable further evaluation of the contact angle and fungal attachment measurements, which are known to be correlated with topography.

It is predicted that Coherence scanning interferometry might be capable of imaging the described translucent surfaces of interest. Subsequently, further work should focus on the development of a methodology to measure surface roughness and the associated parameters

of roughness such as skewness and the average peak to valley height. Ideally this data would be used to further evaluate other data presented within this thesis.

6.1.1.3. High throughput penetration and sorption testing

In this thesis penetration was measured through 5 printed layers of 18 different waxes over 7 days using two solutions of azoxystrobin. The process followed to achieve this was low throughput and challenging to operate due to the risks of breaking the films. Subsequently, while the results collected are useful, they are not extensive and substantially more tests, such as using thinner films, different surfactants and different agrochemicals will need to be carried out to achieve the goal of this thesis.

Subsequently in any future work it would be prudent to develop a high throughput method of preparing and operating the Franz cell assembly in a way that minimising the risk of failure associated with manual preparation. One method this might be achieved is by printing the film directly onto the Franz cell by laying the Franz cell horizontal and printing a wall of wax directly adjacent to the now vertical surface of the acceptor chamber flange.

In addition, it would be ideal to complete the sorption dataset presented in Section 5.3.5 as only one set of sorption data was collected, the sorption of azoxystrobin solubilised in a A20 solution over 7 days. Ideally this experiment would be expanded to test azoxystrobin solubilised in a range of solution such as water, other surfactants, and critically, different concentration of surfactants. The results shown in Section 5.3.5 are predicted to have shown limited sorption for many reasons including excessive partitioning of azoxystrobin into the surfactant micelles due to the high surfactant concentrations. The lack of sorption data makes analysis of the available penetration data challenging as for penetration to occur sorption must also occur.

Subsequently, by expanding the experimental parameters of the sorption experiment it may be possible to identify if the limited permeation observed through the printed films was a result of experimental errors which causing the analyte to not be detected, or if the limited permeation was a result of limited sorption. Additionally, further sorption data will provide more data regarding the interactions of individual waxes and agrochemicals, and agrochemical formulations thus furthering current understanding of the structure-function relationship

6.1.1.4. Development of further printable waxes

In this thesis many waxes were produced. However, they were comprised of only 3 individual waxes each containing a single chemical functionality: alkane, alcohol, and carboxylic acid. The native cuticle contains a range of wax functionalities not represented in the waxes tested such as ketones, aldehydes, esters and critically triterpenoids. Further should seek to expand the library of printable materials to include waxes with new chemical functionalities. Additionally, the impact of chain length should be considered to understand the effect of chain length on interactions with surfactants and agrochemicals as it has been suggested chain length impact penetration.

Unfortunately, waxes that presented different chain lengths or contain the chemical functionalities listed above were expensive. As such, unless a methodology can be developed to produce these waxes initial testing should only include these waxes as a small % w/w of the total film, rather than attempt to print entire films of these new waxes.

Expansion of the printable wax library is critical as the results of this thesis clearly show chemical functionality significantly impacts the films behaviour and properties and thus the printed films can be used to model the behaviour of individual cuticle waxes. Unfortunately, the currently limited library will prevent in depth modelling of the cuticle.

6.1.2. Long term goals

The below suggestions focus on long term research questions spurred from the results of this thesis and could possibly be the basis of a large new body of work

6.1.2.1. Improved printing methodologies

As shown in Chapter 4 it is possible to use 3D printing to produce a range of wax films successfully. Additionally, as shown in Chapter 5, these films show variable properties and behaviours as a function of their chemical composition, and critically the printing parameters used to generate these wax films.

However, there is scope for improvement of the printing methodology. A key improvement would be an expansion of the results presented in Section 4.3.2.4 by using a duel printhead system. In this thesis all multi material films were produced with a single nozzle, which limits the complexity of the structures that can be reliability printed. This is because it is not readily possible to vary the distribution of different waxes on a layer-by-layer basis, which would be

possible using a duel printhead system printing two materials simultaneously. The more complex films that could be produced with a duel printhead would be ideal for modelling the behaviour of a cuticle waxes more accurately, because, as discussed in Section 1.2, the interface between epicuticle and intracuticle is diffuse and the distribution of materials is heterogeneous. Subsequently, it should be a key objective to integrate a duel printhead system into the presented work, enabling the development of more compositionally and structurally complex films.

Some examples of films that could be produced are films wherein there is a gradient of wax composition, reflecting the diffuse intracuticle-epicuticle interface. Additionally, films containing localised regions of increased polar wax and possibly triterpenoids to replicate the polar pores, enable testing of hydrophilic agrochemical penetration. A final suggestion is production of clearly defined wax interfaces within a single print. Section 4.3.6 showed at the print track interfaces there was induced chemical heterogeneity within a film containing only a single binary printed wax. It would be interesting to investigate chemical distribution at the interface of two unique binary waxes to assess if it possible to induce and control chemical distribution within an individual droplet.

6.1.2.2. Fungal attachment measurements and fungal growth imaging

As shown in Section 5.3.7 there was a significant relationship between fungal attachment and the wax composition. However no clear relationship between topography or hydrophobicity and fungal attachment was identified. Subsequently, further work, particularly if the library of waxes is expanded, should include an investigation of *Z. tritici* attachment onto a greater range of wax films printed with different printing parameters to induce physical changes in the printed film.

Further to this there is scope to extent the experimentation beyond evaluation of the interactions of *Z. tritici* and measure the attachment of additional fungal spores. This could then be used to guide decisions regarding the modification of cuticle wax composition in commercial plants to minimise or eliminate fungal attachment.

In addition to this imaging of the fungal spore should be conducted. A simple example would be optical images of the spore infection at set timepoints to observe the visual changes and if a clear site of infection could be identified. Additional work could include the use of Cyro-SEM imaging to investigate the development of hyphae structures. This would provide information

on the nature of the fungal attachment and allow evaluation of the directionality of hyphae growth.

6.1.2.3. Printed wax models compared with alternative methods of modelling the cuticle waxes

In this thesis the focus was the production of wax films using 3D printing to enable the freedom of design, and design flexibility implicit to 3D printing and avoid the use of solvents which might modify the formation of crystalline structures relative to similar structures in native cuticles. However, it must be understood that the printing methodology which introduces the waxes to high pressures and rapid cooling is also not representative of the formation of the wax component in the native cuticle. Subsequently, future work should seek to identify methods to better replicate the natural biosynthesis and distribution of cuticle waxes. The use of a cutin mimic, that the wax could be printed onto and allowed to impregnated might be a method of inducing the formation of crystalline structures that replicate native cuticle waxes.

The primary alternative method for producing a wax film modelling the cuticle wax is evaporation of a solution containing solubilised wax which will leave the wax film of interest. This solvent based method allows the generation of thinner films, compared with 3D printed films, albeit with less design complexity. However, this method also uses a method of delivering the wax that is not representative of natural processes. This means the wax film will not accurately replicate the structures found in the native cuticle. Future work should compare the properties of films produced via 3D printing with solvent evaporation produced films so to identify the methodology that best replicates the physical properties and behaviours of the native cuticle waxes.

6.1.2.4. In depth surface chemical composition analysis

As shown in Section 3.3.1 and Section 4.3.6 it is possible to perform chemical composition analysis of the native cuticle, and the printed films to identify chemical distribution heterogeneity. This work should be expanded in two ways. The first is further evaluation of chemical distribution heterogeneity within the printed films and testing methodologies that might be able to control the development of this heterogeneity. As shown in Section 4.3.6 chemical composition heterogeneity occurred in the same locations as crystalloids formed. This should be further explored to identify if chemical heterogeneity induces crystalloid formation

and if it can be controlled. As shown in Section 4.3.2.3 modifying substrate temperature resulted in changes to the morphology of the printed film through modification of crystalloids density.

An additional area of research should be the use of ToF-SIMs to further investigate permeation. Most of the films tested did not show any permeation over 7 days so it would be beneficial to perform ToF-SIMS depth penetration analyse to elude the fate of the applied agrochemicals. This could be used with further work proposed in Section 6.1.1.3 to determine why penetration in these films was not observed. Once such methodologies have been developed it could be used to explore the fate of other materials exposed to the films such as surfactants.