Cuticular penetration studies

Pathways for diffusion and effects of adjuvants

Dissertation
zur
Erlangung des Doktorgrades (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Moritz Haberstroh

aus

Lörrach

Bonn, 2025

Angefertigt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

Gutachter/Betreuer: Prof. Dr. Lukas Schreiber

Gutachterin: Prof. Dr. Kerstin Koch

Erscheinungsjahr: 2025

Tag der Promotion: 26.08.2025

Table of Contents

Affirmation

I herewith declare that I have written this thesis independently and by myself. I have used no other source than those listed. I have indicated all places where the exact words or analogous text were taken from source. I assure that this thesis has not been submitted for examination elsewhere.

_____ _____

Bonn, date Moritz Haberstroh

List of abbreviations

Physical variables and derived dimensions are abbreviated according to the International System of Units (SI). Chemical elements will be referred to as their symbols and compounds are abbreviated with their chemical formula.

4-NP	4-Nitrophenol
AI	active ingredient
ANOVA	analysis of variance
APG	Alkyl polyglycosides
CA	Contact angle
CM	Cuticular membrane
CMC	Critical micelle concentration
DON	Donor
E	Effect
EO	Ethylene oxide
EtOH	Ethanol
F	Flow
HLB	Hydrophilic-lipophilic-balance
K_{CW}	Cuticle-water-partition coefficient
K_{OW}	Octanol-water partition coefficient
MG	Methyl-glucose
min	minute
MW	Molecular weight
MX	Polymer matrix

n	Sample size (statistics)
P	p-value
P	Permeance
PLS	Phospholipid suspension
PTFE	Polytetrafluorethylene
R	Correlation coefficients
RME	Rapeseed methyl ester
rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SLES	Sodium lauryl ether sulphate
SOFP	Simulation of foliar uptake
TBA	Terbuthylazine
TM-AFM	Tapping mode atomic force microscopy
TMX	Thiamethoxam
UDOS	Unilateral desorption from the outer surface
UV	Ultraviolet radiation
Vx	Characteristic molecular Volume

List of tables

List of figures

Introduction

Protection against uncontrolled water loss of all aerial organs of higher terrestrial plants is provided by a structure that evolved more than 400 million years ago: the plant cuticle (Edwards 1993). Besides its main function of preventing desiccation (Kenrick and Crane 1997, Riederer and Schreiber 2001), the cuticle shields the sensitive photosystems against UV radiation (Grant et al. 1995, Krauss et al. 1997, Liakoura et al. 1999) and provides mechanical stability (Matas et al. 2004, Bargel and Neinhuis 2005). Furthermore, the cuticle forming the interface between the plant and the environment (Schönherr 1976, Bargel et al. 2004, Yeats and Rose 2013) is the first barrier against infestation of insects or pathogens (Lindow and Brandl 2003, Serrano et al. 2014, Gorb and Gorb 2017) and limits leaching of nutrients from the leaf interieur (H. B. Tukey 1970).

Microscopic and chemical investigations of enzymatically isolated cuticular membranes revealed a two-layered structure consisting of cutin, waxes, and polysaccharides (Jeffree 2006, Fernández et al. 2016, Fernández et al. 2021). The outermost layer is the cuticle proper, below the cuticular layer is deposited (Jeffree 2006). The latter is interspersed with polysaccharide microfibrils that build a ramified network (Guzmán et al. 2014a, Segado et al. 2016) and connects the cuticle to the cell wall with a pectinaceous layer (Yeats and Rose 2013). The cuticle proper consists mainly of cutin and associated waxes, whereas polysaccharides are only barely traceable (Guzmán et al. 2014a). Intracuticular waxes embedded in the cutin matrix form the transport-limiting barrier of the plant cuticle (Schönherr and Riederer 1989). Epicuticular waxes located on the surface of the cuticle render the surface hydrophobic and water repellent (Neinhuis and Barthlott 1997).

The surface waxes can exhibit simple amorphous films and granules, but also more complex structures of crystals with various shapes (Barthlott et al. 1998, Jeffree 2006, Koch and Ensikat 2008, Koch and Barthlott 2009, Koch et al. 2009). The commonly known Lotos effect is named after the excellent water repellency and self-cleaning properties of *Nelumbo nucifera* leaves. The leaf surface is very rough leading to a reduced contact area for adhesion of pollutants and water. Impacting raindrops or even dew or fog will run off and take dust or pollutants along with them (Neinhuis and Barthlott 1997). Removal of epicuticular waxes did not alter the

barrier properties of the cuticle against water loss (Zeisler and Schreiber 2016, Zeisler-Diehl et al. 2018). Whereas a total wax extraction led to an 100 to 1000-fold increase in water permeability (Schreiber 2010). The thickness of cuticles can vary widely among species and plant organs. Leaf cuticles of *Arabidopsis thaliana* have only a thickness of 30 nm, whereas fruit cuticles of *Malus domestica* can possess a thickness of 30 µm (Schreiber and Schönherr 2009). There is no correlation between the thickness of a cuticle and the permeability to water and other organic solutes, since the pathway for diffusion across the cuticle can be longer than the cuticular thickness (Schreiber et al. 1996a, Baur et al. 1999a, Riederer and Schreiber 2001).

The uptake of substances into the plant leaf can be described as a process comprising three steps. Firstly, the adsorption of the substance on the plant surface, followed by the diffusion through the cuticle and finally the desorption into the aqueous medium of the apoplast (Kirkwood 1999). The diffusion of molecules through the cuticle is a passive process and not an active transport since the cuticle is not a metabolic membrane. Depending on the lipophilicity of the molecules there are different ways for cuticular permeation (Figure 1). Literature distinguishes between two models: the lipophilic pathway for organic solutes and the polar pathway for small, hydrophilic molecules and ions (Buchholz 2006, Schönherr 2006, Schreiber and Schönherr 2009).

Figure 1: Model of the lipophilic and polar pathway within the cuticle.
Wax domains (dark grey blocks) are accessible by lipophilic solutes, while polysaccharide fibrils (grey lines traversing the cuticle) were used for diffusion of small hydrophilic molecules and ions.

The main barrier to water and lipophilic organic solutes is constituted by cuticular waxes. It is assumed that lipophilic substances permeate through wax domains comprising crystalline and

amorphous fractions. The crystalline fraction in the cuticle is not accessible whereas the amorphous area can be permeated by lipophilic compounds.

Hydrophilic domains within the cuticle are thought to form the polar pathway. These domains comprise polysaccharides and polar groups of the cutin matrix and are presumed to be randomly distributed and clustered (Chamel et al. 1991, Luque et al. 1995). During hydration of the cuticle, water is absorbed mainly by polysaccharides (Chamel et al. 1991). The polysaccharide fibrils are thought to build a ramified network, connecting the surface of the cuticle with inner regions and forming a continuous pathway (Guzmán et al. 2014a, Fernández et al. 2017). This pathway is permeable for hydrophilic compounds, but can be partially blocked by waxes (Popp et al. 2005, Arand et al. 2010, Staiger et al. 2019).

Agrochemicals are most commonly applied diluted in water as foliar sprays. From passing the nozzle of spray droplets to the uptake of the active ingredient into the plant several processes occur (Figure 2, I to V). To influence each of the mentioned processes there are different types of adjuvants in crop protection products integrated that are classified according to where they take effect (Hazen 2000).

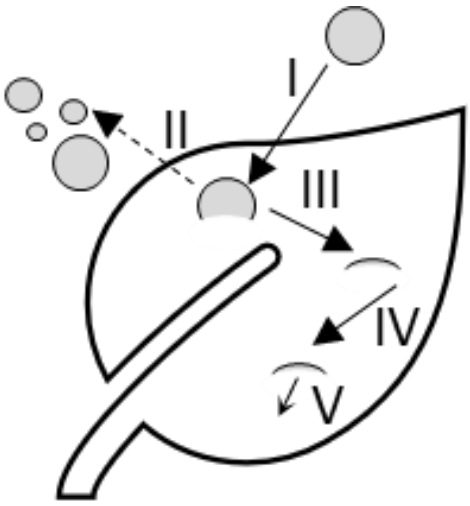

Figure 2: Possible processes of droplets sprayed on leaf surfaces (I).
The droplets can bounce off (II) or stick to the leaf surface and spread (III), subsequently dry (IV) and penetrate into the plant (V).

From passing the spray nozzle of the application device to impacting on the plant surface, it takes less than a second (Figure 2, I) (Wirth et al. 1991). In this time the surface tension has to be sufficiently reduced that the droplet will retain on the plant surface. The sprayed droplet can bounce off or shatter and is, in the worst case, no longer available for uptake (Figure 2, II). For increasing the retention of the droplets, stickers and retention adjuvants are used. These are mainly surface active agents (surfactants), which can decrease the dynamic surface tension in a short period of time. The dynamic surface tension influences the retention of the spray droplet beside the size and the velocity (Wirth et al. 1991, Forster et al. 2012).

Once the droplet is adhering to the leaf, spreading over the surface is beneficial, particularly for active ingredients that do not have to be systemically distributed within the plant because their preferential site of action is on the leaf surface. To enforce this process (Figure 2, III) wetting and spreading adjuvants are used, which further decrease the surface tension and therefore the contact angle of the spray droplet. A low surface tension is perquisite for spreading in particular on leaf surfaces which are hard to wet (Stock and Holloway 1993, Dorr et al. 2014).

After the droplet sticks to the leaf, water will start to evaporate and the droplet will dry out (Figure 2, IV). Humidity and temperature are influencing this process, which can be impeded by the addition of hygroscopic substances. These are called humectants and prevent the droplet from drying out by absorbing moisture from the atmosphere and therefore keeping the active ingredient dissolved (Asmus et al. 2016). Hence, time for diffusion into the plant is prolonged, and efficacy is increased.

The penetration of the active ingredient into the plant is the last process and can be improved by increasing the mobility of the substance in the cuticle (Figure 2, V). Adjuvants for improving cuticular penetration are called accelerators or plasticizers (Schönherr 1993, Baur et al. 1997b). It is assumed that accelerators improve penetration of lipophilic substances by increasing the amorphous wax fraction and/or decreasing the crystalline wax fraction within the cuticle. This result in an increase of the diffusion rate of the agrochemicals and a decreased path length for diffusion (Buchholz 2006, Fagerström et al. 2014).

This dissertation is an attempt to provide a deeper understanding and improved methods for measuring the uptake and diffusion of substances, such as agrochemicals, across the hydrophobic cuticle into plant leaves. In the past, and still common, barrier and transport properties of plant cuticles were largely studied using the so-called cup method and modifications thereof (Schönherr and Lendzian 1981, Schreiber and Schönherr 2009). The experimental setup used for measuring mass flow in transient state is called simulation of foliar penetration (SOFP) whereas steady-state experiments are conducted with the double chamber system (Figure 3).

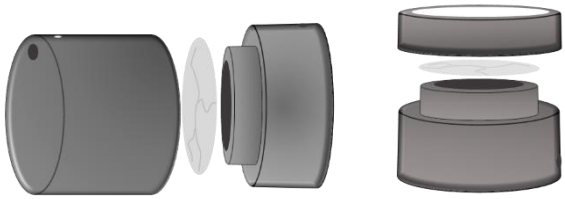

Figure 3: Experimental setup for measuring mass flow.
Shown on the left setup for steady state (double chamber system) and right transient state (SOFP) experiments.

With the latter, cuticular permeances of agrochemicals, nutrients, and environmental pollutants can be determined. The system consists of two half cells with an isolated cuticular membrane mounted in between. The physiological outer surface of the cuticle is facing to the donor compartment which is filled with an aqueous solution containing the compound to be examined. Solutes diffuse across the cuticular membrane from the donor compartment into the receiver. The receiver medium is removed periodically, the content of solutes determined and replaced by fresh one.

Permeances P (m s^{-1}) can be calculated according to the following equation:

$$P = \frac{F}{A} * \Delta c \qquad (1)$$

The slope of the linear regression line fitted to the transport kinetic (amount vs. time) represents the flow F (mol s^{-1}). A (m^2) stands for the area of the cuticle exposed to the donor solution. Due to the frequent exchange of the receiver medium, the solute concentration in the receiver compartment is essentially 0 during the experiment and the driving force for diffusion Δc (mol m^{-3}) is the donor concentration alone.

A method closer to the situation in the field is the above mentioned SOFP, since it reflects the situation after foliar spray application where small aqueous droplets containing the active ingredients sit on the leaf surface and dry out (Schreiber and Schönherr 2009). For this method cuticular membranes are mounted in such a way that the physiological inner side is facing towards the receiver compartment and fixed with a ring shaped lid (Figure 3). The physiological outer side of the cuticle is facing towards the atmosphere and single droplets can be applied. The concentration of penetrated compound in the receiver medium is determined (M_t) at regular intervals (t) and the compartment refilled with fresh medium.

When plotting the penetration of the compound as $-\ln(1-M_t/M_0)$ versus time (t), with M_t being the relative amount of donor left on the cuticle at time t and M_0 being the total amount of the compound applied at time 0, the slope of the fitted regression lines represents the rate constant of penetration k (h^{-1}).

$$k = \frac{-\ln\left(1-\frac{M_t}{M_0}\right)}{t} \quad (2)$$

By adjusting the humidity and the temperature of the atmosphere, different field situations can be simulated.

The effects (E) of air humidity, temperature and adjuvants, acting for example as plasticizers or wetting agents, on rates of cuticular permeability can be quantified by dividing the rate constant obtained with specific treatments (k_2) by the rate constant of the control experiments (k_1).

$$E = \frac{k_2}{k_1} \quad (3)$$

Effects above 1 indicate an increase in permeation, e.g. when adjuvants are added, whereas effects below 1 indicate a negative impact of the specific treatments.

In the first chapter, a newly developed approach is presented to study the plasticizing effects of surfactants on the permeation of a model compound across isolated leaf cuticles of *P. laurocerasus*. It was the intention to establish a cost-effective and straightforward method for measuring cuticular permeability without using radioactive model substances. With the

double chamber system (Figure 3), an experimental setup was chosen that allows the measurement of steady-state diffusion, the calculation of permeances, and their comparison. Fluorescein was used as a model compound because it has similar physiochemical properties in terms of molecular size, molecular volume, water solubility and lipophilicity compared to agrochemicals commonly used in the field. For validation of the new approach permeability determined with radiometry and fluorometry were compared. Both approaches resulted in the same values and it could be shown that the fluorometric approach represents a method which suits well as an alternative for radioactive measurements. The sensitivity of fluorometry allows the detection of minute amounts of penetrated fluorescein, which makes it a reliable and cost-effective method to screen the enhancing effects of surfactants on permeation of agrochemicals. The method was used to study the plasticizing effects on the cuticular permeability of alcohol ethoxylates, varying in their average degree of ethoxylation from 5 to 20 ethylene oxides. It could be shown that with decreasing lipophilicity and increasing size of the surfactants their potential accelerating the diffusion of fluorescein decreased.

In the experiments described in the following two chapters of this thesis SOFP (simulation of foliar penetration) was used as experimental setup. This method was used to measure the diffusion of different solutes through the cuticle and the rate constants of penetration were calculated and compared.

The second chapter describes the permeation of compounds differing in their lipophilicity across isolated cuticular membranes of *Prunus laurocerasus*. It was postulated that lipophilic and polar substances diffuse in the plant cuticle *via* two different pathways. Whereas lipophilic substances can diffuse through the hydrophobic wax and cutin domains, called the lipophilic pathway, polar substances were assumed to diffuse *via* the polar pathway, which may be formed by hydrated polar functional groups of the cutin and polysaccharide fibrils (Schreiber 2005, Buchholz 2006, Fernández et al. 2016). The relative contribution of each pathway on uptake of solutes should be determined by blocking the polar pathway with silver chloride precipitates (Schreiber *et al.* 2006). NaCl needs to be added on the inner side of the isolated cuticle and $AgNO_3$ on the physiological outer side of the cuticle. Thus, Ag^+ and Cl^- ions will diffuse in the polar paths of transport within the cuticle towards each other and once they meet they will form

insoluble AgCl precipitates. These insoluble silver chloride crystals will block the polar paths of transport within the cuticle while the lipophilic path in wax and cutin remaining unaffected. Obtained results in fact confirmed this concept of the two different pathways of transport within the cuticle since rate constants of diffusion of polar methyl-glucose (log K_{OW} : -3.0) were significantly reduced while penetration of lipophilic terbuthylazine (log K_{OW} : 3.4) was not altered.

In chapter three the effects of adjuvants differing significantly in their structure and physicochemical properties on the diffusion of polar, intermediate polar and lipophilic compounds were investigated. Two hydrophilic surfactants (alkyl polyglucoside and sodium lauryl ether sulphate) and a mixture of methylated rapeseed oils were chosen to cover a broad spectrum of adjuvants used in agriculture. Results of the study provide further convincing evidence for the two different pathways of diffusion in cuticles. Polar alkyl polyglucoside was not enhancing the cuticular permeability of any of the used solutes, since it penetrates the cuticle very slowly (Schönherr 2001) and it can be assumed that the major fraction of the applied amount remains on the outer cuticle surface. However, adjuvants that penetrated the cuticle and plasticized the lipophilic pathway increased the penetration of semi-polar and lipophilic compounds.

Chapter 1

Studying cuticular penetration of agrochemicals using a newly developed fluorometric method: the effect of surfactants enhancing the diffusion of fluorescein in cuticles isolated from cherry laurel (*Prunus laurocerasus*)

1.1 Abstract

In the past cuticular permeability of agrochemicals was largely studied using radiolabelled compounds. The aim of this study was to find a non-radioactive, cost-effective and straightforward method with a high sensitivity for screening surfactants potentially enhancing the penetration of a non-radiolabelled model substance across isolated cuticles from *Prunus laurocerasus*.

Physicochemical properties of fluorescein (molecular weight, molar volume, log K_{ow}, predicted and measured log K_{cw} and predicted and measured permeances) fit very well to the well-known systemic fungicide epoxiconazole, which has been used in the past as model compound in many cuticular permeability studies. Therefore, fluorescein is an appropriate model substance representing lipophilic active ingredients of pesticides in general. Permeances of fluorescein were 4.28 (2.33 – 6.91) x 10^{-11} m s^{-1} for the fluorometric experiment and 5.38 (4.38 – 7.30) x 10^{-11} m s^{-1} for the radiometric experiment. Thus, both approaches resulted in the same values and the fluorometric method is a well suited alternative for the radiometric approach in future. Alcohol ethoxylates can accelerate the permeation of fluorescein. With increasing HLB values, decreasing lipophilicity and increasing size of the alcohol ethoxylates their accelerating effects on the diffusion of fluorescein across isolated cuticles of *P. laurocerasus* decreased. Average median effects were 17 ($C_{18}E_5$), 4.6 ($C_{18}E_{10}$) and, 1.7 ($C_{18}E_{20}$).

The results showed (i) that with fluorescein a model substance was found with physicochemical properties characteristic for many common agrochemicals and (ii) that fluorometry represents a highly sensitive and reliable cost-effective method for studying the enhancing effects of surfactants on cuticular permeability of fluorescein. Therefore, this method can be used for screening of surfactants for their potential enhancement of cuticular permeability.

Keywords: cuticular transport, alcohol ethoxylates, diffusion, plant cuticle, surfactants

1.2 Introduction

Leaf surfaces are covered by the hydrophobic plant cuticle forming the interface between leaves and the atmosphere (Schreiber and Schönherr 2009, Fernández et al. 2016). The main function of plant cuticles consists in protecting leaves from uncontrolled water loss if plants are suffering from water deficiency and stomata are closed (Schönherr 1982, Riederer and Schreiber 2001). In addition, they limit the leaching of nutrients from the leaf interior (H. B. Tukey 1970) and they represent the first physical barrier hindering pathogens from infecting leaves (Serrano et al. 2014). Cuticles are composed of the lipophilic cutin polymer and of cuticular waxes, which are deposited as intracuticular waxes within the cutin polymer and as epicuticular waxes on the surface of the cutin polymer (Jeffree 2006). Whereas, intracuticular waxes are largely establishing the cuticular transport barrier (Schönherr and Riederer 1989, Schreiber 2010), epicuticular wax crystallites are rendering leaf surfaces non-wettable (Neinhuis and Barthlott 1997, Koch and Barthlott 2009).

Plant protecting agents are often sprayed onto the plant cuticle covering leaf surfaces (Arand et al. 2018, Krahmer et al. 2021). Consequently, highly impermeable plant cuticles form a major transport barrier for the diffusional foliar uptake of agrochemicals (Bargel et al. 2004). Besides the agrochemical, spray solutions contain adjuvants having a series of various function. They have to ensure adhesion of the spray droplets to the hydrophobic leaf surfaces (Gaskin et al. 2005), they enhance leaf surface wetting and spreading of the spray deposit on the leaf surfaces and they act as plasticizers on the cuticular transport barrier thus enhancing foliar uptake of agrochemicals (Fagerström et al. 2013, Castro et al. 2014). Thus, an optimization of foliar uptake of agrochemicals needs to improve diffusion of agrochemicals through the highly impermeable plant cuticle (Schönherr and Riederer 1989).

In the past diffusion of agrochemicals across isolated plant cuticles was largely studied using radiolabelled compounds (Buchholz and Schönherr 2000, Aponte and Baur 2010, Zeisler-Diehl et al. 2022). This offered an extremely high sensitivity allowing the detection of minute amounts of compounds which had penetrated across the cuticle (Zeisler-Diehl et al. 2017). However, working with radioactivity is costly for several reasons: (i) the availability of a special radionuclide laboratory with high safety standards is mandatory, (ii) radiolabelled compounds

of interest, which need to be obtained by an expensive custom synthesis service of special companies, if not available commercially, are needed and (iii) the disposal of radioactive waste produced in the experiments is cost-intensive. Therefore, the present work was conducted to find a non-radioactive, cost-effective and straightforward method for measuring cuticular permeability of a non-radioactive model substance. This method should provide a high sensitivity for the detection of the amounts of substance, which had penetrated across the cuticle, and it should allow working with a compound, which in fact represents a representative substitute for active ingredients normally used as plant protection agents. Here we present a newly developed approach measuring cuticular diffusion of fluorescein across isolated cuticle from *Prunus laurocerasus* by fluorometry.

1.3 Material and Methods

1.3.1 Plant material

Astomatous adaxial cuticles (CMs) of fully expanded and healthy leaves of *Prunus laurocerasus* (cherry laurel) were isolated enzymatically according to the method of Schönherr and Riederer (Schönherr and Riederer 1986). Leaf discs were punched out with a cork borer and incubated at room temperature in an enzymatic solution containing 2 % cellulase (Erbslöh, Geisenheim, Germany) and 2 % pectinase (Erbslöh, Geisenheim, Germany). The enzyme solution was buffered at pH 3 with citric acid (Carl Roth, Karlsruhe, Germany) and 1 mol l^{-1} sodium azide (Carl Roth, Karlsruhe, Germany) was added to prevent microbiological growth. After 3 to 5 days astomatous cuticles were separated from remaining plant material and kept in fresh enzymatic solution until digestion of the cell debris adhering to the inner side of the cuticle was complete. Since during enzymatic isolation phenolic acids, which are released from the cells, sorb to the lipophilic cuticle (Schönherr and Riederer 1986), isolated cuticles were extensively extracted with borate buffer at pH 9 (Sigma Aldrich, St. Louis, USA). This extracts adsorbed phenolics again, which might interfere with the fluorometric determination of the transport of fluorescein across isolated cuticles. Finally, cuticles were washed with deionised water, dried using a gentle stream of air and stored in petri dishes until further use.

1.3.2 Chemicals

Fluorescein (Sigma Aldrich, St. Louis, USA; 95 % purity) was used as a model compound in cuticular permeation experiments. ^{3}H-labelled fluorescein (radiochemical purity > 99 %, specific radioactivity: 110 Ci $mmol^{-1}$) was obtained from ARC (St. Louis, USA). Genapol O 050, Genapol O 100, and Genapol O 200 (Clariant, Muttenz, Switzerland), are alcohol ethoxylates based on unsaturated fatty alcohols (major component oleyl alcohol abbreviated in the following as C_{18}) differing in their degree of ethoxylation (EO).

Table 1: Characteristic properties of epoxiconazole and fluorescein

	Epoxiconazole	Fluorescein
MW (g mol^{-1})	329.76	332.31
V_X (cm^3 mol^{-1})	222.37[a]	227.97[a]
log K_{OW}	3.44	3.35
log K_{CW} (predicted)	3.39[b]	3.31[b]
log K_{CW} (measured)	3.19[c]	3.36 ± 0.05[d]
P $x10^{-11}$ (m s^{-1}) (predicted)	3.6[e]	3.9[e]
P $x10^{-11}$ (m s^{-1}) (measured)	14 ± 11[f]	4.62 (2.84-6.94)[d]

MW (Molecular weight), V_x (characteristic molecular volume), log K_{OW} (Octanol-water partition coefficient), log K_{CW} (Cuticle-water partition coefficient), P (Permeance)

[a] Calculated according to Abraham and McGowan (1987)

[b] Calculated according to Schönherr and Riederer (1989)

[c] Ballmann (2011), shown is Mean ± standard deviation

[d] Own determination with isolated cuticular membranes of *P. laurocerasus*

[e] Calculated according to Schreiber and Schönherr (2009)

[f] Ballmann (2011), shown is Mean ± 95 % confidence intervals

1.3.3 Transport chambers

Stainless steel chambers were used for transport experiments as described by Schreiber (Schreiber et al. 1995). Isolated cuticles were mounted between the two halves of the chamber system, with the physiological outer side facing towards the donor compartment. Contact areas between cuticles and chambers were sealed with teflon paste (PTFE-Paste, Carl Roth, Karlsruhe, Germany). The two halves of the chambers with the cuticle mounted in between were fixed using adhesive tape (Tesa, Norderstedt, Germany). Donor compartments and sampling of the borate solution in the receiver compartments was done using 1 ml syringes (Hamilton, Reno, USA). Sampling ports were closed by metal stoppers and sealed with adhesive tape (Tesa). Chambers were carefully rotated during the experiments at 10 RPM on a

roller mixer (BTR 10P, Ratek Instruments, Boronia VIC, Australia) and kept constantly at 25 °C in an incubator (MIR-554, Panasonic, Kadoma, Japan).

1.3.4 Fluorometry

A spectrofluorometer (Spark 10M, Tecan, Männedorf, Switzerland) was used to quantify fluorescein. The excitation wavelength was 482 nm and the emission was measured at a wavelength of 527 nm. For the measurements black 96 well-plates (Sterilin Black Microtiter Plates, Thermo Scientific, Waltham, USA) were used. Calibration curves correlating the fluorescence values with the respective concentrations were linear over the observed concentration range (10^{-9} to 10^{-7} mol l^{-1}) used in the experiments. Correlation coefficients r of regression lines fitted to the linear transport kinetics (amount penetrated vs. time) were linear and always better than 0.99.

1.3.5 Radiometry

For quantification of the ^{3}H-radiolabelled fluorescein, diffusing across the cuticles, the samples were dissolved in scintillation cocktail (Ultima Gold XR, PerkinElmer, Waltham, USA) and quantified in a liquid scintillation analyser (Tri-Carb 4910TR, PerkinElmer, Waltham, USA).

1.3.6 Measurement of cuticle/water partition coefficients

Mass of individual cuticles ($mass_{CM}$) was measured with a microbalance (CPA225D, Sartorius Lab Instruments, Göttingen, Germany) and it varied between 1 mg and 2 mg. Cuticles were equilibrated in glass vials with 5 ml ^{3}H-labelled fluorescein solutions at a concentration of 1.43×10^{-11} mol l^{-1} buffered at pH 3. The glass vials were rotated for 24 hours on a roller mixer at 25 °C. CMs were sampled and blotted dry to remove radioactive ^{3}H-fluorescein solution adhering to the outer cuticle surface. Radioactivity in CMs ($amount_{CM}/mass_{CM}$) and the external donor solutions ($amount_{DON}/mass_{DON}$) were determined by liquid scintillation counting (Riederer and Schönherr 1984). Cuticle/water partition coefficients (K_{CW}) between incubated CMs and donor solutions were calculated using the equation:

$$\mathrm{KCW} = \frac{\frac{\mathrm{amount}_{CM}}{\mathrm{mass}_{CM}}}{\frac{\mathrm{amount}_{DON}}{\mathrm{mass}_{DON}}} \quad (1)$$

1.3.7 Measuring transport kinetics

The donor compartment was filled with 3.2 µg fluorescein dissolved in 800 µl citrate buffer (pH 3, 10^{-2} mol l^{-1}, Carl Roth, Karlsruhe, Germany) in case of the fluorometric method. This resulted in a final fluorescein concentration of 1.20 x 10^{-5} mol l^{-1}. 13 kBq ^{3}H-Fluorescein per 800 µl were dissolved in citrate buffer in case of the radioactive method. This resulted in a final fluorescein concentration of 4.10 x 10^{-9} mol l^{-1} in the donor compartment. 800 µl of borate buffer (pH 9, 10^{-2} mol l^{-1}, Sigma Aldrich, St. Louis, USA) were filled in the receiver compartment. Donor solutions were buffered at pH 3.0 to ensure that all fluorescein molecules are in the non-ionized form, since only the non-ionized form of the molecule will diffuse across the lipophilic cuticle (Schreiber et al. 1995). Receiver solutions were buffered at pH 9.0 since this ensure that all fluorescein molecules, which had diffused across the cuticle, are trapped in the receiver in their ionized form. This does not allow fluorescein diffusing back to the donor compartment (Schreiber et al. 1995). Receiver solutions were periodically replaced with fresh borate buffer and the fluorescein content was determined by either fluorometry or scintillation counting. For measuring potential effects of surfactants on cuticular permeation of fluorescein, surfactants were added to the receiver solutions of borate buffer at 0.1 %. Surfactant concentrations were kept constant during the experiments due to the high concentration of surfactant (significantly above the critical micelle concentration) and the periodical replacement of the receiver solutions.

1.3.8 Calculation of permeances

Permeances P (m s^{-1}) were calculated according to the following equation:

$$P = \frac{F}{A} * \Delta c \qquad (2)$$

The flow F (mol s^{-1}) is the slope of the linear regression line fitted to the transport kinetic (amounts vs. time). A (m^2) is the area of the cuticle (1.13 x 10^{-4} m^2) exposed to the donor solution. Δc (mol m^{-3}) represents the driving force, which is given by the concentration difference of fluorescein between donor and receiver (c_{don} - c_{rec}). Since the fluorescein concentration in the receiver is essentially 0, due to the frequent exchange of the receiver solutions during sampling and due to the basic pH of 9 (Kerler et al. 1984), Δc is given by the donor concentration alone.

1.3.9 Sample size and statistics

For measuring fluorescein permeability at least 15 isolated cuticular membranes per experiment were investigated. Cuticle-water partition coefficients were determined with 5 cuticles isolated from upper side of *Prunus laurocerasus* leaves. The statistical analysis was computed using OriginPro (Version 2021, OriginLab, Northampton, USA). The interquartile range method was used to remove outliers. Data for permeances were not normally distributed according to the Shapiro-Wilk test ($P < 0.05$). Wilcoxon signed-rank test was used to determine statistically significant differences.

1.4 Results

Plotting the amounts of fluorescein permeated across the cuticles over time resulted in linear slopes for the fluorometric method with a slight increase after 384 hours (Figure 4, b). For the radiometric method biphasic kinetics were observed (Figure 4, a). An initial steep slope of the transport kinetics within the first 24 to 32 hours was followed by a significantly flatter slope of the transport kinetics, which was constant over 48 to 172 hours (Figure 4, a). Plotting the linear intervals between 48 to 172 hours measured with both, the radioactive and the fluorometric method, resulted in comparable slopes (Figure 4, c).

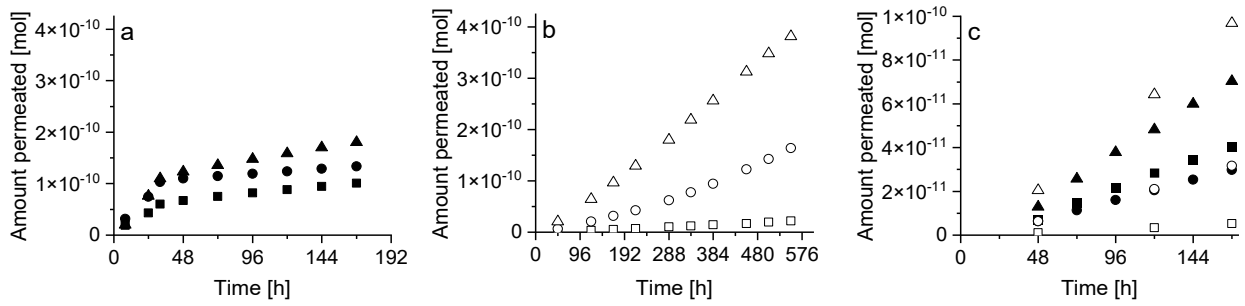

Figure 4: Permeation kinetics.
Amounts of fluorescein diffused across cuticular membranes isolated from *Prunus laurocerasus* vs. time determined by (a) radiometry and (b) fluorometry Three representative examples for highest, lowest and medium permeation rates measured with both experimental systems are shown in (c) Plot of the linear phases of the permeation kinetics between 48 and 168 hours of steady state diffusion of non-labelled fluorescein (white symbols) and ^{3}H-labelled fluorescein (black symbols) across isolated cuticles.

Incremental permeances calculated from the slopes between two neighbouring measuring points of the fluorometric experiment were constant up to 236 hours and then slowly increased (Figure 5, a). With the radioactive method incremental permeances were very high with the first 32 hours and decreased to significantly lower but constant permeances between 32 to 168 hours (Figure 5, b). Total amounts of fluorescein which had diffused across the cuticle between 0 and 384 hours were about 1 % of the amounts added to the donor compartment (Figure 5, c). In the radioactive transport experiment about 1 % of the fluorescein amounts added to the donor compartment had diffused across the cuticle within the first 32 hours, whereas only another 0.5 % were diffusing across the cuticle within 32 to 172 hours (Figure 5, d).

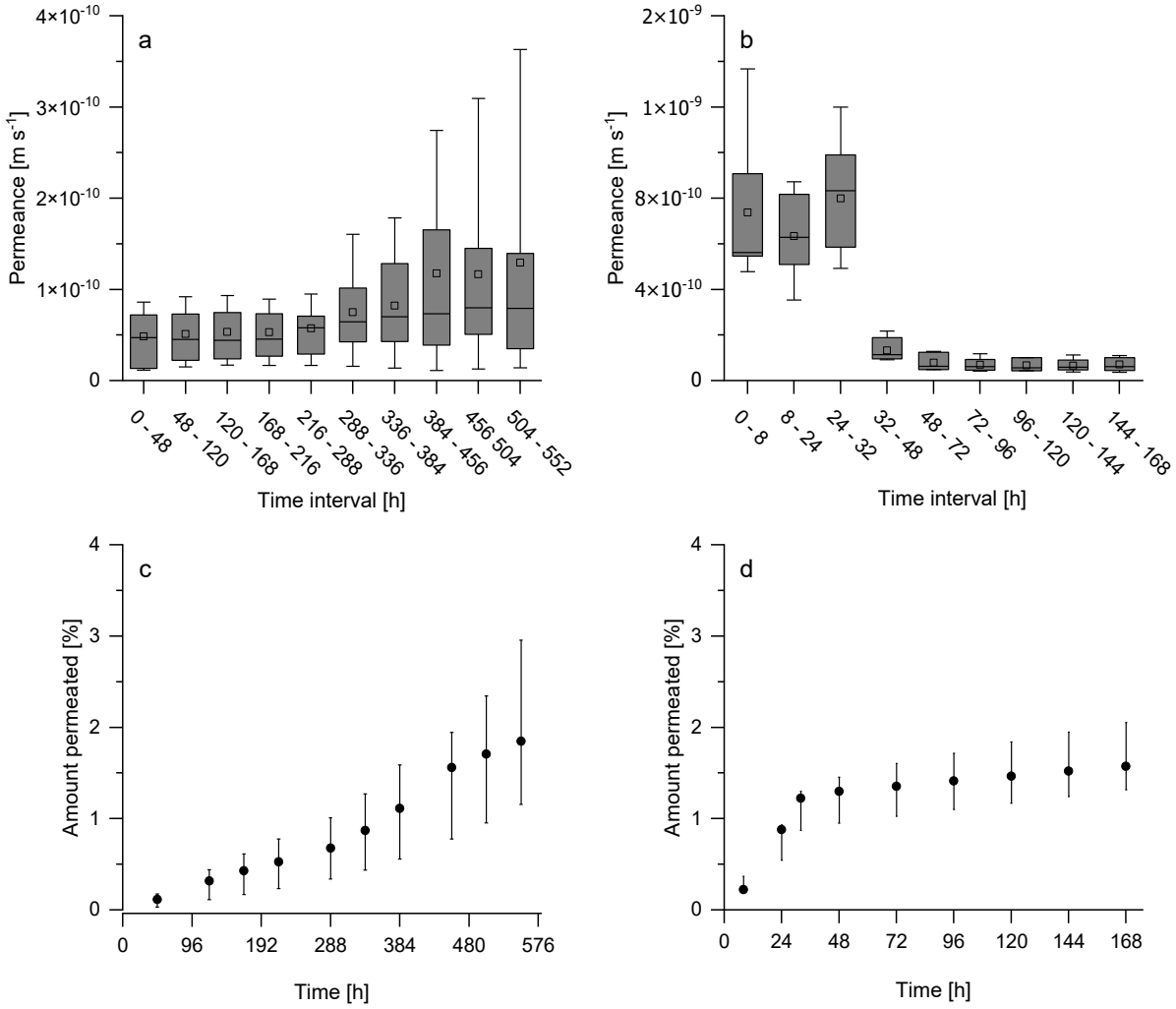

Figure 5: Incremental permeances.
Calculated from the slopes of the figures 4a and 4b between two neighbouring measuring points for (a) the fluorometric method and (b) the radiometric method. Permeances were not different between the two methods comparing the time interval of (a) 0 to 288 hours of the fluorometric experiment with the time interval of (b) 32 to 168 hours of the radiometric experiment. Plots of relative amounts (%) of fluorescein which had diffused across the cuticles (c) in the fluorometric experiment and (d) in the radiometric experiment. Boxes of the box plots in (a) and (b) represent 25th and 75th percentiles. Whiskers indicate 10th and 90th percentiles. Squares and horizontal lines within the boxes represent mean and median, respectively. Medians and quartiles are shown in (c) and (d).

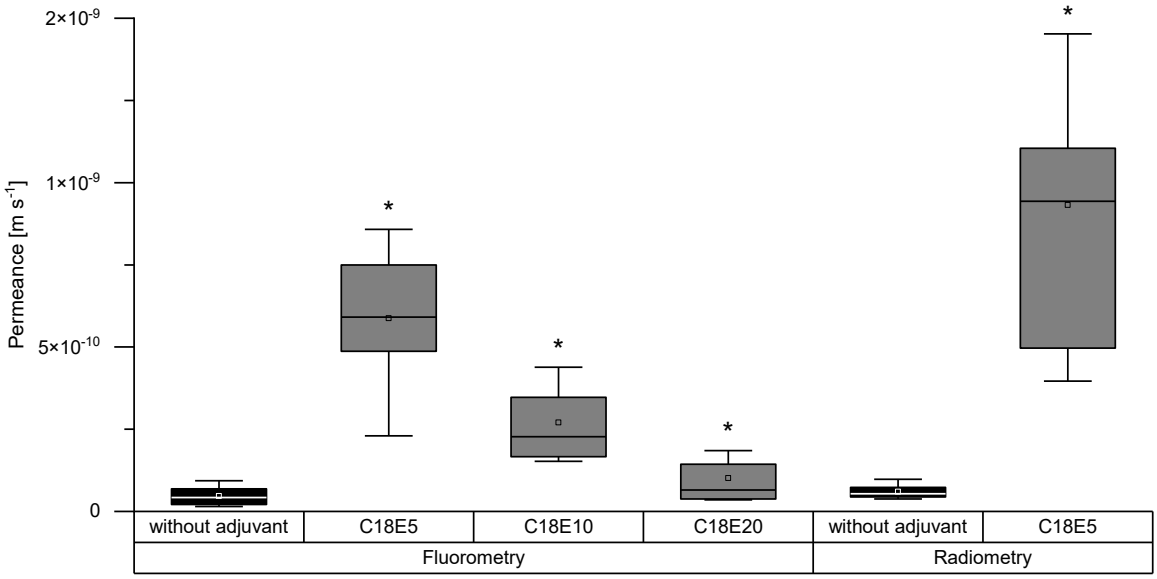

Figure 6: Permeances of fluorescein.
Measured across cuticular membranes isolated from *Prunus laurocerasus* with and without surfactants (0.1 % $C_{18}E_5$, $C_{18}E_{10}$ and $C_{18}E_{20}$) with both the fluorometric and the radiometric method. Boxes of the box plots represent 25th and 75th percentiles. Whiskers indicate 10th and 90th percentiles. Squares and horizontal lines within the boxes represent mean and median, respectively. Asterisks above the boxes indicate significant differences to the corresponding control (Wilcoxon signed-rank test; $P < 0.01$).

During steady-state phases of diffusion, permeances of fluorescein were 4.28 (2.33 – 6.91, 25th to 75th percentile) x 10^{-11} m s^{-1} with the fluorometric (0 to 384 hours) and 5.38 (4.38 – 7.30) x 10^{-11} m s^{-1} with the radioactive method (48 to 172 hours), respectively (Figure 6). Fluorescein permeabilities significantly increased, when different surfactants ($C_{18}E_5$, $C_{18}E_{10}$ and $C_{18}E_{20}$) were added to the receiver compartments (Figure 6). With increasing degree of ethoxylation (EO of 5, 10 and 20) the medians of the effects of added surfactants on permeability of fluorescein across the isolated cuticles of *P. laurocerasus* significantly decreased form about 18-fold, over 5-fold to 2-fold (Figure 7).

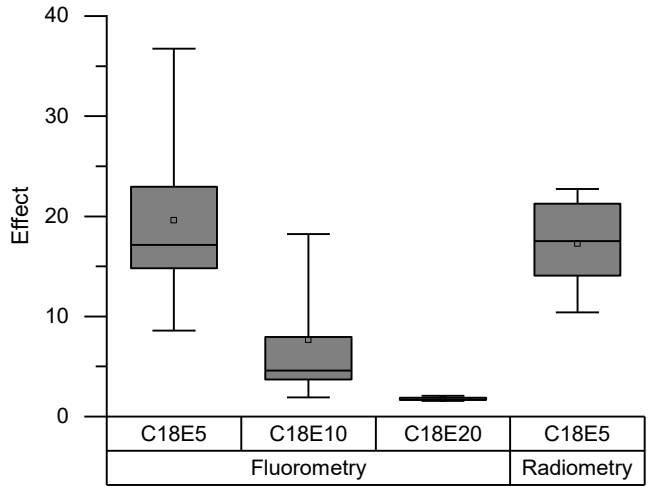

Figure 7: Effects of surfactants.
Fluorescein permeance in presence of surfactants (0.1 % $C_{18}E_5$, $C_{18}E_{10}$ or $C_{18}E_{20}$) across isolated *Prunus laurocerasus* cuticles measured with fluorometry and radiometry. Effects were calculated dividing permeances of fluorescein measured in the presence of the surfactants by the permeances measured as control. Boxes represent 25th and 75th percentiles. Whiskers indicate 10th and 90th percentiles. Squares and horizontal lines within the boxes represent mean and median, respectively.

1.5 Discussion

An older photometric method investigating of cuticular permeability (Schreiber et al. 1995) represents already a good, simple, and inexpensive method, but still had substantial disadvantages. Very high extinction coefficients of the model compounds are needed for spectroscopic detection and potential accelerators must not absorb at the same wavelength as the model compound (Schreiber et al. 1995). Furthermore, 4-Nitrophenol (4-NP) which was used in this publication as model compound for active ingredients, is fairly small (MW: 139 g mol^{-1}) and polar (K_{ow}: 79) and thus not really a representative substitute for the majority of active ingredients on average being significantly larger and more lipophilic.

These disadvantages of the photometric method should be overcome developing a fluorometric method offering an increased sensitivity and specificity. Donor concentrations of 1.39 g l^{-1} were required for 4-nitrophenol used in the photometric method and only 4 mg l^{-1} for fluorescein used in the fluorometric method, in order to be able measuring the penetrated amounts of substances in the receiver compartments with sufficient sensitivity. Thus, 1000-times lower donor concentrations can be used in the fluorometric experiment and this allows experiments with fairly lipophilic model substances (K_{ow} of 1000 or higher), which can be studied below their maximum of water solubility. Of course, compared to the fluorometric approach, highest sensitivity is still achieved with the radiometric method requiring donor concentrations of only 0.14 µg l^{-1}. Here the radiometric method was only used to verify the fluorometric method. For the fluorometric experiment fluorescein was chosen because it is an excellent and well-known fluorescent tracer with a quantum yield of 0.93 at pH 9 (Sjöback et al. 1995). Furthermore, its physicochemical properties (molecular weight, molar volume, log K_{ow}, predicted and measured log K_{cw} and predicted and measured permeances) fit very well to well-known lipophilic active ingredients of common pesticides, e.g. the systemic fungicide epoxiconazole (Table 1), which has been used in the past as model compound in many studies measuring cuticular permeabilities (Gutenberger et al. 2013, Sadler et al. 2016, Zeisler-Diehl et al. 2022).

Using isolated cuticles of *Prunus laurocerasus* as model species, not being a typical crop species, and a double chamber system at a first sight looks like a fairly artificial system, since donor and receiver compartments consist of aqueous solutions. With intact leaves only the receiver (plant interieur) can be assumed being an aqueous environment for efficiently absorbing the penetrated active ingredients, whereas the donor normally is represented by an almost dry spray droplet residue on the outer leaf surface containing the active ingredient

together with the adjuvants. Furthermore, effects like the degree of leaf surface wetting and spreading of applied sprayed droplets and humectancy of adjuvants contribute to rates of foliar uptake of active ingredients (Riederer et al. 1995).

However, the advantage of using this double chamber system is related to the fact that all these boundary effects mentioned above occurring under natural conditions can be excluded due to the following reasons. (i) The model compounds of interest are applied in the donor and they are homogenously dissolved in the donor at a known concentration. (ii) The crystallization of active ingredients will not take place. (iii) A fast diffusion into the cuticle is possible, with a high and constant driving force in the donor. (iv) And finally, this system allows the exclusive investigation of accelerating effects (Schreiber and Schönherr 2009) of surfactants on the permeance of active ingredients across cuticles when surfactants are added only to the receiver side. Surfactants and model compounds are spatially separated (Schreiber et al. 1995) and the concentration of surfactants can be kept constant by repeatedly exchanging the receiver solution. Thus a maximum surfactant concentration in the cuticle is obtained, which allows measuring maximal accelerating effects on diffusion of active ingredients (Schönherr 1993). This is of major advantage, because a combined application of active ingredient together with surfactants leads to the dissolution of the active ingredient within the micelles formed by the surfactants besides their dissolution in the cuticle. As a consequence, the driving force for penetration of active ingredients is lowered and not anymore exactly known (Kerler and Schönherr 1988b, Kerler and Schönherr 1988a, Kirkwood 1993). In the worst case, both effects lowering of the driving force and acceleration of the diffusion of the active ingredient in the cuticle could cancel and an accelerating effect of a potent adjuvant might remain uncovered or at biased.

Most interestingly in both experimental approaches measuring fluorescein diffusion across the cuticles the transport kinetics were characterized by 2 phases (Figure 4, a and b). With the fluorometric method initially linear rates of fluorescein diffusion (0 to 288 hours) slowly but constantly increased with longer measuring times (Figure 4, b and Figure 5, c), which is also reflected by the increase of the incremental permeances after 288 hours (Figure 5, a). This increase in permeability after such a long time of measurement can best be explained as an artefact. Most probably the PTFE-sealing between the cuticle and the stainless steel chambers is becoming leaky, which leads to an increasing diffusion of fluorescein bypassing the cuticle.

A similar observation was already described for this two-chamber-system measuring 4-nitrophenol (Schreiber et al. 1995).

Very different form the fluorometric method, with the radiometric method a steep slope of the transport kinetics was observed within the first 32 to 48 hours (Figure 4, a). Within this time period about 1 % of the ^{3}H-labelled fluorescein rapidly had penetrated the cuticles (Figure 5, d). The second constant phase of ^{3}H-fluorescein diffusion across the cuticle was much slower and stayed linear for the remaining measuring times (Figure 4, a and Figure 5, d). This was also reflected by fairly high initial incremental permeances between 0 and 32 hours and much lower but constant permeances between 48 to 168 hours (Figure 5, b). This very high initial rates of about 1 % of radioactivity diffusing across the cuticle are best explained by a radiochemical impurity of the ^{3}H-labelled fluorescein.

Radiolabelled fluorescein is not commercially available as standard product, but it can be ordered at fairly high costs as a custom synthesized radiolabelled product. This means that non-labelled fluorescein is converted to ^{3}H-labelled fluorescein in the presence of tritium gas and a homogeneous catalyst (2009). The custom synthesized ^{3}H-labelled fluorescein used here had a radiochemical purity of 99 % (Table 1), which is excellent. Nevertheless we assume that the 1 % of "allowed" radiochemical impurity occurring together with the 99% pure ^{3}H-fluorescein must have been an either very small (may be $^{3}H_2O$) and/or very lipophilic compound having a fairly high rate of cuticular diffusion. Once this 1 % contamination had penetrated across the cuticles, the remaining 99 % of pure ^{3}H-fluorecsein in the donor solution lead to much flatter transport kinetics which stayed constant (Figure 4, a). This example here shows, when using radiolabelled compounds in transport studies, increased caution is required with this highly sensitive radioactive method not to measure just the radioactive impurities of the radiolabelled compounds and collect artefacts.

With the reasonable assumptions (i) that the initial phase (0 to 288 hours) in the fluorometric experiment represents the accurate fluorescein permeation across the cuticles, and (ii) that the second phase (32-178 hours) in the radiometric experiment represents the accurate ^{3}H-fluorescein permeation across the cuticles, permeances were calculated from the regression lines fitted to these 2 phases of the transport kinetics (Figure 4, c). Medians of the incremental permeances of the 2 selected phases of the transport kinetics (Figure 5, a and b) were not different from each other. Averaged medians of these 2 phases amounted to 4.28 (2.33 – 6.91) x 10^{-11} m s^{-1} for the fluorometric experiment and 5.38 (4.38 – 7.30) x 10^{-11} m s^{-1} for the

radiometric experiment (Figure 6). This indicates that both approaches resulted in the same values and thus the fluorometric method is well suited replacing the radiometric approach in future. Both permeances measured here are also in good agreement with the predicted fluorescein permeance of 3.9 x 10^{-11} m s^{-1} calculated from a prediction equation correlating measured permeances of various organic compounds with their respective cuticle/water partition coefficients and molar volumes(Schreiber and Schönherr 2009).

The value for the epoxiconazole permeance of isolated P. laurocerasus cuticles published in literature amounts to 14 x 10-11 m s-1(Ballmann et al. 2011), which is about three times higher than that for fluorescein, although all characteristic molecular properties and the 2 molecules were very similar if not identical (Table1). In this cited publication statistical analysis is based on parametric statistics and means are given (Ballmann et al. 2011). Here we decided using non-parametric statistics and show box plots with medians (Figure 6). This is based on the fact that recent studies have shown that the distribution of permeances measured with isolated cuticular membranes often are right skewed not following a normal distribution (Geyer and Schönherr 1990, Schreiber and Riederer 1996, Baur 1997). This tendency was also observed here. Thus, this difference in statistical data analysis best explains the 3-fold difference, since with asymmetric, right-skewed distributions calculated parametric means always tend to be larger than calculated medians.

The main goal of this study was the development of a sensitive fluorometric technique allowing the screening of various surfactants potentially enhancing cuticular penetration (Stock et al. 1993). Therefore, the effect of a set of alcohol ethoxylates, derived from unsaturated oleic alcohol, on cuticular transport of fluorescein was investigated in more detail. With the selection of these alcohol ethoxylates, varying in their average degree of ethoxylation from 5 to 20 EOs, the suitability of this fluorometric methods efficiently discriminating between surfactants enhancing trans-cuticular diffusion from ineffective surfactants should be verified. With an increasing degree of ethoxylation, the ratio between the hydrophilic parts (EOs) and the lipophilic fatty alcohol (oleyl alcohol) of the amphiphilic surfactants increases leading to higher HLB values of 8.4 ($C_{18}E_5$), 12.0 ($C_{18}E_{10}$) and, 15.1 ($C_{18}E_{20}$) (Asmus et al. 2016) and thus decreased lipophilicity. In addition, surfactants increase in molecular weight ($C_{18}E_5$: 488.7 g mol^{-1} , $C_{18}E_{10}$: 709.0 g mol^{-1} , $C_{18}E_{20}$: 1149.5 g mol^{-1}) and consequently in size.

Permeances of non-radioactive fluorescein in *P. laurocerasus* cuticles were significantly (Wilcoxon signed-rank test; $12 \leq n \leq 14$; $P < 0.01$) increased for all 3 surfactants tested

(Figure 6). Effects, which were calculated by dividing the permeance measured after addition of the surfactants by the permeance obtained without surfactant (Figure 7), continuously decreased from 17 ($C_{18}E_5$) over 4.6 ($C_{18}E_{10}$) to 1.7 ($C_{18}E_{20}$). Thus, with increasing HLB values, decreasing lipophilicity and increasing size of the surfactants tested their potential accelerating the diffusion of non-labelled fluorescein across isolated cuticles of *P. laurocerasus* decreased (Figure 6). In the radioactive experiment the effect of $C_{18}E_5$ on permeance of ^{3}H-fluorescein was investigated. The increase in permeance of ^{3}H-fluorescein in the presence of $C_{18}E_5$ (Figure 6) as well as the calculated effect of $C_{18}E_5$ on ^{3}H-fluorescein (Figure 7) were identical when compared to the results obtained with the fluorometric experiment (Figure 6 and Figure 7). This allows to conclude that the fluorometric approach represents a good and reliable non-radioactive alternative for screening the effect of surfactants potentially enhancing rates of cuticular permeability of organic molecules.

Thus, results obtained here also fit to experimental results published for alcohol ethoxylates in the past. Accelerating effects of a series of different alcohol ethoxylates on mobility of various lipophilic solutes have been studied (Schönherr 1993, Baur et al. 1999b, Schönherr et al. 2001, Gutenberger et al. 2013). There it was also described that effects enhancing cuticular diffusion of organic molecules decreased with an increasing degree of ethoxylation of the surfactants (Riederer et al. 1995, Perkins et al. 2005a, Shi et al. 2005a). It was shown for example that surfactants derived from coconut oil (mean chain length of the fatty alcohol: C_{12}) with the same degrees of ethoxylation studied here (EO_5, EO_{10} and EO_{20}) enhanced rates of cuticular permeability of radiolabelled agrochemicals in a similar way (Baur et al. 1997b, Cronfeld et al. 2001). The data presented here provide further more evidence for the general principles of alcohol ethoxylates accelerating the diffusion of lipophilic solutes in isolated cuticular membranes.

1.6 Conclusion

The data presented here allow to conclude (i) that fluorescein represents a good model substance with physicochemical properties characteristic for many common agrochemicals and (ii) that fluorometry represents highly sensitive and reliable cost-effective method studying the enhancing effects of surfactants on cuticular permeability of fluorescein across isolated cuticles. Thus, the screening of surfactants for their potentially enhancing effects on rates of cuticular permeability is feasible in future using this method.

Chapter 2

Blockage of polar pores in cuticles leads to reduced penetration of polar methyl-glucose and neutral thiamethoxam but not of lipophilic terbuthylazine

2.1 Abstract

Lipophilic and polar substances have been postulated to diffuse in the plant cuticle *via* lipophilic and polar pathways. The relative contribution of the two different pathways on diffusion of organic solutes was never investigated so far. It was the aim of this study to determine the contribution of the polar pathway on diffusion of solutes significantly differing in their lipophilicity in isolated leaf cuticles of *Prunus laurocerasus*.

Rate constants of penetration were measured using ethanol or water as solvent for the application of the donor solution as droplet. Utilization of ethanol led to an improved surface wetting and spreading, which resulted in increased rate constants of penetration for methyl-glucose and thiamethoxam, whereas no significant alteration was observed for terbuthylazine. Penetration rates of the tested compounds increased with their lipophilicity. Rate constants determined with ethanol as donor solvent were 5.45 (2.54 – 9.22) x 10^{-4} h^{-1} (methyl-glucose), 1.91 (1.32 – 3.69) x 10^{-3} h^{-1} (thiamethoxam) and 1.72 (0.80 – 2.76) x 10^{-2} h^{-1} (terbuthylazine). Blockage of the polar pathway reduced the penetration of methyl-glucose and thiamethoxam 0.51-fold and 0.62-fold, respectively, while the penetration of terbuthylazine was not altered at all.

These findings presented here show that (i) intense surface wetting and coverage of cuticular membranes is of high importance for hydrophilic and neutral compounds, (ii) diffusion of compounds across the cuticle takes place *via* two different pathways depending on their lipophilicity and (iii) the presented method can be used to characterize the contribution of the polar pathway on diffusion of solutes through isolated cuticles.

Keywords: cuticle, diffusion, foliar uptake, silver chloride precipitates, polar pathway, transport

2.2 Introduction

The plant cuticle is a hydrophobic layer covering all primary above-ground plant organs (Riederer 2006). It forms the interface between the plant and the environment (Yeats and Rose 2013) and prevents desiccation (Schönherr 1982). Furthermore, it attenuates photosynthetically active and UV radiation and protects the sensitive photosynthesis processes against excessive light (Krauss et al. 1997). The cuticle is composed of the polyester cutin with the associated waxes (Samuels et al. 2008), but it also contains polysaccharides extending from the cell wall into the cutin polymer (Guzmán et al. 2014a, Segado et al. 2016). The waxes are embedded in the cutin matrix (intracuticular waxes) or deposited on the outer surface (epicuticular waxes) (Buschhaus and Jetter 2011). Epicuticular wax crystallites render the plant surface water repellent (Neinhuis and Barthlott 1997), while intracuticular waxes form the limiting barrier for the uptake of pollutants (Schönherr and Riederer 1989) and leaching of nutrients (H. B. Tukey 1970).

The majority of agrochemicals are applied as diluted sprays on the aerial parts (mainly leaves) of the plant covered by the cuticle (Rodham 2000). The uptake of the active ingredients takes place from the spray droplet residue across the highly impermeable plant cuticle (Zabkiewicz 2007). Active ingredients exhibit a large spectrum of lipophilicity (Baur et al. 1997c), which is the decisive factor for cuticular diffusion. Lipophilic substances can diffuse through the wax and cutin domains of the cuticle, which is called the lipophilic pathway (Buchholz 2006). Whereas ionic and polar substances have to diffuse across the cuticle *via* the so-called polar pathway, which may be formed by hydrated polar functional groups of the cutin and polysaccharide fibrils (Schreiber 2005, Fernández et al. 2016). Since lipophilic (octanol-water partition coefficient (log K_{OW}) > 3) and polar substances (log K_{OW} < -3), in particular charged molecules and ions, are thought to be limited to their corresponding pathways and have been studied intensively (Schönherr and Baur 1994, Baur et al. 1997b, Schönherr and Luber 2001, Schönherr 2006), we were interested in the question which of the two pathways are preferentially used by neutral substances with log K_{OW} of around 0?

In the past, it was shown that silver chloride precipitates within isolated cuticular membranes of 13 species reduce the rates of cuticular transpiration (Schreiber et al. 2006). Cuticular membranes of *Prunus laurocerasus* exhibited a pronounced reduction in water permeability after the blockage of the polar pathway and were therefore selected as model species for the experiments. For the transport studies, organic substances were selected based on their

differences in lipophilicity: (i) terbuthylazine (log K_{OW}: 3.4) (ii) thiamethoxam (log K_{OW}: -0.1) and (iii) methyl-glucose (log K_{OW}: -3.0). Rate constants of penetration were determined with silver chloride precipitates present within the cuticle and without precipitates serving as control. The results presented here reveal the relative contributions of the polar and lipophilic pathway on the uptake of solutes differing in their lipophilicity.

2.3 Material and Methods

2.3.1 Plant material

Adaxial astomatous cuticular membranes (CM) of *Prunus laurocerasus* leaves were used in the experiments. The membranes were obtained by enzymatic isolation according to Schönherr and Riederer (1986).

2.3.2 Chemicals

As radiolabelled substances ^{3}H-labelled methyl-glucose (ARC Inc., St. Louis, USA), ^{14}C-labelled thiamethoxam (Selcia Ltd, Essex, UK) and ^{14}C-labelled terbuthylazine (Selcia Ltd, Essex, UK) were used in the experiments. Radiochemical purity of all substances was greater than 98.6 %. Silver nitrate ($AgNO_3$, ≥ 99.9 % purity) and sodium chloride (NaCl, 99.9 % purity) were obtained from Carl Roth (Karlsruhe, Germany) and Gerbu Biotechnik (Heidelberg, Germany), respectively, and used as aqueous solutions at concentrations of 0.01 mol l^{-1}.

Table 2: Characteristic properties of methyl-glucose, thiamethoxam and terbuthylazine

	Methyl-glucose	Thiamethoxam	Terbuthylazine
MW (g mol^{-1})	194[a]	292[b]	230[b]
V_X (cm^3 mol^{-1})	140[c]	181[c]	176[c]
Molecular radius (nm)	0.38	0.42	0.41
log K_{OW}	-3.0[a]	-0.1[b]	3.4[b]
log K_{CW}	-0.87 ± 0.10[d]	0.53 ± 0.08[e]	3.39 ± 0.02[e]
Specific activity (MBq mg^{-1})	11443	5.2	4.2

MW (Molecular weight), V_x (characteristic molecular volume), log K_{OW} (Octanol-water partition coefficient), log K_{CW} (Cuticle-water partition coefficient)

[a] Values taken from Schreiber and Schönherr (2009)

[b] Values for thiamethoxam (PubChem CID: 5485188) and terbuthylazine (CID: 22206) taken from PubChem

[c] Calculated according to Abraham and McGowan (1987)

[d] Baur *et al.* 1997c

[e] Own determination with isolated cuticular membranes of *P. laurocerasus*

2.3.3 Measurement of cuticle/water partition coefficients

Cuticles were equilibrated in glass vials with 3 ml of aqueous solution containing the respective radioactive labelled compound for 24 h on a roller mixer (BTR 10P, Ratek Instruments, Boronia VIC, Australia) at 25 °C. Concentrations of the solutions were 2.63 x 10^{-11} mol l^{-1} (methyl-glucose), 3.85 x 10^{-8} mol l^{-1} (thiamethoxam), and 6.06 x 10^{-8} mol l^{-1} (terbuthylazine). Mass of the individual cuticles varied between 1 mg and 2 mg. Radioactivity in cuticles and aqueous donor solutions were determined by liquid scintillation counting.

Cuticle-water partition coefficient (K_{CW}) is defined by the equation (Riederer and Schönherr 1984) and calculated accordingly:

$$K_{CW} = \frac{equilibirum\ concentration\ in\ cuticle\ (\frac{Bq}{kg})}{equilibirum\ concentration\ in\ water\ (\frac{Bq}{kg})} \quad (1)$$

2.3.4 Penetration experiments

Cuticular penetration rates were measured using SOFP (simulation of foliar penetration) as described by Schönherr and Baur (1994). Cuticles were mounted on stainless steel chambers using PTFE-Paste (Carl Roth, Karlsruhe, Germany) and fixed with a ring-shaped lid. Membranes were orientated that the physiological outer side was facing towards the atmosphere. The two parts of the chamber system were fixed with adhesive tape (Tesa, Norderstedt, Germany). Each CM was tested for leaks by applying a 10 µl droplet of absolute ethanol (≥ 99.8 %, Sigma-Aldrich, Steinheim, Germany). Light refraction will change and holes become visible as dark spots. Only intact membranes were used for the experiments.

The compounds were dissolved in absolute ethanol or water and applied to the outer surface of the cuticle as 10 µl droplets. Concentrations of the donor solutions were 167 Bq μl^{-1} for methyl-glucose and 67 Bq μl^{-1} for thiamethoxam and terbuthylazine. After drying of the droplet, 800 µl PLS (Phospholipid suspension, 1 % soybean lecithin, Carl Roth, Karlsruhe, Germany) was added to the chamber as receiver solution. The chambers were placed on scintillation vials and kept at 25 °C in an incubator (MIR-554, Panasonic, Kadoma, Japan). 250 µl glycerol (≥ 99 % purity, Fisher Chemical, Loughborough, UK) was filled in each scintillation vial to ensure a relative humidity of 2 %. The receiver solutions were replaced by fresh PLS in predetermined

time intervals using a 1 ml syringe (Hamilton, Reno, USA). To quantify the radioactive labelled compounds desorbed from the cuticle, the receiver solutions were dissolved in scintillation cocktail (Ultima Gold XR, PerkinElmer, Waltham, USA) and counted by a Tri-Carb 4910TR liquid scintillation analyser (PerkinElmer, Waltham, USA).

After 48 hours the exposed area of the cuticles was cut out and the residual amount of radioactivity in the cuticles was determined by scintillation counting. The amount on the outer surface, in the cuticle and desorbed by the receiver solution over time (M_t) were added up and denoted as M_0 and represented the total amount of substance which was applied at time 0. By plotting the negative logarithm of the amounts remaining on the outer cuticle surface $-\ln(1-M_t/M_0)$ vs. time (t) desorption kinetics could be linearized. Therefore, penetration was a first-order process and can defined by the following equation:

$$\frac{M_t}{M_0} = 1 - e^{-kt} \qquad (2)$$

Rate constants k (h^{-1}) were determined by calculation of the slope of the regression lines.

The method described by Schreiber *et al.* (2006) to investigate the effect of AgCl precipitates on cuticular transpiration was slightly adapted to SOFP (Schreiber et al. 2006).

NaCl solution (800 µl) was filled in the receiver compartment, while 800 µl $AgNO_3$ solution was pipetted on the outer side. Sealing the upper part of the chamber was done by using parafilm (Bemis Company, Neenah, USA) and the sampling port was closed with adhesive tape. To ensure that both surfaces of the cuticle were exposed homogeneously to the added solutions, the chambers were rotated horizontally at 10 RPM on a roller mixer (BTR 10P) at 25 °C. Ag^+ and Cl^- ions were diffusing into the cuticle from the physiological outer and inner side, respectively. Insoluble silver chloride (AgCl) crystals were formed when the ions met within the membrane. Silver nitrate and sodium chloride solutions were completely withdrawn after 24 hours. Residues of the salt solutions were removed by repeatedly rinsing the compartments carefully with deionised water.

2.3.5 Sample size and statistics

Partition coefficients were determined with at least 5 adaxial astomatous cuticular membranes isolated from *P. laurocerasus*. SOFP experiments were conducted with at least 10 up to 70 isolated CMs. OriginPro (Version 2021, OriginLab, Northampton, USA) was used for the statistical analysis. Distribution of rate constants was not normal according to the Shapiro-Wilk test ($P < 0.05$). Outliers were removed according to the interquartile range method. Statistical significance of differences were tested with Kruskal-Wallis analysis of variance (ANOVA) and post-hoc Dunn´s test ($P < 0.05$).

2.4 Results

Determination of K_{CW} was only possible for thiamethoxam and terbuthylazine (Table 2). Measured values of log K_{CW} were -0.1 and 3.4 for thiamethoxam and terbuthylazine, respectively (Table 2). The radioactive method was not suitable for measuring K_{CW} of the highly water soluble methyl-glucose. There was no measurable decrease in the concentration of donor solution after incubating cuticles for 24 hours in the donor solution. In addition, even after dipping the cuticles several times shortly in water and gently blotting with tissue paper, in order to remove radioactive donor solutions of methyl-glucose superficially adhering to the incubated cuticles, the amount of radioactivity still associated the cuticle was unreasonably high.

Plotting $-\ln(1-M_t/M_0)$ versus time showed a flatter initial slope in the first 24 hours followed by a linear steeper slope after 24 hours for thiamethoxam and terbuthylazine (Figure 8, b and c), whereas penetration of methyl-glucose showed a linear slope from the beginning intersecting the origin of the x- and y-axis (Figure 8, a). Therefore, rate constants were calculated for all compounds in the linear intervals between 24 hours and 48 hours. Highest rates of penetration were measured with terbuthylazine, intermediate rates with thiamethoxam and lowest rates with methyl-glucose (Figure 8). Using water as solvent for the application of the radiolabelled compounds (squares in Figure 8), the rate constants ranged from 8.12 (5.00 – 10.2, 25th to 75th percentile) x 10^{-5} h^{-1} (methyl-glucose) over 5.59 (2.91 – 8.52) x 10^{-4} h^{-1} (thiamethoxam) to 1.23 (0.54 – 1.63) x 10^{-2} h^{-1} (terbuthylazine). Using ethanol as solvent for the application of the radiolabelled compounds (circles in Figure 8), led to increased rate constants of 5.45 (2.54 – 9.22) x 10^{-4} h^{-1} (methyl-glucose), 1.91 (1.32 – 3.69) x 10^{-3} h^{-1} (thiamethoxam) and 1.72 (0.80 – 2.76) x 10^{-2} h^{-1} (terbuthylazine). Effects of ethanol increasing rate constants (Figure 10) were highest for methyl-glucose (6.7), intermediate for thiamethoxam (3.4) and lowest for terbuthylazine (1.4). Using ethanol as solvent for methyl-glucose and thiamethoxam resulted in a statistically significant increase of their rate constants (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$). In all further desorption experiments ethanol was used as solvent for applying the model compounds to the cuticle surface.

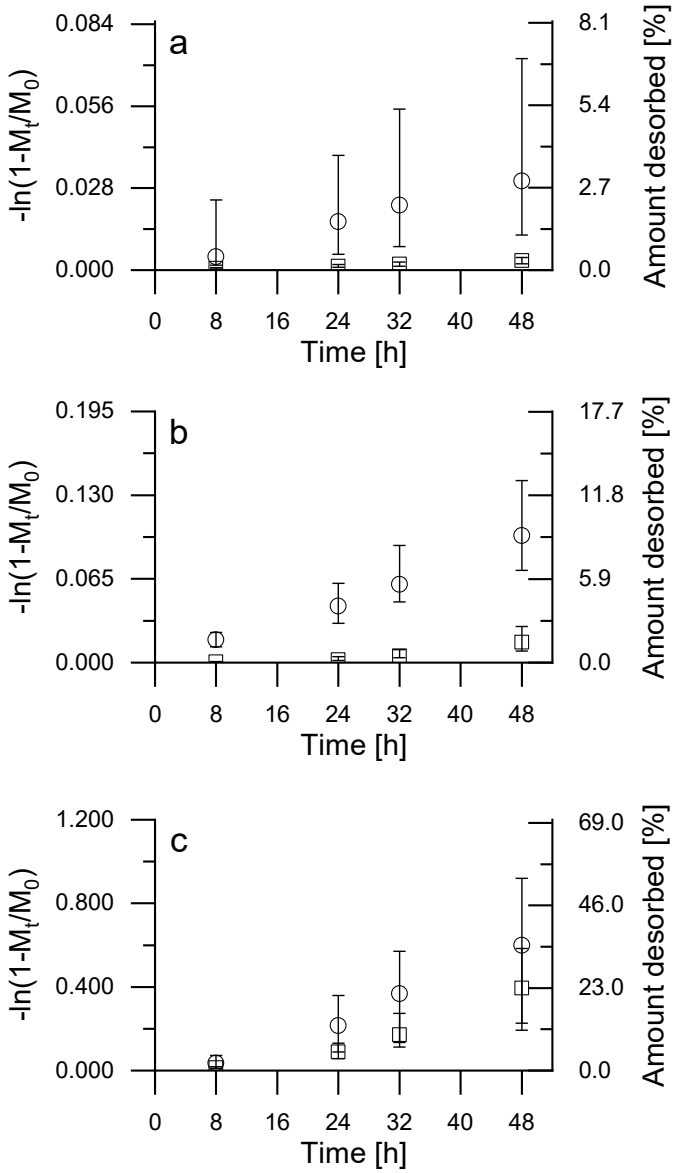

Figure 8: Time courses of penetration.
Methyl-glucose (a), thiamethoxam (b) and terbuthylazine (c) penetration across isolated cuticles of *Prunus laurocerasus* using water (squares) or ethanol (circles) as donor solvent. For each individual CM $-\ln(1-M_t/M_0)$ was calculated and median values with quartiles were plotted.

The diffusion of the 3 model compounds (methyl-glucose, thiamethoxam and terbuthylazine) across isolated cuticular membranes of *Prunus laurocerasus* was measured with cuticles carrying silver chloride precipitates within the cuticle (squares in Figure 9) and compared to untreated cuticles (circles in Figure 9). Rate constants ranged from 2.76 (2.20 – 4.79) x 10^{-4} h^{-1} (methyl-glucose) over 1.18 (0.87 – 1.36) x 10^{-3} h^{-1} (thiamethoxam) to 2.24 (1.02 – 2.62) x 10^{-2} h^{-1} (terbuthylazine) when AgCl precipitates were present in the cuticles (Figure 9). Rate constants for methyl-glucose and thiamethoxam were significantly decreased in isolated cuticles with AgCl precipitates (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$; Figure 11). For terbuthylazine the formation of AgCl precipitates within the cuticle did result in a rate constant, which was not statistically different from the rate constant measured in untreated cuticles (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$; Figure 11).

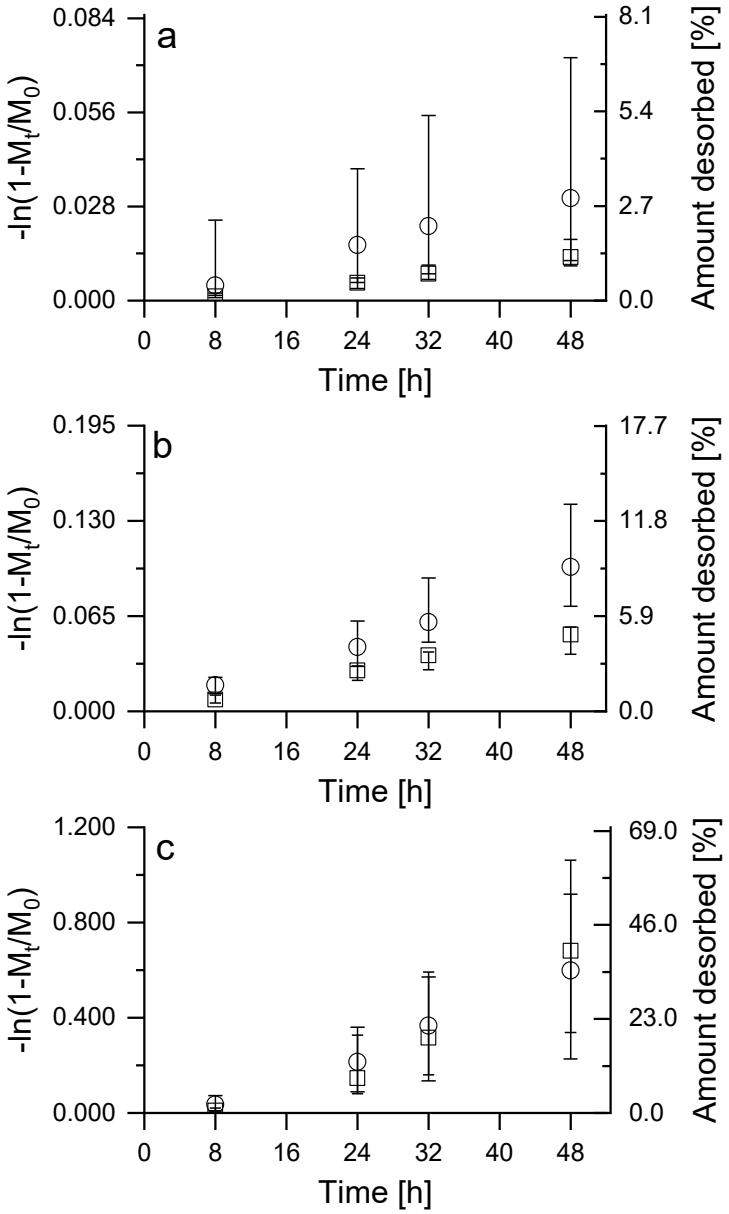

Figure 9: Time courses of penetration.
Methyl-glucose (a), thiamethoxam (b) and terbuthylazine (c) penetration with (squares) and without (circles) silver chloride precipitates within the cuticle of *P. laurocerasus*. For each individual CM $-\ln(1-M_t/M_0)$ was calculated and median values with quartiles were plotted.

2.5 Discussion

The cuticle-water partition coefficient K_{CW} has been described as useful parameter characterizing the affinity of substances to diffuse in cuticles (Kerler and Schönherr 1988b, Kerler and Schönherr 1988a). It has been shown that K_{CW} is numerically generally very similar to the octanol-water partition coefficient (K_{OW}), which is often used to predict the environmental behaviour of chemicals (Hermens et al. 2013). K_{CW} measured here for terbuthylazine (log K_{CW}: 3.39) was in good accordance with the published log K_{OW} of 3.4 (Table 2) as well with the estimated log K_{CW} (3.36) using a prediction equation (Schönherr and Riederer 1989). For thiamethoxam a K_{CW} of 0.85 was predicted, but a four times higher K_{CW} of 3.39 was measured here. One reason for this discrepancy is that the prediction equation was developed using substances with K_{OW} values ranging from 10^2 to 10^8 and is therefore not appropriate for substances with K_{OW} values exceeding this range. Another reason are probably methodological limitations. The physiological inner side of the cuticle is easy to wet and a thin film of adhering donor solution will result in overestimation of the amount absorbed by the lipophilic cuticle (Schreiber and Schönherr 2009). Although, inner sides of the cuticles were gently blotted with tissue paper, the possibility that small amounts were still sticking to the surface cannot fully be excluded. For substances with an even more hydrophilic character, as methyl-glucose, a sufficiently accurate determination of K_{CW} was not at all possible. The concentration of polar methyl-glucose in an adhering water film of donor solution would be significantly higher compared to the low amounts dissolved within the cuticle (Schreiber and Schönherr 2009).

Foliar penetration takes place in three steps: (i) sorption into the cuticle, followed by (ii) diffusion through and finally (iii) desorption from the cuticle (Kirkwood 1999). Sorption only occurs when solutes are in direct contact with the cuticle proper, which represents the transport limiting barrier of cuticular membranes (Schönherr and Riederer 1989). The morphology of epicuticular waxes, located on the surface of the cuticle, depends on plant species. The structures can range from simple amorphous wax films, granules or plates to highly complex geometries like tubes with hollow centres or flattened ribbons with various forms of edge decoration (Barthlott et al. 1998, Jeffree 2006). These crystallites may exhibit an height larger than the thickness of the cuticle (Jeffree 2006). Barley, as an example for an agronomical relevant crop, exhibits surface waxes arranged as platelets. The average height of these crystallites is 1 µm as shown by scanning electron microscopic investigations (Baales et al. 2022). The surface of *P. laurocerasus* cuticles is characterized by wax platelets with an loose

and irregular, but broad coverage (Perkins et al. 2005b, Zeisler and Schreiber 2016). The application of aqueous droplets on cuticular membranes covered with epicuticular waxes in crystallite conformation will result in no or only rare wetting of the surface. Droplets sit and evaporate on the tips of the crystallites (Schönherr 2000, Schulte et al. 2011). The solutes are not directly in contact with the cuticle proper. Thus, sorption will be reduced or not taking place.

The current model describing the diffusion of solutes across the plant cuticle suggests the existence of two parallel paths of diffusion (Buchholz 2006, Schönherr 2006). Non-ionic lipophilic compounds preferentially diffuse through lipophilic wax and cutin domains of the cuticle, what has been named the lipophilic pathway. Cuticular permeability of these compounds can best be predicted by two parameters (lipophilicity and mobility). Lipophilicity describes the solubility of the compound within the cuticle proper and is given by the partition coefficient K_{CW} (Schönherr and Riederer 1989). Mobility describes the diffusion of the compounds across the transport limiting barrier of the cuticle and it is depending on the size of the molecules (Baur et al. 1997a, Buchholz et al. 1998).

Polar solutes and especially charged ions have been suggested to preferentially diffuse across the cuticle on a different pathway, since they are hardly (polar organic compounds) or not at all (charged ions) soluble in the lipophilic domains of the cuticles. The polar pathway is associated with hydrophilic domains within the cuticle, which are probably randomly distributed and clustered (Luque et al. 1995). Chamel *et al.* (1991) investigated the water sorption of cuticles and dewaxed cuticular membranes and suggested that the absorption sites for water were mainly made up of polar polysaccharides (Chamel et al. 1991). The water content for seven investigated species ranged from 1.1 % to 7.7 % of the dehydrated cuticular weight. Two third of the total absorbed water was attributed to polysaccharides, whereas cutin was accountable for one third and wax around 1 %. Ester, carboxyl and hydroxyl groups were assumed to absorb water in the cutin matrix. These domains may build up a ramified network (Schreiber 2005, Guzmán et al. 2014a, Fernández et al. 2017).

Cellulose and pectin were found not only on the innermost cuticle surface, but have also been described to extend up to the epicuticular wax layer for several species (Guzmán et al. 2014a, Guzmán et al. 2014b). Those carbohydrates may connect the surface of the cuticle with the inner regions and establish a continuous polar pathway for the diffusion of polar solutes. (Domínguez and Heredia 1999, Fernández et al. 2017). This pathway can be blocked partially by waxes, as suggested by several authors, since total wax extraction of cuticular membranes

showed an increased permeability for polar solutes (Popp et al. 2005, Arand et al. 2010, Staiger et al. 2019).

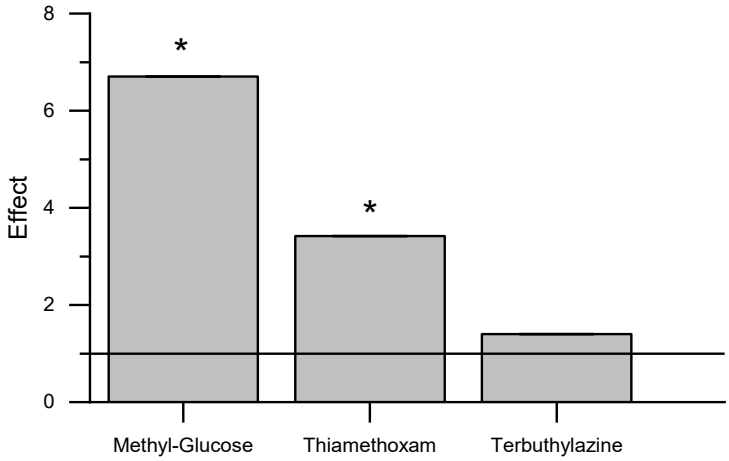

Figure 10: Effects of ethanol as donor solvent on the rate constants.
Methyl-glucose, thiamethoxam and terbuthylazine desorbed from the physiological inner side of isolated *Prunus laurocerasus* cuticles. Rate constants were calculated for each single CM. Effects on desorption were calculated by dividing median values of rate constants measured with ethanol as donor solvent by median values of rate constants with water as solvent. Asterix above the boxes indicate significant effects (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$).

Here it was investigated if the donor solvent has an influence on the penetration of the model compounds and rate constants of penetration for the model compounds were measured using ethanol or water as donor solvent. Only very low penetration of methyl-glucose was observed when the compound was dissolved in water, whereas the rate constant was significantly increased when ethanol was used as donor solvent (Figure 10). This must be attributed to a better wettability of the lipophilic cuticular surface. With ethanol, fully wetting the cuticle surface the polar paths of transport obviously become accessible for methyl-glucose due to an intimate contact with the surface. The hydrophilic domains within the cuticle are randomly distributed and represent only a minor fraction of the cuticle (Fernández et al. 2017). The total pore area per cm^2 cuticle was reported to be in the range of 10^{-4} cm^2 for dewaxed *Citrus aurantium* cuticles and 10^{-5} cm^2 for cuticular membranes of *Populus* × *canescens* (Schönherr 1976, Remus-Emsermann et al. 2011). With ethanol as donor solution the direct contact of the polar methyl-glucose with the polar pores on or near to the surface of the cuticle is significantly improved. This was also shown to be relevant for the diffusion of charged ions across polar paths of transport in cuticles (Schönherr 2000, Schönherr 2001). Whereas, the effect of ethanol as donor solvent on the rate constant of the neutral thiamethoxan was only intermediate

(Figure 10), with the lipophilic terbuthylazine there was no difference between water and ethanol used as donor (Figure 10). Lipophilic compounds, like terbuthylazine, having a high K_{CW} can obviously easily establish a close contact with lipophilic domains of the cuticle.

For the neutral thiamethoxam (log K_{OW}: -0.1) a statistical significant increase of the rate constant by a factor of 3.5 was determined (Figure 10). The accessibility of the polar domains also seems to contribute a significant proportion to the penetration of thiamethoxam. These results are in good accordance with previous studies, showing an increase in penetration of polar solutes when surface wetting and coverage is increased by a wetting agent (Schönherr 2000, Schönherr 2002, Shi et al. 2005a). Furthermore, the results underline the utilisation of an appropriate solvent as donor solution leading to a consistent coverage of the cuticular surface, when penetration rates of solutes differing in their lipophilicity are investigated.

It could be argued that ethanol as an organic solvent might remove or alter epicuticular waxes, establishing the cuticular transport barrier, and therefore the penetration of methyl-glucose and thiamethoxam might be increased. However, it was shown that the application of ethanol on cuticular surfaces will not remove waxes and had no effect on transpiration (Zeisler and Schreiber 2016, Zeisler-Diehl et al. 2018). Water is a polar but very small molecule and can penetrate the cuticle *via* both pathways (Schreiber et al. 2006). Therefore, alteration of lipophilic or polar pathway should result in changes in the water permeability of cuticular membranes, which was not the effect with ethanol.

In previous studies, mean radii of aqueous polar pores have been determined for cuticular membranes of *Hedera helix* (0.3 nm- 0.5 nm), intact leaves of *Coffea arabica* (2.0 nm), *Populus* × *canadensis* (2.4 nm) and dewaxed *Citrus aurantium* membranes (0.45 nm) (Schönherr 1976, Popp et al. 2005, Eichert and Goldbach 2008). Ions cannot diffuse through the lipophilic domains of the cuticle due to their hydration shell, thus they have to take the polar pathway for diffusion (Schönherr 2000, Schönherr and Schreiber 2004b, Schönherr and Schreiber 2004a). The hydrated ion radii of Ag^+ and Cl^- were 0.34 nm and 0.33 nm, respectively (Volkov et al. 1997). Therefore, diffusion through the polar pores should be possible. The functionality of the blockage of polar pathway with silver chloride precipitates was demonstrated in experiments investigating the cuticular transpiration. The water permeability of isolated cuticles of 13 different species was reduced when the polar pores were blocked. An effect of 0.69 was reported for *Prunus laurocerasus* cuticles (Schreiber et al. 2006). This significant reduction of transpiration can be seen as an evidence of a pronounced polar pathway

across the cuticle of *P. laurocerasus* for diffusion of polar solutes. Assuming a spherical shape, the radius of the diffusing molecules used here can be estimated using the molar volume. Calculated radii are 0.38 nm, 0.42 nm and 0.41 nm for methyl-glucose, thiamethoxam and terbuthylazine, respectively. Hence, diffusion of the neutral thiamethoxam and of the polar methyl-glucose across the cuticle *via* the polar pathway should be possible.

Rate constants of penetration of the three solutes differed by two orders of magnitude. Terbuthylazine showed the fastest penetration (1.72 (0.80 – 2.76) x 10^{-2} h^{-1}), followed by thiamethoxam (1.91 (1.32 – 3.69) x 10^{-3} h^{-1}) and methyl-glucose (5.45 (2.54 – 9.22) x 10^{-4} h^{-1}).

Due to the low solubility of methyl-glucose and thiamethoxam in the wax and cutin of the cuticle, expressed by a negative log K_{OW}, the sorption into these domains can be expected to be low. Penetration of methyl-glucose (log K_{OW}: -3.0) across the lipophilic pathway seems unlikely.

Silver chloride precipitates within the cuticular membrane led to a statistically significant reduction of the rate constants of methyl-glucose and thiamethoxam, while the penetration of terbuthylazine was not altered (Figure 9). This indicates, that the polar domains within the cuticle are not important for terbuthylazine diffusion. Therefore, a blockage of the polar pathway is not affecting the penetration of lipophilic compounds. Blocking the polar pathway had the biggest effect on methyl-glucose. The rate constant was reduced by a factor of two. Hence, it can be assumed that at least 50 % of the methyl-glucose molecules were diffusing through the polar pathway across the cuticle. This assumption is in accordance with the contribution of the polar pathway in *Pyrus communis* cuticles to the uptake of methyl-glucose determined by Shi *et al.*(2005). Rate constants for calcium chloride and methyl-glucose were compared, revealing that the rate constants for methyl-glucose were occasionally higher than for calcium chloride. It was suggested that methyl-glucose can additionally to some extent also use the lipophilic pathway for diffusion which is not accessible for calcium chloride. Due to their hydration shells ions were excluded from the lipophilic pathway, while non-electrolytes can shed their hydration shell and therefore can diffuse in the lipid phase and in the polar phase of the cuticle. A contribution of 60 % was attributed to the polar pathway for the penetration of methyl-glucose. Water permeability for *Pyrus communis* was 7.5 times larger than for *Prunus laurocerasus* (Schreiber et al. 2006), indicating a more pronounced polar pathway. Thiamethoxam has a slight hydrophilic character (K_{OW}: 0.74), but is almost equally soluble in the lipophilic and the hydrophilic phase, therefore thiamethoxam is presumably penetrating the

cuticle *via* both pathways. Diffusion of thiamethoxam across the lipophilic domains of the cuticle can be assumed to be more significant, since the pore area is only a thousandth of the whole cuticle area (Schönherr 2006). It was shown for charged calcium salts, which cannot diffuse in the lipophilic pathway, that the rate constants were in the same order of magnitude as the observed rate constants for polar compounds (Baur et al. 1997b, Schönherr 2001). The blockage of the polar pathway showed an effect of 0.6 on the rate constant of penetration for thiamethoxam. Thus, at most 60 % of the molecules were taking the lipophilic pathway for diffusing across the cuticle. The polar and the lipophilic pathway were contributing nearly the same proportion to the uptake of thiamethoxam.

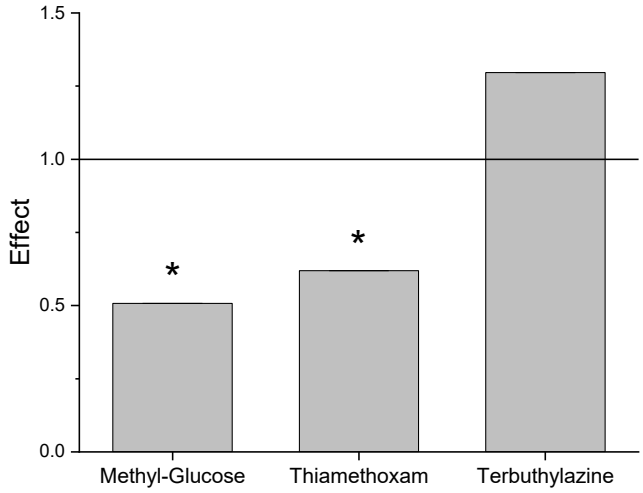

Figure 11: Effects of silver chloride precipitates.
Calculated with rate constants of methyl-glucose, thiamethoxam and terbuthylazine across cuticular membranes of *Prunus laurocerasus*. Rate constants were calculated for each CM and effects were calculated by dividing median values of rate constants measured with silver chloride precipitates within the cuticle by median values of rate constants without silver chloride precipitates. Asterix above the boxes indicate significant effects (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$).

Interestingly, when the polar pathway was blocked, the variability of the individual rate constants, expressed by the 25th and 75th percentile, decreased by factor of 2.6 and 4.5 for methyl-glucose and thiamethoxam, respectively, while for terbuthylazine a factor of 1.2 was determined. The decrease in variability of rate constants determined for methyl-glucose and thiamethoxam can be interpreted as an indication that the penetration of these solutes became independent of the accessibility of the heterogeneously distributed polar domains of the cuticle and diffusion will take place mainly *via* the lipophilic pathway. Trichomes and stomata were identified as preferential sites for uptake of polar solutes in *Vicia faba* (Schlegel et al. 2005). Furthermore, veins of isolated astomatous *Populus* × *canescens* cuticles, where formerly

trichomes were present, were associated with the uptake of polar solutes (Schreiber et al. 2006, Remus-Emsermann et al. 2011). These findings revealed clustered appearing spots on the surface of cuticles, which were more permeable for polar solutes. Although, adaxial cuticular membranes of *P. laurocerasus* exhibit no trichomes and stomata, it can be expected that the pores were randomly allocated on the surface and they may appear in clusters within the cuticle (Fernández et al. 2017). Microscopic studies with TM-AFM and phase imaging using a hydrophilic probe indicated two distinct surface components on the nanoscale in isolated cuticles of *P. laurocerasus* (Perkins et al. 2005b). The authors assigned the darker appearing regions to be most hydrophilic and the lighter appearing regions the least hydrophilic regions of the surface. These regions were heterogeneously distributed and not following a regular pattern. It was suggested that the differences in hydrophilicity were caused by crystalline and amorphous formations of the waxes and the regions differ in their likeliness for retention and penetration of agrochemicals or fungal attachment (Perkins et al. 2005b). It seems likely that the polar pores were present in the more hydrophilic area of the surface of *Prunus laurocerasus* located within the amorphous wax.

2.6 Conclusion

The findings presented reveal (i) the importance of wetting and consistent coverage of cuticular membranes when penetration of hydrophilic and lipophilic solutes are investigated and compared, (ii) a strong experimental evidence for the two different pathways of diffusion across the plant cuticle, and (iii) the contribution of the polar pathway on uptake of solutes. In future, results of this study might help improve the foliar uptake of polar active ingredients of crop protection products or fertilizers.

Chapter 3

Determination of foliar penetration of organic chemicals in isolated cuticles of *Prunus laurocerasus*: Effects of adjuvants

3.1 Abstract

The plasticizing effect of adjuvants on the diffusion of compounds within the lipophilic pathway of cuticles is well investigated. However, studies of adjuvant effects on the polar pathway are missing. Therefore, this study was conducted to reveal potential accelerating effects of adjuvants on penetration of polar and intermediate polar compounds preferentially diffusing along the polar path of transport in cuticles.

Penetration of three compounds across isolated cuticles of *Prunus laurocerasus* differing in their polarity was measured in absence and presence of adjuvants. Rate constants of penetration were highest for lipophilic terbuthylazine (1.72 (0.80 – 2.76, 25th to 75th percentile) x 10^{-2} h^{-1}) and lowest for hydrophilic methyl-glucose (5.45 (2.54 – 9.22) x 10^{-4} h^{-1}). For thiamethoxam representing an intermediate polar substance determined rate constant was 1.91 (1.32 – 3.69) x 10^{-3} h^{-1}. Effects of the structurally and physiochemically different adjuvants on cuticular penetration varied. Alkyl polyglucoside was not statistically significant altering the penetration of any of the compound used in this study. But a slight decrease was observed for penetration of methyl-glucose (0.8) and thiamethoxam (0.7). Penetration of methyl-glucose, thiamethoxam and terbuthylazine was enhanced in presence of sodium lauryl ether sulphate. Effects were highest for terbuthylazine (2.8) and lowest for methyl-glucose (1.2). Methylated rapeseed oil accelerated penetration of all compounds, although effects were statistically significant only for semi-hydrophilic thiamethoxam (2.8) and lipophilic terbuthylazine (4.0).

This study shows that (i) plasticizing of cuticular wax has no effect on diffusion of hydrophilic substances. (ii) Diffusion of semi-hydrophilic and lipophilic compounds was enhanced by plasticizers. (iii) Finally, the penetration rates of the plasticizing adjuvants and agrochemicals in the cuticle must be adjusted to enhance uptake by the leaves.

Keywords: cuticular transport, diffusion, foliar uptake, adjuvants, plasticizer effect, agrochemicals

3.2 Introduction

The interface between primary parts of higher terrestrial plants and the environment is formed by a thin hydrophobic layer, called the plant cuticle (Schönherr 1976, Fernández et al. 2008). Cuticular membranes provide protection against biotic and abiotic stresses (Yeats and Rose 2013). As major physiological role the cuticle prevents desiccation due to uncontrolled water loss (Schönherr and Schmidt 1979, Kerstiens 2006), but also shielding the sensitive photosynthesis systems from excess UV radiation (Grant et al. 1995, Liakoura et al. 1999) and constituting the first barrier against infestation of insects or pathogens (Lindow and Brandl 2003, Gorb and Gorb 2017). The lipophilic biopolymer cutin with cuticular waxes are the main components of plant cuticles (Zeisler-Diehl et al. 2020). Cutin is forming the matrix and basis for embedded intracuticular and deposited epicuticular waxes (Schreiber 2010), respectively. Although plant cuticles are interpreted as lipophilic membranes, a distinct heterogeneity in their structure exists (Schönherr and Riederer 1988, Fernández et al. 2017). On the physiological inner side substantial amounts of hydrophilic cell wall carbohydrates were present within the cutin polymer (Lopez-Casado et al. 2007, Fernández et al. 2021). Whereas on the outer side the cutin is sealed by deposited lipophilic crystalline waxes forming the limiting barrier for cuticular transport (Schönherr and Riederer 1988).

Foliar spray application of agrochemicals is commonly used for plant protection. Almost all crop protection products contain adjuvants besides the active ingredient. They make important contributions to the improvement of the spray application as well as the enhancement of biological efficacy (Kirkwood 1993, Penner 2000, Zabkiewicz 2000). Adjuvants can be categorized in two groups: (i) activator and (ii) utility adjuvants (McMullan 2000). The latter are influencing the properties of the spray solution and they are not directly affecting the efficacy of the applied agrochemical. Whereas, activator adjuvants can directly enhance the uptake from the foliar spray deposit (McMullan 2000). The enhancement can be mediated by improved (i) spreading of the spray droplet and wettability of the leaf surface (ii) adhering of dry residues on the foliage, (iii) dry-down rate (decelerated) of the spray droplet and therefore prevention of crystallization and (iv) stomatal infiltration or cuticular penetration (Hazen 2000).

Cuticular penetration can be enhanced by adjuvants which diffuse into cuticular wax rendering them more fluid (Schreiber et al. 1996b, Schreiber 2006, Fagerström et al. 2014, Zeisler-Diehl et al. 2022). Plasticizing of the transport-limiting barrier of the plant cuticle is primarily beneficial for lipophilic substances. Depending on the polarity of active ingredients there are

two different pathways available for diffusion. Lipophilic agrochemicals diffuse across the cuticle *via* the lipophilic pathway, which is made up of amorphous wax (Buchholz 2006). Whereas the polar pathway, which has been suggested to be formed by polysaccharide fibrils and polar wax and cutin domains, should preferentially be available for diffusion of hydrophilic molecules and ions (Schreiber 2005). Therefore, uptake enhancement of polar substances has to be based on other adjuvant induced mechanisms. In the past, there have been many studies investigating adjuvant effects on diffusion of lipophilic compounds by plasticizing the lipophilic pathway (Riederer et al. 1995, Baur et al. 1997b, Fagerström et al. 2013, Zeisler-Diehl et al. 2022). Studies of adjuvant effects on the polar pathway are missing. Therefore, the objective of this study was to uncover potential accelerating effects of adjuvants on penetration of polar and intermediate polar compounds. Sodium lauryl ether sulphate, alkyl polyglucoside and rapeseed oil methyl ester were chosen to cover a broad spectrum of adjuvants used in agriculture. The three adjuvants selected vary significantly in structure and physicochemical properties. The model compounds for investigating accelerating effects of adjuvants on penetration were methyl-glucose, thiamethoxam and terbuthylazine. With (i) the variety of compounds mainly differing in their polarity and (ii) the adjuvants differing in their physicochemical properties, potential effects on diffusion *via* the lipophilic and the polar pathway should be compared.

3.3 Material and Methods

3.3.1 Plant material

Adaxial astomatous cuticular membranes (CM) were isolated enzymatically from fully expanded leaves of cherry laurel (*Prunus laurocerasus*), dried and stored in petri dishes until further use (Schönherr and Riederer 1986).

3.3.2 Chemicals

Radiolabelled model compounds varied widely in their lipophilicity. From hydrophilic methyl-glucose (log K_{OW}: -3.0, specific activity: 60 Ci mmol^{-1}) to lipophilic terbuthylazine (log K_{OW}: 3.4, 26.08 mCi mmol^{-1}) with thiamethoxam (log K_{OW}: -0.1, 40.82 mCi mmol^{-1}) in between. ^{3}H-labelled methyl-glucose was obtained from ARC Inc. (St. Louis, USA), ^{14}C-labelled thiamethoxam and ^{14}C-labelled terbuthylazine from Selcia Ltd (Essex, UK).

Table 3: Characteristic properties of methyl-glucose, thiamethoxam and terbuthylazine

	Methyl-Glucose	Thiamethoxam	Terbuthylazine
MW (g mol^{-1})	194[a]	292[b]	230[b]
V_X (cm^3 mol^{-1})	140[c]	181[c]	176[c]
log K_{OW}	-3.0[a]	-0.1[b]	3.4[b]
Radiochemical purity	< 99 %	98.6 %	99.2 %

MW (Molecular weight), V_x (characteristic molecular volume), log K_{OW} (Octanol-water partition coefficient)

[a] Values taken from Schreiber and Schönherr (2009)

[b] Values for thiamethoxam (PubChem CID: 5485188) and terbuthylazine (CID: 22206) taken from PubChem

[c] Calculated according to Abraham and McGowan (1987)

The non-ionic surfactant alkyl polyglucoside (APG 8107) was obtained from BASF (Ludwigshafen, Germany). The anionic surfactant sodium lauryl ether sulphate (SLES SL200, 200 g l^{-1}) and the mixture of methylated fatty acids (RME EW400, 400 g l^{-1}) were obtained formulated from Syngenta Crop Protection AG (Münchwilen, Switzerland). Details of the model compounds and adjuvants are listed in Table 3 and 4.

Table 4: Characteristic properties of the different adjuvants used

	APG	SLES	RME
Chemical name	Alkyl polyglucoside	Sodium lauryl ether sulphate	Rapeseed oil methyl ester
Average MW (g mol^{-1})	450	360	300
Polar head group	1-2 glucose	Sulphate, 1-4 EO	-
Most frequent alkyl length	8-10	10-16	16-18
HLB	~13.6[a]	~40[a]	-

MW (Molecular weight), HLB (Hydrophilic-lipophilic balance), EO (Ethylene oxide)

[a] calculated according to Davies (1957)

3.3.3 Penetration experiments

To study effects of adjuvants on rates of cuticular penetration Simulation of Foliar Penetration (SOFP) experiments were conducted (Schönherr and Baur 1994, Gutenberger et al. 2013). Cuticular membranes were mounted on the receiver side of the chamber system, facing the physiological outer side towards the atmosphere and fixed with a ring-shaped lid. Lid and receiver chamber were fixed with adhesive tape (Tesa, Norderstedt, Germany). Cuticles were tested on integrity by application of 10 µl droplets of ethanol. Holes would appear as dark spots after the ethanol had penetrated. Damaged cuticles were replaced by intact ones.

Radioactive labelled compounds dissolved in ethanol (10 µl) were applied with or without an adjuvant to the center of the membranes. Droplets contained 1667 Bq (methyl-glucose) or 667 Bq (thiamethoxam and terbuthylazine) of the radiolabelled compound, while adjuvant concentration was 1 g l^{-1}. After evaporation of ethanol, the chambers were filled with 800 µl phospholipid suspension (PLS, 1 % soybean lecithin in water, Carl Roth, Karlsruhe, Germany) as receiver medium and placed upside down on scintillation vials filled with 250 µl glycerol (≥ 99 %, Fisher Chemical, Loughborough, UK). This ensured a relative humidity of 2 % over the donor residues. The receiver medium was replaced by fresh PLS at regular intervals. Radioactivity in the receiver medium was counted by a liquid scintillation analyser (Tri-Carb 4910TR, PerkinElmer, Waltham, USA) after adding scintillation cocktail (Ultima Gold XR, PerkinElmer, Waltham, USA). The temperature of the transport chambers was kept constant at

25 °C in an incubator (MIR-554, Panasonic, Kadoma, Japan). After the last sampling, the exposed area of the cuticles was excised and the radioactive residue was determined.

Penetration was plotted as $-\ln(1-M_t/M_0)$ versus time (t). $(1-M_t/M_0)$ is the relative amount of donor left on the cuticle at time t. M_t is the desorbed amount at time t and M_0 is total amount of applied radioactive labelled compound. Regression lines were fit to the transport kinetics. The slope of the regression line represents the rate constant of penetration k (h^{-1}).

3.3.4 Sample size and statistics

Rates of cuticular penetration were measured of at least 20 isolated cuticular membranes for each treatment. Statistical analysis was carried out using OriginPro (Version 2021, OriginLab, Northampton, USA). Shapiro-Wilk test ($P < 0.05$) showed non-normal distribution for the data of rate constants. In accordance with the interquartile range method outliers were removed. Significant differences were determined statistically using Kruskal-Wallis analysis of variance (ANOVA) with post-hoc Dunn´s test ($P < 0.05$). For each individual CM $-\ln(1-M_t/M_0)$ was calculated and median values with 25^{th} and 75^{th} interquartile ranges were plotted.

3.4 Results

Time courses of penetration plotted as $-\ln(1-M_t/M_0)$ over time resulted in linear slopes for methyl-glucose in presence and absence of adjuvants. After 48 hours less than 3 % of the initially applied amounts of methyl-glucose penetrated the cuticles (Figure 12, a). Trans-cuticular diffusion of methyl-glucose was linear and constant during the whole experiment and ranged from 4.47 (2.97 – 8.46) x 10^{-4} h^{-1} (1 g l^{-1} APG) to 7.05 (4.79 – 13.5) x 10^{-4} h^{-1} (1 g l^{-1} SLES). Penetration rates of thiamethoxam and terbuthylazine without adjuvant were linear after 24 hours (Figure 12, b and c). After 48 hours around 9 % and 45 % of the initially applied amount of thiamethoxam and terbuthylazine, respectively, were desorbed from the physiological inner side of the cuticle. For thiamethoxam rate constants were determined and varied from 1.33 (0.75 – 1.86) x 10^{-3} h^{-1} (1 g l^{-1} APG) to 5.31 (2.41 – 7.20) x 10^{-3} h^{-1} (1 g l^{-1} RME). Highest rate constants of terbuthylazine were determined when RME was used as adjuvant (6.87 (5.96 – 8.17) x 10^{-2} h^{-1}) and lowest when no adjuvant was used (1.72 (0.80 – 2.76) x 10^{-2} h^{-1}). The penetration rates of thiamethoxam and terbuthylazine levelled off after 32 hours in the presence of RME (Figure 12, b and c).

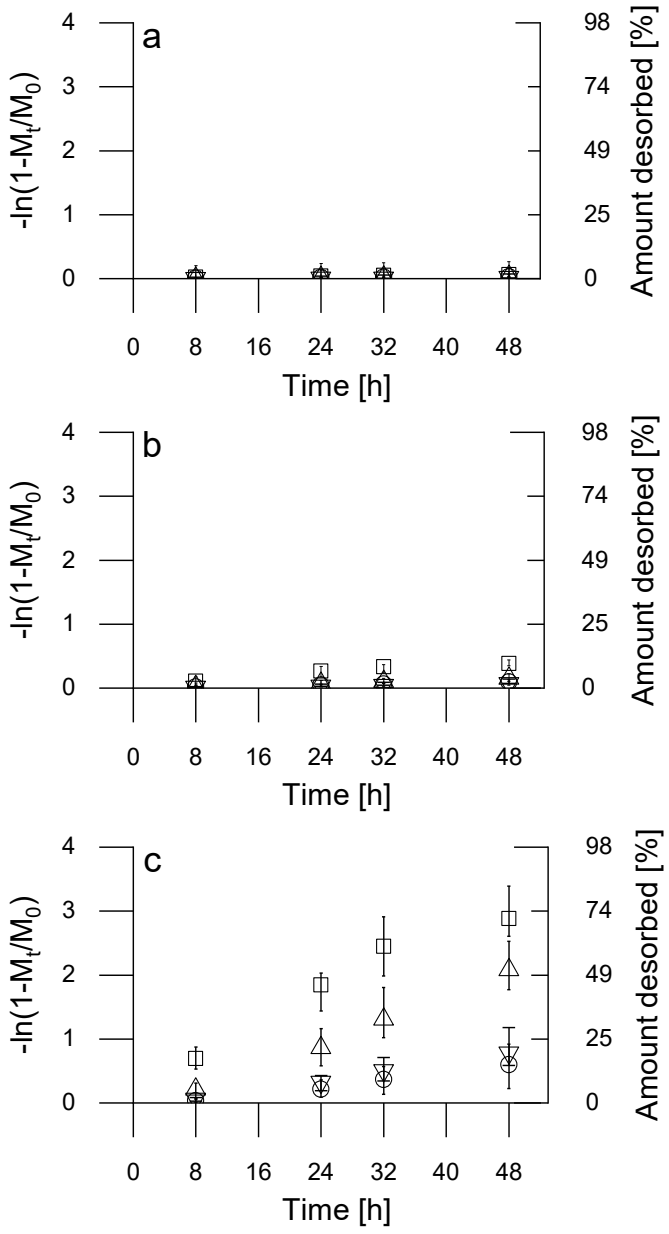

Figure 12: Time courses of penetration.
Methyl-glucose (a), thiamethoxam (b) and terbuthylazine (c) penetration across isolated cuticles of *Prunus laurocerasus*. The investigated compounds were dissolved in ethanol and applied as 10 µl droplets on the physiological outer side of the cuticle. Additionally, the donor droplets contained 1 g l-1 of APG (triangles facing down), SLES (triangles facing up), RME (squares) or no adjuvant (circles) serving as control. For each individual CM −ln(1−Mt/M0) was calculated and median values with quartiles were plotted.

Addition of APG led to a decreased penetration of methyl-glucose and thiamethoxam, while penetration of terbuthylazine was slightly increased (Figure 13). Slight, but statistically not significant, enhancing effects on the penetration of methyl-glucose and thiamethoxam was observed when SLES was used as adjuvant (Figure 13). The effect on diffusion of

terbuthylazine was higher (2.8) and of statistical significance (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$). RME increased the penetration of all tested compounds (Figure 13), but only for thiamethoxam (2.8) and terbuthylazine (4.0) the enhancement was statistically significant (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$).

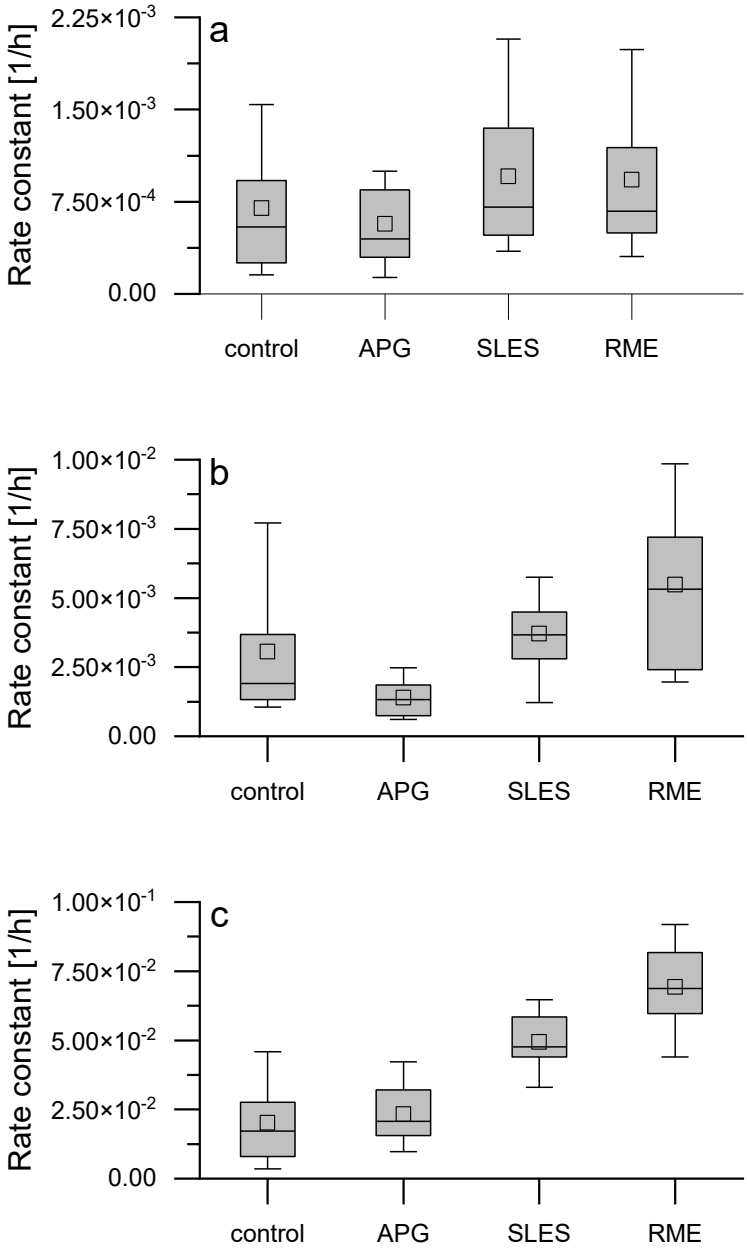

Figure 13: Rate constants of methyl-glucose (a), thiamethoxam (b) and terbuthylazine (c).
Penetration across isolated leaf cuticles of *Prunus laurocerasus* with and without adjuvants (0.1 % APG, SLES and RME). Regression lines were fit to the transport kinetics (Figure 12). The slope of the regression line represents the rate constant of penetration k (h-1). Boxes of the box plots represent 25th and 75th percentiles. Whiskers indicate 10th and 90th percentiles. Squares and horizontal lines within the boxes represent mean and median, respectively.

3.5 Discussion

In this study adjuvant effects on cuticular penetration of three model compounds differing in their lipophilicity were investigated. Log octanol-water partition coefficients ranged from -3.0 (methyl-glucose) to -0.1 (thiamethoxam) and 3.4 (terbuthylazine). The used adjuvants were two hydrophilic surfactants (SLES and APG) and a mixture of lipophilic methylated rapeseed oils (RME). Penetration enhancing effects were determined by applying 10 µl droplets of the radiolabelled compounds, with and without adjuvant, on the physiological outer side of cuticular membranes.

Rate constants of penetration determined for the three model compounds without adjuvant differed by two orders of magnitude. Fastest penetration was observed for lipophilic terbuthylazine and slowest for hydrophilic methyl-glucose (Figure 12). Polar molecules as well as charged ions are suggested to diffuse across the plant cuticle *via* the polar pathway (Schönherr 2000, Schönherr 2002). This pathway is formed by polysaccharides, which build up a ramified network in the cuticle (Guzmán et al. 2014a, Fernández et al. 2017). Polysaccharide fibrils are extending from the cell wall up to the cuticle proper, which represents the transport-limiting barrier of the plant cuticle (Guzmán et al. 2014a, Guzmán et al. 2014b). The cuticle proper consists of crystalline and amorphous wax associated with the cutin matrix (Reynhardt and Riederer 1991) and is located on the physiological outer side of the cuticle (Wattendorff and Holloway 1984). Lipophilic molecules can sorb into and diffuse through the amorphous waxes, which is called the lipophilic pathway (Buchholz 2006). Hydrophilic compounds might fully or partially be excluded from the lipophilic pathway, due to their low solubility in the cutin and wax domains of the cuticle (Schönherr 2006).

In the presence of APG, penetration was statistically not altered for all three tested compounds (Figure 12). APG is a non-ionic surfactant with an HLB (Hydrophilic-lipophilic balance) value of 13.6. The polar head consists out of hydrophilic glucose molecules (1.7 in average), while the lipophilic part is formed by an alkyl chain with 8 to 10 carbons. Due to the distinct wetting and spreading properties APG is used as an adjuvant in agricultural products (Garst 1996, Castro et al. 2014). It was shown in experiments, that APG can enhance the cuticular penetration of polar substances, which was attributed to the improved wetting of the surface (Schönherr 2001, Schönherr 2002, Schönherr and Schreiber 2004a, Rodriguez-Lucena et al. 2010). Effects of droplet spreading or enhanced wetting of the cuticle surface due to APG can be excluded in the conducted experiments, since with ethanol already complete coverage and wetting is

achieved. It was shown in previous experiments (unpublished results), that with ethanol as donor solvent the covered area of the cuticle was increased by factor of 7 compared to water as donor solvent. Ethanol is a fairly uncommon solvent for the application of agrochemicals. It is used here only to investigate potential accelerating effects on diffusion independent from wetting and spreading effects. In unilateral desorption from the outer side (UDOS) experiments it was shown that APGs did not have accelerating effects on the mobility of lipophilic molecules (Shi et al. 2005b). For this method compounds were applied to the physiological inner side of the cuticle and desorption from the physiological outer side was measured. The receiver compartment was containing an aqueous solution of the potential accelerator adjuvant. Effects on polar substances were not investigated, since this method is not suitable for polar substances due to the low solubility in the sorption compartment (Schreiber and Schönherr 2009).

Surprisingly, penetration of hydrophilic methyl-glucose and semi-hydrophilic thiamethoxam was even slightly reduced in the presence of APG (Figure 13, a and b), while a slight increase was observed for lipophilic terbuthylazine (Figure 13, c). Alkyl polyglucosides penetrate the cuticle very slowly (Schönherr 2001) if at all and it can be assumed that the major fraction of the applied amount remain on the cuticle surface. During the experiment, water molecules are diffusing from the aqueous receiver compartment across the cuticle. The diffused water can be retained, at least partially, by the hydrophilic part of the APGs (Hughes and Lew 1970). From the water permeance of isolated *Prunus laurocerasus* leave cuticles (1.1×10^{-10} m s^{-1}) the amount of water penetrating the cuticular membrane can be calculated (Schreiber et al. 2006). Over 24 hours around 1.1 µl of water is diffusing across the cuticle. At a humidity of 2 % crystallization of the applied compounds is likely, but since penetration occurs even in absence of adjuvants the amount of penetrated water is apparently sufficient to form a humid deposit layer on the cuticular surface. In this deposit layer the compounds may dissolved at their maximum water solubility.

Methyl-glucose and thiamethoxam are more soluble in these possibly humid residues of APGs than in the cuticle. Water sorption decreases the concentration of the compounds in the deposit and therefore driving force for diffusion is lower and sorption into the cuticle is ceased (Baur 1999). For the lipophilic terbuthylazine the situation is very different. The water potentially present in the residue will solubilise terbuthylazine and prevent crystallisation. The partitioning of lipophilic molecules in cuticular waxes is higher than in the hydrophilic residue on the cuticle surface, therefore sorption into the cuticle is increased (Buchholz 2006). This explains the slight

increase in penetration of terbuthylazine, while for methyl-glucose and thiamethoxam a decrease was observed.

SLES enhanced the penetration of all compounds (Figure 13). The highest effect was determined for terbuthylazine (2.8) and the only one of statistical significance (Figure 14, b). The HLB value of the anionic surfactant SLES is around 40. The lipophilic aliphatic tail has a length of 10 to 16 C-atoms and the polar head is made up of sulphate and 1 to 4 ethylene oxides. Water can be retained by the polar head due to water adsorption to the sulphate group (Berthold et al. 1996) or to the ethylene oxides (Stevens and Bukovac 1987b, Stevens and Bukovac 1987a). SLES showed improved herbicide efficacy in field and semi-field trials (Pannacci et al. 2010, Javaid and Tanveer 2017, Tanveer et al. 2018, Machado et al. 2019). Enhancement was attributed to the improved retention and rainfastness and reduced spray drift of the applied agrochemicals. Due to the negative charge of the polar fraction of SLES, it can be assumed that diffusion into the cuticle is low, since the plant cuticle is carrying a negative charge at a pH above 3 (Schönherr and Bukovac 1973). Therefore, SLES is, like APG, remaining on the cuticular surface during the experiment. The ability of water sorption of surfactants among different classes can be ranked by the HLB (Asmus et al. 2016). With a HLB value of 40 SLES is presumably able to retain more water than APG (HLB of 13.6).

Riederer and Schönherr (1990) reported a decrease in water permeability of isolated cuticles when sodium dodecyl sulphate (SDS) was applied to the physiological outer side (Riederer and Schönherr 1990). This decrease was explained that SDS is lying on the outer cuticle surface and thus could form an additional diffusional barrier for water on the cuticle. However, alternatively this decrease could also be explained by SDS retaining diffused water and since water loss was gravimetrically determined the amounts of water lost would be underestimated leading to the conclusion that cuticular permeability is decreased. SLES contains 1 to 4 EO, while SDS possess no EOs. With increasing EO content molecules can retain more water as it was reported for alcohol ethoxylates (Stock and Holloway 1993). With EO numbers of 10 and higher alcohol ethoxylates will enhance uptake of hydrophilic compounds due to humectancy effects, while EO numbers below 6 will lead to increased uptake of lipophilic compounds (Stock et al. 1993, Burghardt et al. 1998). Other authors showed that humectancy increases with increasing oxygen content even at lower EO numbers than 10 (Asmus et al. 2016). Therefore, water sorption of SLES can be even more pronounced than the ability of SDS (Lee et al. 2014) and APG.

As further possibility, it can also be postulated that the lipophilic fraction of the SLES molecules interacts with cuticular waxes and facilitate diffusion of lipophilic molecules (Coret and Chamel 1994, Schreiber et al. 1996b). This interaction is assumed to separate crystalline regions and thus rendering the wax more amorphous (Fagerström et al. 2014). Additionally, it was shown that surfactants can have even in absence of water plasticizing effects on waxes (Fagerström et al. 2014). Methyl-glucose is restricted to penetrate the cuticle *via* the polar pathway, due to the high hydrophilicity. Therefore, plasticizing of waxes will not lead to an increased rate of penetration. The fact, that a slight enhancement effect by SLES is observed can be explained by a plasticization effect on the waxes surrounding the polar pores that broadens the pore radius rendering them more accessible for methyl-glucose. Since thiamethoxam is a semi-hydrophilic molecule, it can diffuse through the cuticle on the lipophilic and the polar pathway. Addition of SLES led to a higher effect (1.9) on the rate constant of penetration of thiamethoxam than for methyl-glucose (1.3) (Figure 14, b). Highest effects on penetration was observed for terbuthylazine, which is diffusing across the cuticle only *via* the lipophilic pathway. Therefore, the enhancement effect of SLES can be attributed to plasticizing of the lipophilic pathway.

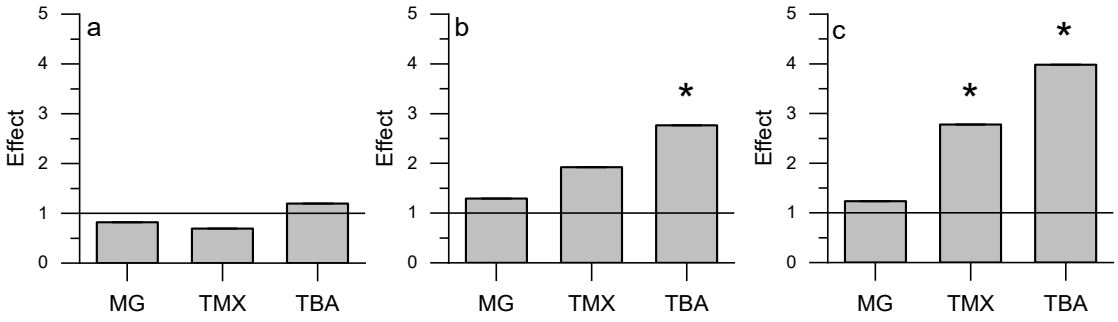

Figure 14: Effects of adjuvants.
Penetration of methyl-glucose (MG), thiamethoxam (TMX) and terbuthylazine (TBA) across cuticular membranes of *Prunus laurocerasus* affected by APG (a), SLES (b) or RME (c). Concentration of adjuvants was 1 g l-1. Effects were calculated dividing median rate constants of penetration in the presence of the adjuvants by the median rate constants of penetration measured as control. The line indicates no effect (ratio of median with and without adjuvant = 1). Asterix above the boxes indicate significant effects (Kruskal-Wallis ANOVA with post-hoc Dunn´s test, $P < 0.05$).

Addition of RME to the donor solution led to a statistical significant enhancement of penetration for thiamethoxam and terbuthylazine, while penetration of methyl-glucose was only slightly increased (Figure 12). RME is a heterogeneous mixture of methylated saturated and unsaturated fatty acids derived from rapeseed oil (mainly C_{16} and C_{18}). Methylated seed oils are known as accelerator adjuvants (Hart and Wax 1996, Knezevic et al. 2010, Zhang et al. 2013, Brabham et al. 2019), which can enhance the uptake of herbicides by plasticizing cuticular wax (Gauvrit

and Cabanne 1993, Santier and Chamel 1996). Since polar substances are not soluble or only to a minor degree in cuticular waxes, plasticizing of wax seems unlikely to enhance the diffusion of polar molecules. In previous studies, it was shown that a total wax extraction led to an increase by only a factor 2 to 3 of cuticular permeability for polar substances (Schönherr 2000), while a factor of 273 was determined for permeability of lipophilic substances (Popp et al. 2005). It was suggested that waxes partially block the polar pathway and the removal of them opened new pathways for diffusion. When a plasticizer is present, waxes blocking the pores may become more permeable for the polar solutes or the pore radii increase and therefore diffusion is facilitated. This may explain the slight, but not significant, effect (1.2) on penetration of methyl-glucose (Figure 13, a).

Penetration of lipophilic terbuthylazine and semi-hydrophilic thiamethoxam was significantly increased by a factor of 4.0 and 2.8, respectively (Figure 14, c), when applied together with RME. Interestingly, rate of penetration of both substances levelled off after 32 hours (Figure 12). At first sight this phenomenon can attributed to the decreasing amount of terbuthylazine on the cuticular surface available for diffusion, since approximately 63 % of the initially applied amount was already desorbed after 32 hours. However, since it occurs likewise with thiamethoxam that had penetrated the cuticle only to 27 % after 32 hours, there have to be another explanation. Enhancing effects of adjuvants on penetration depend on their concentration in the cuticle (Riederer et al. 1995, Santier and Chamel 1996). Since the initially applied amount of RME is finite and RME is penetrating the cuticle as well, it is dissolved in the receiver solution and removed with each sample taken. Thus, the concentration of RME in the cuticle is decreasing over the duration of the experiment. Therefore, with a decreasing concentration of RME in the cuticle resulting in a continuously decreasing plasticizing effect on wax towards the end of the experiment the enhancing effect will also decrease. These results are in accordance with experiments where penetration of isoxaflutole (log K_{OW}: 2.3) was increased by a factor of 2 when applied with methylated seed oil and likewise levelled off (Young and Hart 1998).

The data presented here may suggest that hydrophilic adjuvants are not appropriate for enhancing uptake of agrochemicals, whereas lipophilic RME increased penetration of at least two of the three investigated model compounds. For the uptake of foliar applied agrochemicals different processes are involved and the actions of investigated adjuvants are differing (Kirkwood 1993). Furthermore, environmental conditions (e.g. temperature and humidity) in

the field are variable and maybe others than in the conducted experiments. The aim of this mechanistic study was to reveal accelerating effects on diffusion of compounds differing in their polarity. Therefore, other adjuvant effects like humectancy, wetting and spreading were completely excluded by the experimental procedure.

However, in the field when agrochemicals are applied to leaves the sprayed droplets have to stick on the leaves in the first instance before any uptake can take place (Wirth et al. 1991, Taylor 2011). For retention of the droplets and subsequent spreading adjuvants like APG and SLES are very useful. These adjuvants reduce the surface tension and thus improve adhesion of the droplets and wetting of the leave surfaces. For the sorption from the spray droplet residue into the cuticle the use of these adjuvants potentially only show benefits when humidity is increased and therefore more water can be absorbed, crystallization is prevented and time for uptake is prolonged (Baur et al. 1999b). At the investigated low humidity only RME and SLES can accelerate uptake of lipophilic and semi-hydrophilic substances, since they plasticize cuticular waxes.

3.6 Conclusion

This study shows that the polar surfactant APG did not enhance cuticular permeability of the three solutes varying in their polarity (polar, semi-hydrophilic and lipophilic). Enhanced penetration of semi-hydrophilic and lipophilic substances which were diffusing *via* the lipophilic pathway can be achieved by adjuvants which plasticize the lipophilic path of diffusion. Furthermore, for optimizing uptake of agrochemicals the penetration rates of plasticizer adjuvant and agrochemical across cuticles should be adjusted. For intermediate polar (semi-hydrophilic) substances a rather slow penetrating accelerator would be beneficial.

Summary

In future the growing world population will result in an increased demand for food. To satisfy this future demand without destroying habitats deserving protection, food production on current used arable land has to be more sustainable and efficient. In modern agriculture high efficiency is obtained by applying crop protection products. To further increase the sustainability of these products their efficacy has to be improved. One great opportunity is to study the uptake of the active ingredients of these products and provide a better understanding of this process.

Here a newly developed approach is introduced to measure potentially enhancing effects of surfactants on the permeability of isolated leaf cuticles of *Prunus laurocerasus*. With fluorescein, a representative model compound was found showing physicochemical properties of the majority of active ingredients used in crop protection products. The newly established method could be verified by radiometric control measurements. Hence, fluorometry is a valid method with sufficient sensitivity and specificity allowing experiments with fairly lipophilic model substances (K_{OW} of 1000 or higher), which can be studied below their maximum of water solubility. A set of alcohol ethoxylates varying in their average degree of ethoxylation, derived from unsaturated oleic alcohol, was selected to study potential effects on cuticular transport of fluorescein. All three studied alcohol ethoxylates enhanced the permeance of fluorescein significantly. With increasing HLB (hydrophilic-lipophilic balance) value, decreasing lipophilicity and increasing size of the surfactants their potential of accelerating the diffusion of fluorescein across isolated cuticles of *Prunus laurocerasus* decreased. This indicates that the presented new method allows to discriminate between surfactants enhancing trans-cuticular diffusion and ineffective surfactants. Thus, the fluorometric approach represents a good and reliable non-radioactive alternative for screening the efficiency of surfactants in the future.

Furthermore, penetration studies showed the importance of wetting and consistent coverage of cuticular surfaces, especially if compounds differing in their lipophilicity where investigated and compared. The influence is rather negligible for lipophilic substances while penetration of hydrophilic substances can be impeded or not occur at all. Accessibility of the polar pathway of transport is not given for polar compounds applied as aqueous donor droplets. With ethanol as the donor solution, polar pores on or close to the outer surface of the cuticle became more reachable due to an intimate contact with the surface. Regarding the situation in the field, this is of high importance since target plants are often exhibiting leaf surfaces which are hard to wet. The rate constants of solute penetration increased with their lipophilicity and differed by

two orders of magnitude. Silver chloride precipitates were used to block the polar pathway and rate constants of the solutes were determined. Blockage of the pores resulted in a 50 % and 40 % lower penetration rate for the hydrophilic and semi-hydrophilic compound, respectively, while the penetration of the lipophilic solute was not altered. These results provide strong experimental evidence for the two different pathways of diffusion across plant cuticles and may help to improve foliar uptake of polar active ingredients or fertilizers.

At last, the potential accelerating effects of adjuvants on the diffusion of compounds differing in their polarity across isolated leaf cuticles of *Prunus laurocerasus* were investigated. In the presence of alcohol polyglucosides (APG), penetration was statistically not altered for all three tested compounds. Surprisingly, penetration of the polar and intermediate polar compounds was even slightly reduced in the presence of APG, while a slight increase was observed for the lipophilic substance. A humid deposit formed by the water adsorbing adjuvant APG could potentially prevent the lipophilic compound from crystallization which will increase the penetration. Whereas a humid deposit will constitute a polar phase on the leaf surface dissolving polar substances and consequently penetration across the cuticle will be impeded. Sodium lauryl ether sulphate (SLES) and the mixture of methylated rapeseed oils (RME) enhanced the penetration of all compounds. Both adjuvants can be assumed to exhibit plasticizing effects on cuticular waxes which leads to accelerated penetration of lipophilic solutes. The accelerating effect on the polar pathway can be explained by widening the radii of the pores within the cuticular waxes and rendering partially blocked pores more accessible for polar and intermediate polar compounds. The data presented here may suggest that hydrophilic adjuvants are not appropriate for enhancing the uptake of agrochemicals, but different processes are involved in the uptake and the actions of the investigated adjuvants differ. Adjuvants like APG and SLES are very useful adjuvants for improving retention of the droplets and subsequent spreading, while methylated seed oils are known to plasticize the lipophilic pathway of diffusion.

Based on the results presented in this dissertation future experiments should be conducted. Linking relative humidity, blockage of the polar pores and the use of adjuvants can provide further fundamental insights into the complex and diverse processes of cuticular penetration of substances differing in their lipophilicity. The results of the work presented here provide enhanced understanding of the process of cuticular penetration and new methods are suggested to measure the diffusion of substances like agrochemicals into plant leave surfaces. Identifying

and selecting appropriate adjuvants with regards to the pathway and the velocity of diffusion will improve the efficacy of crop protection products could lead to reduced application rates and a more sustainable agriculture.

Bibliography

(2009). "American Radiolabeled Chemicals, Inc: Custom Synthesis." from https://www.arcincusa.com/custom-synthesis.

Abraham, M. H. and J. C. McGowan (1987). "The Use of Characteristic Volumes to Measure Cavity Terms in Reversed Phase Liquid Chromatography." Chromatographia **23**.

Aponte, J. and P. Baur (2010). Penetration and translocation of coleoptileapplied systemic agrochemicals. Proceedings of the 9th International Symposium on Adjuvants for Agrochemicals, ISAA Society Wageningen.

Arand, K., E. Asmus, C. Popp, D. Schneider and M. Riederer (2018). "The Mode of Action of Adjuvants-Relevance of Physicochemical Properties for Effects on the Foliar Application, Cuticular Permeability, and Greenhouse Performance of Pinoxaden." Journal of Agricultural and Food Chemistry **66**(23): 5770-5777.

Arand, K., D. Stock, M. Burghardt and M. Riederer (2010). "pH-dependent permeation of amino acids through isolated ivy cuticles is affected by cuticular water sorption and hydration shell size of the solute." Journal of Experimental Botany **61**(14): 3865-3873.

Asmus, E., C. Popp, A. A. Friedmann, K. Arand and M. Riederer (2016). "Water Sorption Isotherms of Surfactants: A Tool To Evaluate Humectancy." Journal of Agricultural and Food Chemistry **64**(26): 5310-5316.

Baales, J., V. V. Zeisler-Diehl, Y. Malkowsky and L. Schreiber (2022). "Interaction of surfactants with barley leaf surfaces: time-dependent recovery of contact angles is due to foliar uptake of surfactants." Planta **255**(1): 1-77.

Ballmann, C., S. De Oliveira, A. Gutenberger, F. Wassmann and L. Schreiber (2011). "A radioactive assay allowing the quantitative measurement of cuticular permeability of intact Arabidopsis thaliana leaves." Planta **234**(1): 9-20.

Bargel, H., W. Barthlott, K. Koch, L. Schreiber and C. Neinhuis (2004). Plant cuticles: multifunctional interfaces between plant and environment. The Evolution of Plant Physiology. A. R. Hemsley and I. Poole, Academic Press London**:** 171-194.

Bargel, H. and C. Neinhuis (2005). "Tomato (Lycopersicon esculentum Mill.) fruit growth and ripening as related to the biomechanical properties of fruit skin and isolated cuticle." Journal of Experimental Botany **56**(413): 1049-1060.

Barthlott, W., F. Ditsch, C. Neinhuis, D. Cutler, I. Meusel, I. Theisen and H. Wilhelmi (1998). "Classification and terminology of plant epicuticular waxes." Botanical Journal of the Linnean Society **126**: 237-260.

Baur, P. (1997). "Lognormal distribution of water permeability and organic soiute mobility in plant cuticles." Plant, Cell & Environment **20**: 167-177.

Baur, P. (1999). "Surfactant Effects on Cuticular Penetration of Neutral Polar Compounds: Dependence on Humidity and Temperature." Journal of Agricultural and Food Chemistry **47**(2): 753-761.

Baur, P., A. Buchholz and J. Schönherr (1997a). "Diffusion in plant cuticles as affected by temperature and size of organic solutes: similarity and diversity among species." Plant, Cell & Environment **20**(8): 982-994.

Baur, P., B. T. Grayson and J. Schönherr (1997b). "Polydisperse Ethoxylated Fatty Alcohol Surfactants as Accelerators of Cuticular Penetration. 1. Effects of Ethoxy Chain Length and the Size of the Penetrants." Pesticide Science **51**(2): 131-152.

Baur, P., H. Marzouk and J. Schönherr (1999a). "Estimation of path lengths for diffusion of organic compounds through leaf cuticles." Plant, Cell & Environment **22**: 291-299.

Baur, P., H. Marzouk, J. Schönherr and B. T. Grayson (1997c). "Partition Coefficients of Active Ingredients between Plant Cuticle and Adjuvants As Related to Rates of Foliar Uptake." Journal of Agricultural and Food Chemistry **45**(9): 3659-3665.

Baur, P., J. Schönherr and B. T. Grayson (1999b). "Polydisperse Ethoxylated Fatty Alcohol Surfactants as Accelerators of Cuticular Penetration. 2: Separation of Effects on Driving Force and Mobility and Reversibility of Surfactant Action." Pesticide Science **55**(8): 831-842.

Berthold, J., M. Rinaudo and L. Salmefi (1996). "Association of water to polar groups; estimations by an adsorption model for ligno-cellulosic materials." Colloids and Surfaces A: Physicochemical and Engineering Aspects **112**(2-3): 117-129.

Brabham, C., J. K. Norsworthy, C. A. Sandoski, V. K. Varanasi and L. M. Schwartz-Lazaro (2019). "Spray deposition, adjuvants, and physiochemical properties affect benzobicyclon efficacy." Weed Technology **33**(2): 258-262.

Buchholz, A. (2006). "Characterization of the diffusion of non-electrolytes across plant cuticles: properties of the lipophilic pathway." Journal of Experimental Botany **57**(11): 2501-2513.

Buchholz, A., P. Baur and J. Schönherr (1998). "Differences among plant species in cuticular permeabilities and solute mobilities are not caused by differential size selectivities." Planta **206**(2): 322-328.

Buchholz, A. and J. Schönherr (2000). "Thermodynamic analysis of diffusion of non-electrolytes across plant cuticles in the presence and absence of the plasticiser tributyl phosphate." Planta **212**(1): 103-111.

Burghardt, M., L. Schreiber and M. Riederer (1998). "Enhancement of the Diffusion of Active Ingredients in Barley Leaf Cuticular Wax by Monodisperse Alcohol Ethoxylates." Journal of Agricultural and Food Chemistry **46**(4): 1593-1602.

Buschhaus, C. and R. Jetter (2011). "Composition differences between epicuticular and intracuticular wax substructures: how do plants seal their epidermal surfaces?" Journal of Experimental Botany **62**(3): 841-853.

Castro, M. J. L., C. Ojeda and A. F. Cirelli (2014). "Advances in surfactants for agrochemicals." Environmental Chemistry Letters **12**(1): 85-95.

Chamel, A., M. Pineri and M. Escoubes (1991). "Quantitative determination of water sorption by plant cuticles." Plant, Cell & Environment **14**(1): 87-95.

Coret, J. and A. Chamel (1994). "Effect of some ethoxylated alkylphenols and ethoxylated alcohols on the transfer of [^{14}C]chlorotoluron across isolated plant cuticles." Weed Research **34**: 445-451.

Cronfeld, P., K. Lader and P. Baur (2001). Classification of adjuvants and adjuvant blends by effects on cuticular penetration. Pesticide Formulations and Application Systems. A. K. Viets, R. S. Tann and J. C. Mueninghoff, ASTM Special Technical Publication, West Conshohocken, PA. **20:** 81-94.

Davies, J. T. (1957). A quantitative kinetic theory of emulsion type, I. Physical chemistry of the emulsifying agent. Gas/Liquid and Liquid/Liquid Interface. Proceedings of the International Congress of Surface Activity, Citeseer.

Domínguez, E. and A. Heredia (1999). "Water hydration in cutinized cell walls: a physico-chemical analysis." Biochimica et Biophysica Acta **1426**(1): 168-176.

Dorr, G. J., D. M. Kempthorne, L. C. Mayo, W. A. Forster, J. A. Zabkiewicz, S. W. McCue, J. A. Belward, I. W. Turner and J. Hanan (2014). "Towards a model of spray–canopy interactions: Interception, shatter, bounce and retention of droplets on horizontal leaves." Ecological Modelling **290**: 94-101.

Edwards, D. (1993). "Cells and tissues in the vegetative sporophytes of early land plants." New Phytologist **125**(2): 225-247.

Eichert, T. and H. E. Goldbach (2008). "Equivalent pore radii of hydrophilic foliar uptake routes in stomatous and astomatous leaf surfaces--further evidence for a stomatal pathway." Physiologia Plantarum **132**(4): 491-502.

Fagerström, A., V. Kocherbitov, T. Ruzgas, P. Westbye, K. Bergström and J. Engblom (2013). "Effects of surfactants and thermodynamic activity of model active ingredient on transport over plant leaf cuticle." Colloids and Surfaces B: Biointerfaces **103**: 572-579.

Fagerström, A., V. Kocherbitov, P. Westbye, K. Bergström, T. Arnebrant and J. Engblom (2014). "Surfactant softening of plant leaf cuticle model wax--a Differential Scanning Calorimetry (DSC) and

Quartz Crystal Microbalance with Dissipation (QCM-D) study." Journal of Colloid and Interface Science **426**: 22-30.
Fernández, V., H. A. Bahamonde, J. J. Peguero-Pina, E. Gil-Pelegrín, D. Sancho-Knapik, L. Gil, H. E. Goldbach and T. Eichert (2017). "Physico-chemical properties of plant cuticles and their functional and ecological significance." Journal of Experimental Botany **68**(19): 5293-5306.
Fernández, V., E. Gil-Pelegrín and T. Eichert (2021). "Foliar water and solute absorption: an update." The Plant Journal **105**(4): 870-883.
Fernández, V., P. Guzmán-Delgado, J. Graça, S. Santos and L. Gil (2016). "Cuticle Structure in Relation to Chemical Composition: Re-assessing the Prevailing Model." Frontiers in Plant Science **7**: 427.
Fernández, V., V. D. Río, L. Pumariño, E. Igartua, J. Abadía and A. Abadía (2008). "Foliar fertilization of peach (Prunus persica (L.) Batsch) with different iron formulations: Effects on re-greening, iron concentration and mineral composition in treated and untreated leaf surfaces." Scientia Horticulturae **117**(3): 241-248.
Forster, W. A., G. N. Mercer and W. C. Schou (2012). "Spray droplet impaction models and their use within AGDISP software to predict retention." New Zealand Plant Protection **65**: 85-92.
Garst, R. (1996). Alkyl Polyglycosides—New Solutions for Agricultural Applications. Alkyl Polyglycosides**:** 131-137.
Gaskin, R. E., K. D. Steele and W. A. Forster (2005). "Characterising plant surfaces for spray adhesion and retention." New Zealand Plant Protection **58**: 179-183.
Gauvrit, C. and F. Cabanne (1993). "Oils for Weed Control: Uses and Mode of Action." Pesticide Science **37**(2): 147-153.
Geyer, U. and J. Schönherr (1990). "The effect of the environment on the permeability and composition of Citrus leaf cuticles: I. Water permeability of isolated cuticular membranes." Planta **180**(2): 147-153.
Gorb, E. V. and S. N. Gorb (2017). "Anti-adhesive effects of plant wax coverage on insect attachment." Journal of Experimental Botany **68**(19): 5323-5337.
Grant, R. H., M. A. Jenks, P. J. Rich, P. J. Peters and E. N. Ashworth (1995). "Scattering of ultraviolet and photosynthetically active radiation by sorghum bicolor: influence of epicuticular wax." Agricultural and Forest Meteorology **75**(4): 263-281.
Gutenberger, A., V. V. Zeisler, R. Berghaus, H. Auweter and L. Schreiber (2013). "Effects of poly- and monodisperse surfactants on 14C-epoxiconazole diffusion in isolated cuticles of Prunus laurocerasus." Pest Management Science **69**(4): 512-519.
Guzmán, P., V. Fernández, M. L. Garcia, M. Khayet, A. Fernández and L. Gil (2014a). "Localization of polysaccharides in isolated and intact cuticles of eucalypt, poplar and pear leaves by enzyme-gold labelling." Plant Physiology and Biochemistry **76**: 1-6.
Guzmán, P., V. Fernández, J. Graça, V. Cabral, N. Kayali, MohamedKhayet and L. Gil (2014b). "Chemical and structural analysis of Eucalyptus globulus and E. camaldulensis leaf cuticles: a lipidized cell wall region." Frontiers in Plant Science **5**: 481.
H. B. Tukey, J. (1970). "The Leaching of Substances from Plants." Annual Review of Plant Physiology **21**(1): 305-324.
Hart, S. E. and L. M. Wax (1996). "Dicamba Antagonizes Grass Weed Control with Imazethapyr by Reducing Foliar Absorption." Weed Technology **10**(4): 828-834.
Hazen, J. L. (2000). "Adjuvants—Terminology, Classification, and Chemistry1." Weed Technology **14**(4): 773-784.
Hermens, J. L. M., J. H. M. d. Bruijn and D. N. Brooke (2013). "The octanol-water partition coefficient: strengths and limitations." Environmental Toxicology and Chemistry **32**(4): 732-733.
Hughes, F. A. and B. W. Lew (1970). "Physical and functional properties of some higher alkyl polyglucosides." Journal of the American Oil Chemists' Society **47**(5): 162-167.
Javaid, M. M. and A. Tanveer (2017). "Optimization of Application Efficacy for POST Herbicides with Adjuvants on Three-Cornered Jack (Emex australis Steinheil) in Wheat." Weed Technology **27**(3): 437-444.

Jeffree, C. E. (2006). The fine structure of the plant cuticle. Annual Plant Reviews. M. Riederer and C. Müller. Biology of the plant cuticle, Blackwell Publishing Ltd. **23:** 11-125.
Kenrick, P. and P. R. Crane (1997). "The origin and early evolution of plants on land." Nature **389**(6646): 33-39.
Kerler, F., M. Riederer and J. Schönherr (1984). "Non-Electrolyte Permeability of Plant Cuticles - a Critical-Evaluation of Experimental Methods." Physiologia Plantarum **62**(4): 599-606.
Kerler, F. and J. Schönherr (1988a). "Accumulation of Lipophilic Chemicals in Plant Cuticles: Prediction From Octanol/Water Partition Coefficients." Archives of Environmental Contamination and Toxicology **17**(1): 1-6.
Kerler, F. and J. Schönherr (1988b). "Permeation of Lipophilic Chemicals across Plant Cuticles - Prediction from Partition-Coefficients and Molar Volumes." Archives of Environmental Contamination and Toxicology **17**(1): 7-12.
Kerstiens, G. (2006). "Water transport in plant cuticles: an update." Journal of Experimental Botany **57**(11): 2493-2499.
Kirkwood, R. C. (1993). "Use and Mode of Action of Adjuvants for Herbicides: A Review of some Current Work." Pesticide Science **38**(2-3): 93-102.
Kirkwood, R. C. (1999). "Recent developments in our understanding of the plant cuticle as a barrier to the foliar uptake of pesticides." Pesticide Science **55**(1): 69-77.
Knezevic, S. Z., A. Datta, J. Scott and L. D. Charvat (2010). "Application timing and adjuvant type affected saflufenacil efficacy on selected broadleaf weeds." Crop Protection **29**(1): 94-99.
Koch, K. and W. Barthlott (2009). "Superhydrophobic and superhydrophilic plant surfaces: an inspiration for biomimetic materials." Philosophical Transactions of the Royal Society A **367**(1893): 1487-1509.
Koch, K., B. Bhushan, H.-J. Ensikat and W. Barthlott (2009). "Self-healing of voids in the wax coating on plant surfaces." Philosophical Transactions of the Royal Society A **367**(1894): 1673-1688.
Koch, K. and H.-J. Ensikat (2008). "The hydrophobic coatings of plant surfaces: epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly." Micron **39**(7): 759-772.
Krahmer, H., H. Walter, P. Jeschke, K. Haaf, P. Baur and R. Evans (2021). "What makes a molecule a pre- or a post-herbicide - how valuable are physicochemical parameters for their design?" Pest Management Science **77**(11): 4863-4873.
Krauss, P., C. Markstädter and M. Riederer (1997). "Attenuation of UV radiation by plant cuticles from woody species." Plant, Cell & Environment **20**(8): 1079-1085.
Lee, S. W., K. E. Tettey, Y. Yarovoy and D. Lee (2014). "Effects of anionic surfactants on the water permeability of a model stratum corneum lipid membrane." Langmuir **30**(1): 220-226.
Liakoura, V., S. Stavrianakou, G. Liakopoulos, G. Karabourniotis and Y. Manetas (1999). "Effects of UV-B radiation on Olea europaea: comparisons between a greenhouse and a field experiment." Tree Physiology **19**(13): 905-908.
Lindow, S. E. and M. T. Brandl (2003). "Microbiology of the Phyllosphere." Applied and Environment Microbiology **69**(4): 1875-1883.
Lopez-Casado, G., A. J. Matas, E. Dominguez, J. Cuartero and A. Heredia (2007). "Biomechanics of isolated tomato (Solanum lycopersicum L.) fruit cuticles: the role of the cutin matrix and polysaccharides." Journal of Experimental Botany **58**(14): 3875-3883.
Luque, P., R. Gavara and A. Heredia (1995). "A study of the hydration process of isolated cuticular membranes." New Phytologist **129**(2): 283-288.
Machado, W. A., S. M. Silva, S. M. Carvalho and J. P. A. R. d. Cunha (2019). "Effect of Nozzles, Application Rates, and Adjuvants on Spray Deposition in Wheat Crops." Engenharia Agrícola **39**(1): 83-88.
Matas, A. J., E. D. Cobb, J. A. Bartsch, J. Dominick J. Paolillo and K. J. Niklas (2004). "Biomechanics and anatomy of Lycopersicon esculentum fruit peels and enzyme-treated samples." American Journal of Botany **91**(3): 352-360.
McMullan, P. M. (2000). "Utility Adjuvants." Weed Technology **14**(4): 792-797.

Neinhuis, C. and W. Barthlott (1997). "Characterization and Distribution of Water-repellent, Self-cleaning Plant Surfaces." Annals of Botany **79**(6): 667-677.
Pannacci, E., S. K. Mathiassen and P. E. R. Kudsk (2010). "Effect of adjuvants on the rainfastness and performance of tribenuron-methyl on broad-leaved weeds." Weed Biology and Management **10**(2): 126-131.
Penner, D. (2000). "Activator Adjuvants." Weed Technology **14**(4): 785-791.
Perkins, M. C., C. J. Roberts, D. Briggs, M. C. Davies, A. Friedmann, C. Hart and G. Bell (2005a). "Macro and microthermal analysis of plant wax/surfactant interactions: plasticizing effects of two alcohol ethoxylated surfactants on an isolated cuticular wax and leaf model." Applied Surface Science **243**(1-4): 158-165.
Perkins, M. C., C. J. Roberts, D. Briggs, M. C. Davies, A. Friedmann, C. A. Hart and G. A. Bell (2005b). "Surface morphology and chemistry of Prunus laurocerasus L. leaves: a study using X-ray photoelectron spectroscopy, time-of-flight secondary-ion mass spectrometry, atomic-force microscopy and scanning-electron microscopy." Planta **221**(1): 123-134.
Popp, C., M. Burghardt, A. Friedmann and M. Riederer (2005). "Characterization of hydrophilic and lipophilic pathways of Hedera helix L. cuticular membranes: permeation of water and uncharged organic compounds." Journal of Experimental Botany **56**(421): 2797-2806.
Remus-Emsermann, M. N. P., S. d. Oliveira, L. Schreiber and J. H. J. Leveau (2011). "Quantification of lateral heterogeneity in carbohydrate permeability of isolated plant leaf cuticles." Frontiers in Microbiology **2**: 1-7.
Reynhardt, E. C. and M. Riederer (1991). "Structure and molecular dynamics of the cuticular wax from leaves of Citrus aurantium L." Journal of Physics D: Applied Physics **24**(3): 478-486.
Riederer, M. (2006). Introduction: biology of the plant cuticle. Annual Plant Reviews. M. Riederer and C. Müller. Biology of the plant cuticle, Blackwell Publishing Ltd. **23:** 1-10.
Riederer, M., M. Burghardt, S. Mayer, H. Obermeier and J. Schönherr (1995). "Sorption of Monodisperse Alcohol Ethoxylates and Their Effects on the Mobility of 2,4-D in Isolated Plant Cuticles." Journal of Agricultural and Food Chemistry **43**(4): 1067-1075.
Riederer, M. and J. Schönherr (1984). "Accumulation and Transport of (2,4-Dichlorophenoxy)acetic Acid in Plant Cuticles: I. Sorption in the Cuticular Membrane and Its Components." Ecotoxicology and Environmental Saftey **8**(3): 236-247.
Riederer, M. and J. Schönherr (1990). "Effects of Surfactants on Water Permeability of Isolated Plant Cuticles and on the Composition of their Cuticular Waxes." Pesticide Science **29**(1): 85-94.
Riederer, M. and L. Schreiber (2001). "Protecting against water loss: analysis of the barrier properties of plant cuticles." Journal of Experimental Botany **52**(363): 2023-2032.
Rodham, D. K. (2000). "Colloid and interface science in formulation research for crop protection products." Current Opinion in Colloid & Interface Science **5**(5-6): 280-287.
Rodriguez-Lucena, P., E. Ropero, L. Hernández-Apaolaza and J. J. Lucena (2010). "Iron supply to soybean plants through the foliar application of IDHA/Fe3+: effect of plant nutritional status and adjuvants." Journal of the Science of Food and Agriculture **90**(15): 2633-2640.
Sadler, C., B. Schroll, V. Zeisler, F. Wassmann, R. Franke and L. Schreiber (2016). "Wax and cutin mutants of Arabidopsis: Quantitative characterization of the cuticular transport barrier in relation to chemical composition." Biochimica et Biophysica Acta **1861**(9 Pt B): 1336-1344.
Samuels, L., L. Kunst and R. Jetter (2008). "Sealing plant surfaces: cuticular wax formation by epidermal cells." Annual Review of Plant Biology **59**: 683-707.
Santier, S. and A. Chamel (1996). "Penetration of triolein and methyl oleate through isolated plant cuticles and their effect on penetration of [^{14}C]quizalofop-ethyl and [^{14}C]fenoxaprop-ethyl." Weed Research **36**: 167-174.
Schlegel, T. K., J. Schönherr and L. Schreiber (2005). "Size selectivity of aqueous pores in stomatous cuticles of Vicia faba leaves." Planta **221**(5): 648-655.

Schönherr, J. (1976). "Water Permeability of Isolated Cuticular Membranes: The Effect of pH and Cations on Diffusion, Hydrodynamic Permeability and Size of Polar Pores in the Cutin Matrix." Planta **128**(2): 113-126.
Schönherr, J. (1982). Resistance of Plant Surfaces to Water Loss: Transport Properties of Cutin, Suberin and Associated Lipids. Physiological Plant Ecology II: Water Relations and Carbon Assimilation. O. L. Lange, P. S. Nobel, C. B. Osmond and H. Ziegler, Springer, Berlin, Heidelberg**:** 153-179.
Schönherr, J. (1993). "Effects of Alcohols, Glycols and Monodisperse Ethoxylated Alcohols on Mobility of 2,4-D in Isolated Plant Cuticles." Pesticide Science **39**(3): 213-223.
Schönherr, J. (2000). "Calcium chloride penetrates plant cuticles via aqueous pores." Planta **212**(1): 112-118.
Schönherr, J. (2001). "Cuticular penetration of calcium salts: effects of humidity, anions, and adjuvants." Journal of Plant Nutrition and Soil Science **164**(2): 225-231.
Schönherr, J. (2002). "A mechanistic analysis of penetration of glyphosate salts across astomatous cuticular membranes." Pest Management Science **58**(4): 343-351.
Schönherr, J. (2006). "Characterization of aqueous pores in plant cuticles and permeation of ionic solutes." Journal of Experimental Botany **57**(11): 2471-2491.
Schönherr, J. and P. Baur (1994). "Modelling Penetration of Plant Cuticles by Crop Protection Agents and Effects of Adjuvants on their Rates of Penetration." Pesticide Science **42**(3): 185-208.
Schönherr, J. and M. J. Bukovac (1973). "Ion Exchange Properties of Isolated Tomato Fruit Cuticular Membrane: Exchange Capacity, Nature of Fixed Charges and Cation Selectivity." Planta **109**(1): 73-93.
Schönherr, J. and K. J. Lendzian (1981). "A Simple and Inexpensive Method of Measuring Water Permeability of Isolated Plant Cuticular Membranes." Zeitschrift für Pflanzenphysiologie **102**(4): 321-327.
Schönherr, J. and M. Luber (2001). "Cuticular penetration of potassium salts: Effects of humidity, anions, and temperature." Plant and Soil **236**(1): 117-122.
Schönherr, J. and M. Riederer (1986). "Plant cuticles sorb lipophilic compounds during enzymatic isolation." Plant, Cell & Environment **9**(6): 459-466.
Schönherr, J. and M. Riederer (1988). "Desorption of Chemicals from Plant Cuticles: Evidence for Asymmetry." Archives of Environmental Contamination and Toxicology **17**(1): 13-19.
Schönherr, J. and M. Riederer (1989). Foliar Penetration and Accumulation of Organic Chemicals in Plant Cuticles. Reviews of Environmental Contamination and Toxicology. G. W. Ware. New York, NY, Springer New York**:** 1-70.
Schönherr, J. and H. W. Schmidt (1979). "Water Permeability of Plant Cuticles: Dependence of Permeability Coefficients of Cuticular Transpiration on Vapor Pressure Saturation Deficit." Planta **144**: 391-400.
Schönherr, J. and L. Schreiber (2004a). "Interactions of Calcium Ions with Weakly Acidic Active Ingredients Slow Cuticular Penetration: A Case Study with Glyphosate." Journal of Agricultural and Food Chemistry **52**(21): 6546-6551.
Schönherr, J. and L. Schreiber (2004b). "Size selectivity of aqueous pores in astomatous cuticular membranes isolated from Populus canescens (Aiton) Sm. leaves." Planta **219**(3): 405-411.
Schönherr, J., L. Schreiber and A. Buchholz (2001). "Effects of temperature and concentration of the accelerators ethoxylated alcohols, diethyl suberate and tributyl phosphate on the mobility of [^{14}C]2,4-dichlorophenoxy butyric acid in plant cuticles." Pest Management Science **57**(1): 17-24.
Schreiber, L. (2005). "Polar paths of diffusion across plant cuticles: new evidence for an old hypothesis." Annals of Botany **95**(7): 1069-1073.
Schreiber, L. (2006). "Review of sorption and diffusion of lipophilic molecules in cuticular waxes and the effects of accelerators on solute mobilities." Journal of Experimental Botany **57**(11): 2515-2523.
Schreiber, L. (2010). "Transport barriers made of cutin, suberin and associated waxes." Trends in Plant Science **15**(10): 546-553.

Schreiber, L., S. Bach, T. Kirsch, D. Knoll, K. Schalz and M. Riederer (1995). "A simple photometric device analysing cuticular transport physiology: surfactant effect on permeability of isolated cuticular membranes of Prunus laurocerasus L." Journal of Experimental Botany **46**(293): 1915-1921.
Schreiber, L., S. Elshatshat, K. Koch, J. Lin and J. Santrucek (2006). "AgCl precipitates in isolated cuticular membranes reduce rates of cuticular transpiration." Planta **223**(2): 283-290.
Schreiber, L., T. Kirsch and M. Riederer (1996a). "Transport properties of cuticular waxes of Fagus sylvatica L. and Picea abies (L.) Karst.: Estimation of size selectivity and tortuosity from diffusion coefficients of aliphatic molecules." Planta **198**: 104-109.
Schreiber, L. and M. Riederer (1996). "Ecophysiology of cuticular transpiration: comparative investigation of cuticular water permeability of plant species from different habitats." Oecologia **107**(4): 426-432.
Schreiber, L., M. Riederer and K. Schorn (1996b). "Mobilities of Organic Compounds in Reconstituted Cuticular Wax of Barley Leaves : Effects of Monodisperse Alcohol Ethoxylates on Diffusion of Pentachlorophenol and Tetracosanoic Acid." Pesticide Science **48**(2): 117-124.
Schreiber, L. and J. Schönherr (2009). Water and Solute Permeability of Plant Cuticles, Springer-Verlag Berlin Heidelberg.
Schulte, A. J., D. M. Droste, K. Koch and W. Barthlott (2011). "Hierarchically structured superhydrophobic flowers with low hysteresis of the wild pansy (Viola tricolor) - new design principles for biomimetic materials." Beilstein Journal of Nanotechnology **2**: 228-236.
Segado, P., E. Domínguez and A. Heredia (2016). "Ultrastructure of the Epidermal Cell Wall and Cuticle of Tomato Fruit (Solanum lycopersicum L.) during Development." Plant Physiology **170**(2): 935-946.
Serrano, M., F. Coluccia, M. Torres, F. L'Haridon and J.-P. Métraux (2014). "The cuticle and plant defense to pathogens." Frontiers in Plant Science **5**: 1-8.
Shi, T., J. Schönherr and L. Schreiber (2005a). "Accelerators Increase Permeability of Cuticles for the Lipophilic Solutes Metribuzin and Iprovalicarb but Not for Hydrophilic Methyl Glucose." Journal of Agricultural and Food Chemistry **53**(7): 2609-2615.
Shi, T., E. Simanova, J. Schönherr and L. Schreiber (2005b). "Effects of Accelerators on Mobility of 14C-2,4-Dichlorophenoxy Butyric Acid in Plant Cuticles Depends on Type and Concentration of Accelerator." Journal of Agricultural and Food Chemistry **53**(6): 2207-2212.
Sjöback, R., J. Nygren and M. Kubista (1995). "Absorption and fluorescence properties of fluorescein." Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy **51**(6): L7-L21.
Staiger, S., P. Seufert, K. Arand, M. Burghardt, C. Popp and M. Riederer (2019). "The permeation barrier of plant cuticles: uptake of active ingredients is limited by very long-chain aliphatic rather than cyclic wax compounds." Pest Management Science **75**(12): 3405-3412.
Stevens, P. J. G. and M. J. Bukovac (1987a). "Studies on Octylphenoxy Surfactants. Part 1: Effects of Oxyethylene Content on Properties of Potential Relevance to Foliar Absorption." Pesticide Science **20**: 19-35.
Stevens, P. J. G. and M. J. Bukovac (1987b). "Studies on Octylphenoxy Surfactants. Part 2: Effects on Foliar Uptake and Translocation." Pesticide Science **20**: 37-52.
Stock, D. and P. J. Holloway (1993). "Possible Mechanisms for Surfactant-Induced Foliar Uptake of Agrochemicals." Pesticide Science **38**(2-3): 165-177.
Stock, D., P. J. Holloway, B. T. Grayson and P. Whitehouse (1993). "Development of a Predictive Uptake Model to Rationalise Selection of Polyoxyethylene Surfactant Adjuvants for Foliage-applied Agrochemicals." Pesticide Science **37**(3): 233-245.
Tanveer, A., N. Abbas, M. E. Safdar and R. M. Ikram (2018). "Alkyl Ether Sulfate Improves Efficacy of Herbicides Against Common Goosefoot and Field Bindweed in Wheat." Planta Daninha **35**(0).
Taylor, P. (2011). "The wetting of leaf surfaces." Current Opinion in Colloid & Interface Science **16**(4): 326-334.
Volkov, A. G., S. Paula and D. W. Deamer (1997). "Two mechanisms of permeation of small neutral molecules and hydrated ions across phospholipid bilayers." Bioelectrochemistry and Bioenergetics **42**(2): 153-160.

Wattendorff, J. and P. J. Holloway (1984). "Periclinal Penetration of Potassium-Permanganate into Mature Cuticular Membranes of Agave and Clivia Leaves - New Implications for Plant Cuticle Development." Planta **161**(1): 1-11.
Wirth, W., S. Storp and W. Jacobsen (1991). "Mechanisms Controlling Leaf Retention of Agricultural Spray Solutions." Pesticide Science **33**(4): 411-420.
Yeats, T. H. and J. K. C. Rose (2013). "The formation and function of plant cuticles." Plant Physiology **163**(1): 5-20.
Young, B. G. and S. E. Hart (1998). "Optimizing foliar activity of isoxaflutole on giant foxtail (Setaria faberi) with various adjuvants." Weed Science **46**(4): 397-402.
Zabkiewicz, J. A. (2000). "Adjuvants and herbicidal efficacy - present status and future prospects." Weed Research **40**: 139-149.
Zabkiewicz, J. A. (2007). "Spray formulation efficacy—holistic and futuristic perspectives." Crop Protection **26**(3): 312-319.
Zeisler-Diehl, V., Y. Müller and L. Schreiber (2018). "Epicuticular wax on leaf cuticles does not establish the transpiration barrier, which is essentially formed by intracuticular wax." Journal of Plant Physiology **227**: 66-74.
Zeisler-Diehl, V. V., J. Baales, B. Migdal, K. Tiefensee, M. Weuthen, I. Fleute-Schlachter, Kremzow-Graw and L. Schreiber (2022). "Alcohol Ethoxylates Enhancing the Cuticular Uptake of Lipophilic Epoxiconazole Do Not Increase the Rates of Cuticular Transpiration of Leaf and Fruit Cuticles." Journal of Agricultural and Food Chemistry **70**(3): 777-7784.
Zeisler-Diehl, V. V., W. Barthlott and L. Schreiber (2020). Plant Cuticular Waxes: Composition, Function, and Interactions with Microorganisms. Hydrocarbons, Oils and Lipids: Diversity, Origin, Chemistry and Fate. H. Wilkes, Springer, Cham**:** 123-138.
Zeisler-Diehl, V. V., B. Migdal and L. Schreiber (2017). "Quantitative characterization of cuticular barrier properties: methods, requirements, and problems." Journal of Experimental Botany **68**(19): 5281-5291.
Zeisler, V. and L. Schreiber (2016). "Epicuticular wax on cherry laurel (Prunus laurocerasus) leaves does not constitute the cuticular transpiration barrier." Planta **243**(1): 65-81.
Zhang, J., O. Jaeck, A. Menegat, Z. Zhang, R. Gerhards and H. Ni (2013). "The mechanism of methylated seed oil on enhancing biological efficacy of topramezone on weeds." PLoS One **8**(9): e74280.

Acknowledgements

I would like to express my heartfelt thanks to all the people who have supported and accompanied me on my journey.

My special thanks go to Dr. Lukas Schreiber, who provided invaluable scientific advice and always had an open door and a listening ear for me.

I would also like to thank Dr. Viktoria Zeisler, who not only helped me in the lab with her expertise but also supported me outside of the research group.

I am very grateful to Dr. Christian Popp for enabling my PhD studies by facilitating contacts and organizing the funding for my studies through Syngenta Group.

I would like to thank my office colleagues for the intense scientific exchange and the valuable interpersonal relationships, which made the collaboration so pleasant.

A big thank you to the Ecophysiology group for their constant support, stimulating scientific discussions, and lively idea exchange, which greatly enriched my work.

A special thank you to my girlfriend Jessica, who supported me in every possible way. Without her patience, love, and help, much of this would not have been possible.

Finally, I would like to thank my family for their constant support and encouragement – without them, I would not have been able to embark on this journey.