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Development of a Method for the Analysis of Neonicotinoid Insecticides using Thin-Layer Chromatography

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Idzaid Bin Idros

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Supervising Professor:

Prof. Dr. rer. nat. Bernd Spangenberg

Second Examiner:

Prof. Dr. rer. nat. Klemens Lorenz

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Sulz-Lahr Idzaid Bin Idros

Abstract

The objective of this thesis is the quantification and qualification of neonicotinoid insecticides using thin-layer chromatography (TLC). Neonicotinoids are a relatively new form of pesticides, which have been proven to be extremely lethal to the honey bee, Apis mellifera. In this paper six forms of neonicotinoid insecticides (i.e. Acetamiprid, Thiacloprid, Imidacloprid, Clothianidin, Thaimethoxam, and Nitenpyram) are analysed. The initial steps are to first find a suitable mobile phase eluent, followed by the search for a reagent causing a luminescence effect of the neonicotinoids on a TLC plate. Subsequently, a calibration method is then used to find the detection limit of this TLC experiment.

The aim is, therefore, to achieve a standard method of quantifying and qualifying neonicotinoids via TLC.

Whilst a suitable mobile phase has been established, an optimal fluorescent reagent has yet to be found and more research on the subject must be carried out.

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List of Abbreviations

Ac	Acetamiprid
ACN	Acetonitrile
CH_2Cl_2	Dichloromethane
Cl	Clothianidin
Dq	Diquat
EPA	Environmental Protection Agency
EtOH	Ethanol
H_2O dist.	Distilled Water
Im	Imidacloprid
LD_{50}	Lethal Dose, 50%
LM	“Laufmittel”, translation of the German word for “eluent”
MEK	Methyl ethyl ketone
MeOH	Methanol
MTBE	tert-butyl methyl ether
NH_4OH	Ammonium hydroxide
Np	Nitenpyram
R_f	Retardation Factor
RP	Reverse Phase
RTP	Room Temperature and Pressure
Tc	Thiacloprid

List of Abbreviations

THF	Tetrahydrofuran
TLC	Thin-Layer Chromatography
Tx	Thiamethoxam
UV	Ultra-violet
WHO	World Health Organisation

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1. Introduction

1.1 Theory

Neonicotinoids are a relatively new class of insecticide specifically acting on the central nervous system of insects. Initially to be used against aphids they have, however, also had a negative effect on the bee population. Widespread colony collapse disorder in Europe has been attributed to the use of Clothianidin (a type of neonicotinoid). It has been suggested that the European honey bee (Apis mellifera) may come into contact with the Neonicotinoids via their presence in pollen, nectar and guttation liquid (1).

In this thesis, the usage of TLC as a method for the quantifying and qualifying these insecticides is discussed. The initial stage of experimentation dealt with the analysis of various types of fluorescent reagents used in aiding the quantification of samples. The latter stages involved the analysis of different eluent solutions to be used as the mobile phase in TLC.

In earlier studies it has been suggested that the LD_{50} (the median lethal dose required to kill 50% of a population of bees) of the neonicotinoids range from as low as 18 ng/bee to as high as 14.6 mg/bee (2).

1.2 Thin Layer Chromatography

1.2.1 Definition

Thin-Layer Chromatography (TLC) is a method used in the separation of mixtures. The adsorbent layer in TLC is known as the stationary phase, whilst the solvent (or mixture of solvents) travelling through the stationary phase is known as the mobile phase. The mobile phase is drawn up the stationary phase via capillary action. Because of the ability of different analytes to ascend the stationary phase at different rates, separation can be achieved.

1.2.2 History of TLC

Chromatography

The words “chromatogram” and “chromatography” comprise of the Greek words “chroma” meaning “colour” and “graphein” meaning “to write”. It was first used by the Russian botanist Mikhail Tswett in 1906. It was also Tswett who made the distinction between sample, mobile, and stationary phase, as well as the single application of a sample and the permanent effect of the mobile phase (3). It was in the first of his three articles to the German Botanical Society in 1906 that Tswett described his use of chromatography in the separation of dyes by using using a calcium carbonate column and a petrol ether solution. His work on chromatography, however, remained largely unrecognised until 1931 (4).

It was then in 1930 that German scientist Willstädter translated Tswett’s work into German, and presented this copy to Richard Kuhn. Kuhn was later awarded the Nobel Prize for his work on column chromatographic separations of carotenoids (3).

Thin-Layer Chromatography

In 1938, Russian scientists Izmailov and Shraiber searched for a separation method by transferring the results of column chromatography to so-called open columns. This method of using open columns was constantly being improved until 1949, when Meinhardt and Hall introduced a significant technical improvement to “surface chromatography”. On glass plates, they fixed aluminium oxide by using starch as a bonding agent. Fluorescence indicators were then added, and under short-wavelength UV-light (254 nm), fluorescent zones were observed. The advantage of performing the experiments in this manner, is that substances applied to the plate can be observed without dyeing or destroying them (3).

The standardization of TLC devices and materials from the companies Merck and Desaga, has enabled thin-layer chromatography to be an accepted and recognised process of chemical analysis.

1.2.3 Theory

The principle method of performing a TLC experiment is relatively easy. The steps necessary firstly include the application of the samples onto a TLC plate. Once the samples have been applied, the plate is then placed into a chamber where the mobile phase is found. Once inside the chamber, the chamber is then closed with a lid, and the mobile phase is left to carry the samples up the stationary phase. After travelling for a distance of approximately 7 cm, the lid is removed and the plate is withdrawn from the chamber and left to dry.

Depending on what samples are being analysed, and the type of plate being used, it is sometimes also necessary to apply a fluorescent reagent to the plate in order to detect the samples. Samples can then be detected with the help of UV-light.

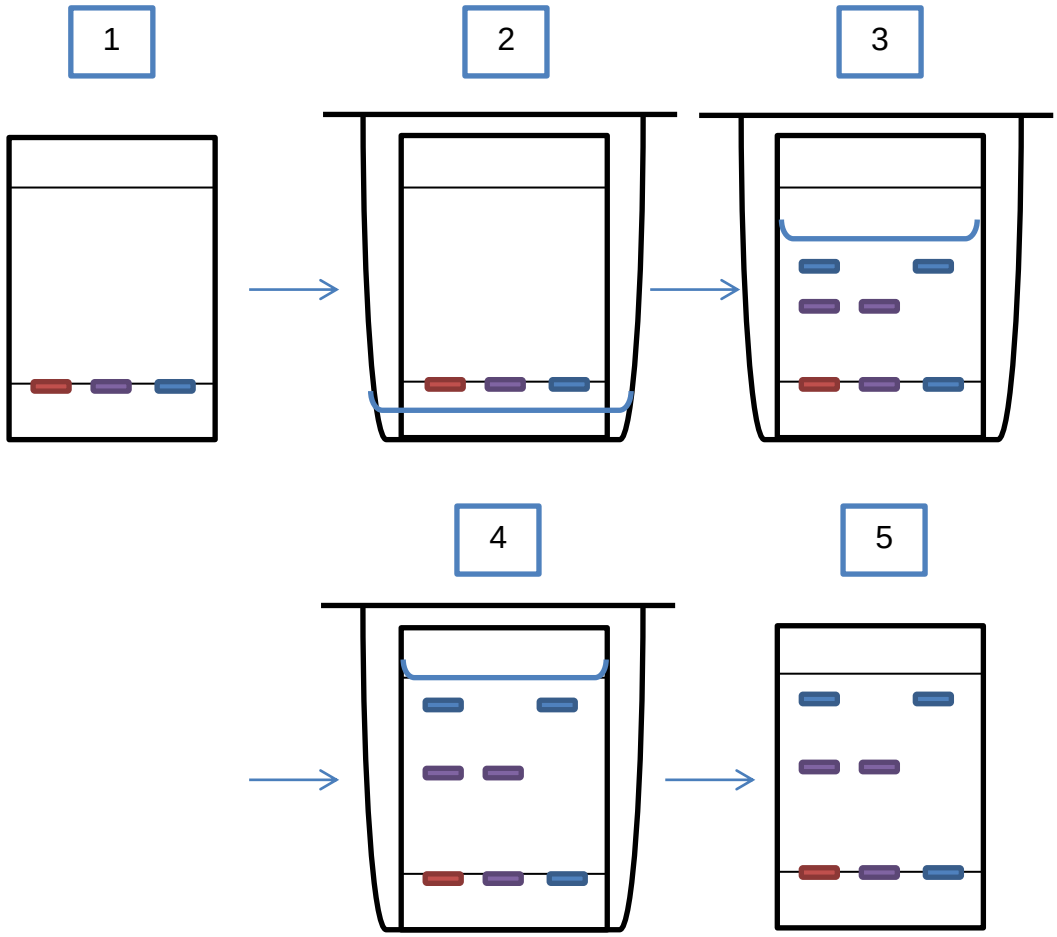


Fig. 1.1 *Schematic design of a basic TLC experiment*

1. The unknown sample on the left is applied on the plate. Two known substances are applied in the middle and the right sides.

2. Inside the chamber, a solvent has already been added. The TLC plate is placed in this vertical chamber. The lid is then closed.

3. The solvent travels up the plate, and brings the separated compounds along with it.

4. The solvent front has now reached the 7 cm limit. The plate is then removed

5. After analysing under UV-light (assuming the compounds are invisible under visible light), it can be deduced that the unknown sample is made up of the two known substances. This method of qualitative analysis is only possible as the R_f (Retardation factor) values are the same.

The Mobile Phase

The most important factor for method development in TLC is the mobile phase composition (3).

In normal TLC, the mobile phase chosen is usually an un-polar eluent solution. In RP-TLC, the opposite is true, and a polar eluent solution is more often used. By adjusting the ratio of the solvents in the eluent solution, the polarity of the solution can be controlled.

The eluotropic table contains a list of solvents arranged according to their polarity and plays a helpful role in deciding which solvents to use.

The Stationary Phase

In TLC, the most commonly used stationary phase is uncoated silica gel. Due to the polarity of silica gel, non-polar components tend to elute before more polar components. In this case, this form of chromatography is known as normal phase chromatography. When hydrophobic groups, however, are attached to the silica gel then polar components elute first. This form of chromatography is known as reverse phase chromatography (5). It is this second method of chromatography that is carried out with the analysis of neonicotinoids, as neonicotinoids are known to be highly polar (6).

The Retardation Factor (R_f)

The R_f value is used in the qualitative analysis of TLC. By determining the R_f value of analytes on a TLC-plate, it is possible to distinguish one sample from another. R_f values below 0.1 and above 0.85 should be avoided (3).

The following diagram (Fig. 1.2) describes how the R_f value is calculated.

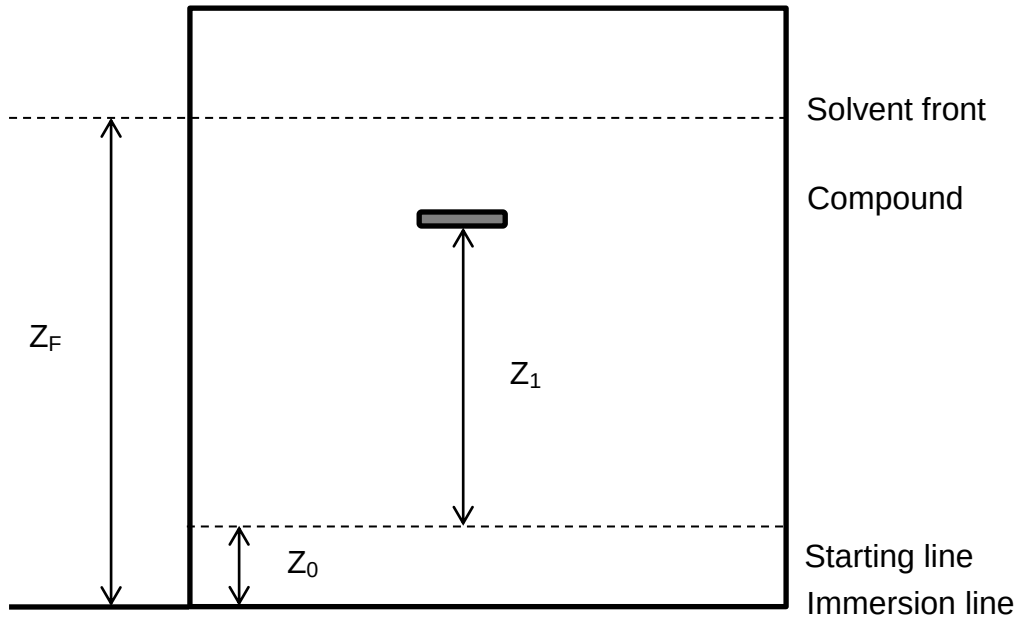


Fig. 1.2 *Calculation of R_f values*

$$R_f \equiv \frac{Z_1}{Z_F - Z_0}$$

$$= \frac{Distance\ travelled\ by\ compound}{Distance\ travelled\ by\ the\ solvent\ front - Distance\ from\ starting\ line\ to\ immersion\ line}$$

Densitogram

The quantitative analysis of a TLC plate can be done by using a densitogram and TLC scanner. This device allows for measurement in both visible light and UV-light wavelengths.

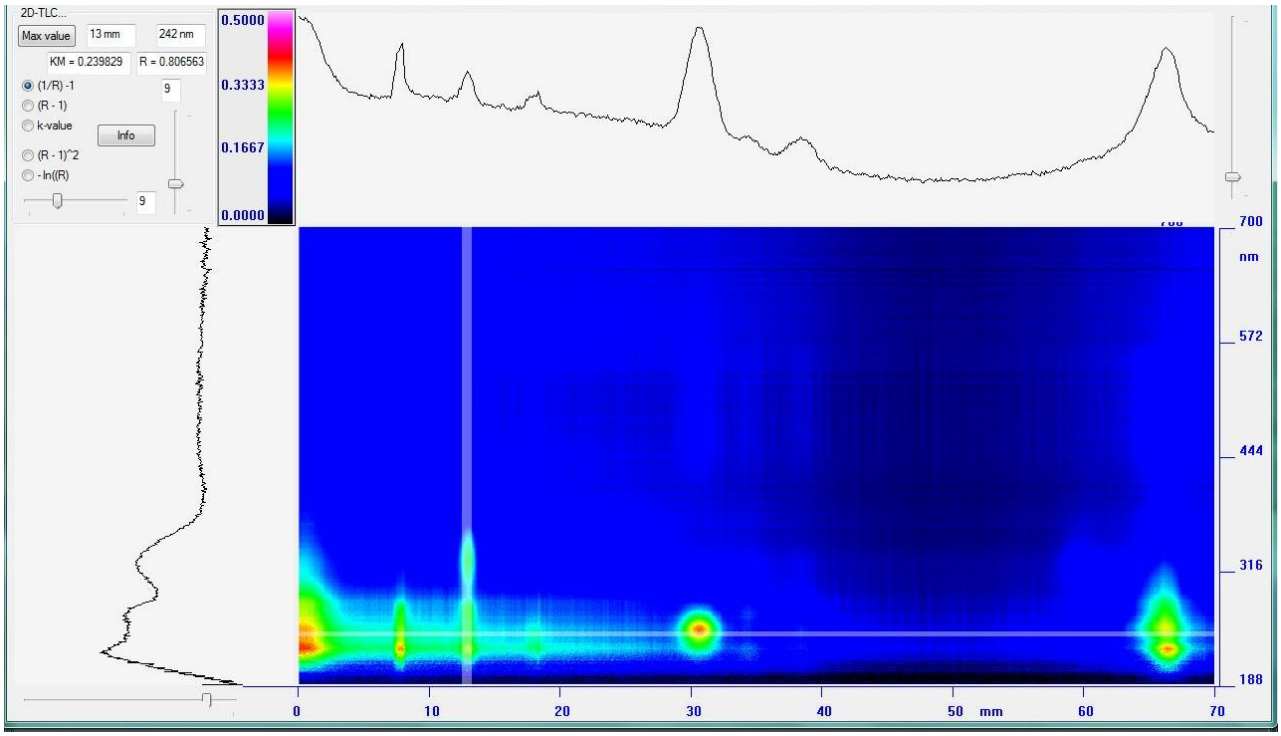


Fig. 1.3 Densitogram of the 5th track from calibration plate 3

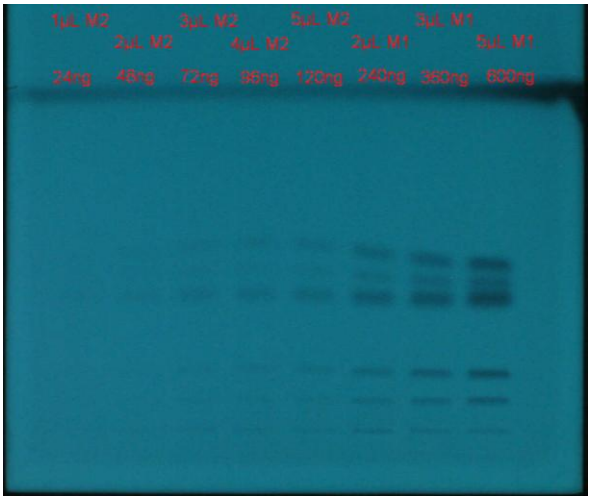


Fig. 1.4 Calibration plate 3

1.2.4 Statistics of TLC

Introduction

The goal of analytical measurements in TLC is the quantification of the analyte. In order to achieve this, the conversion of the observed signal into absolute sample amounts is possible through calibration. Calibration is, therefore, achieved through statistical calculations of results. These statistical calculations shall now be discussed (3).

The Mean Value

Random fluctuations in measurements are what are known as noise. This noise comprises all effects caused by the measurement environment. The goal of statistics in TLC is to eliminate as much noise as possible, or to reduce it down to a point where it has a minimal effect on calculations. In order to first estimate the noise in observations, a series of tests or measurements should be performed. The German mathematician, Carl Friedrich Gauss (1777-1855), proved that through multiple observations, a better interpretation of results could be achieved. It was then said that the best representative value to describe a whole range of values is the mean value (3).

$$\overline{Y} = \frac{1}{n}\sum_{i} y_i$$

Where,

$\overline{Y}$ = Mean value (the mean value shall always be written in capital letters)

n = total number of measurements taken

i = measurement number

y = measurement, i, taken

The Standard Deviation, σ

After the mean value has been established, the next hurdle to overcome is to measure the amount of noise.

Each value taken from the series (if differing from the mean value) has a certain amount of variance from the mean value. This variance can be described by the equation:

$$y = 0 \pm \sigma$$

Assuming that with *n* data measured, after squaring the above equation, it can be calculated that all values will have a positive noise. This can be written as:

$$\sum_{i=1}^{n} y_i^2 = n\sigma^2$$

Assuming that all measurements are independent of one another, it can be said that the products of various different measurements are always zero. This can be described by this equation:

$$\left(\sum_{i=1}^{n} y_i\right)^2 = \sum_{i=1}^{n} y_i^2 = n\sigma^2$$

The sum of squared residuals in this particular measurement system can be written as:

$$\sum_{i=1}^{n} (y_i - \overline{Y})^2 = \sum_{i=1}^{n} y_i^2 - 2\sum_{i=1}^{n} y_i \overline{Y} + \left(\sum_{i=1}^{n} \overline{Y}\right)^2$$

With $\bar{Y} = \frac{1}{n}\sum_i y_i$ the following equation is obtained:

$$\sum_{i=1}^{n}(y_i - \bar{Y})^2 = n\sigma^2 - 2\sum_{i=1}^{n} y_i \frac{1}{n}\sum_{i=1}^{n} y_i + \left(\sum_{i=1}^{n}\frac{1}{n}\sum_{i=1}^{n} y_i\right)^2$$

$$= n\sigma^2 - \frac{2}{n}\left(\sum_{i=1}^{n} y_i\right)^2 + n\frac{1}{n^2}\left(\sum_{i=1}^{n} y_i\right)^2$$

$$\sum_{i=1}^{n}(y_i - \bar{Y})^2 = n\sigma^2 - \frac{1}{n}\left(\sum_{i=1}^{n} y_i\right)^2 = n\sigma^2 - \sigma^2.$$

After resolving for noise,

$$\sigma^2 = \frac{1}{n-1}\sum_{i=1}^{n}(y_i - \bar{Y})^2.$$

The standard deviation, σ, can then be calculated by the square root of this equation. Giving:

$$\sigma = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(y_i - \bar{Y})^2}$$

The standard deviation can be used as an indication of the precision or uncertainty (3).

The relative standard deviation (%RSD)

The relative standard deviation is the absolute value of the coefficient of variation. It is widely used in analytical chemistry to express the precision and repeatability of an assay. It is multiplied by 100 and expressed as a percentage (7):

$$\%RSD = 100 \cdot \left(\frac{\sigma}{\bar{Y}}\right)$$

1.3 Neonicotinoids

1.3.1 Definition

Neonicotinoids are a relatively new class of insecticides designed specifically to act on the central nervous systems of insects, causing paralysis and eventually leading to death. The name “Neonicotinoid” comes from the similarity of their chemical structure with that of nicotine. Both neonicotinoids and nicotine belong to the Group 4 insecticide modes of action (8). As a group, neonicotinoids are most effective against sucking insects and some Heteroptera, Coleoptera and Lepidoptera (2).

Based on their chemical structure, neonicotinoids can be divided into two groups: nitro-substituted compounds (i.e. Imidacloprid, Clothianidin, and Thiamethoxam) and cyano-substituted compounds (i.e. Acetamiprid and Thiacloprid). It is this first group of nitro-substituted compounds that are most lethal to honey bees (2).

1.3.2 Mode of Action

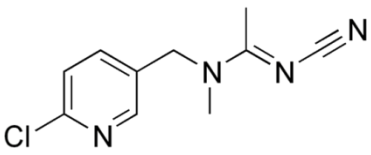
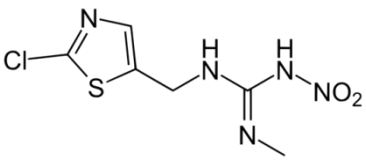
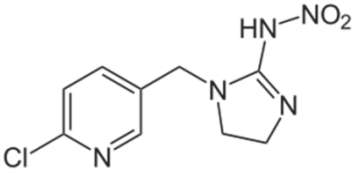
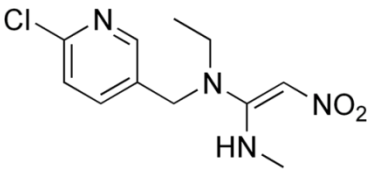
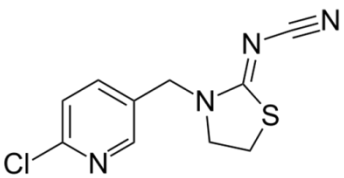
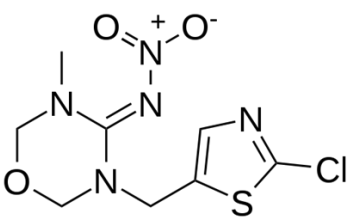
Neonicotinoids act on the insect nicotinic (acetylcholine) receptor (nAChR) (2). According to the WHO/EPA, these compounds are placed in the toxicity classes II or III. However, due to neonicotinoids blocking a specific pathway found more abundant in insects rather than warm-blooded animals, they are considered to be more toxic to insects than mammals (9).

Neonicotinoids have been proven to be fatal to the honey bee, Apis mellifera, with LD_{50} levels ranging from as low as 18 ng/bee to 14.6 µg/bee (Table 1.1) (2). It is because of this high toxicity to the honey bee, that neonicotinoids are implicated in cases of CCD (Colony Collapse Disorder).

Table 1.1 *Neonicotinoids: IUPAC names and their LD_{50} per bee*

Neonicotinoid	IUPAC Name	LD_{50}/bee (2)
Acetamiprid	(E)-N1-[(6-chloro-3-pyridyl)methyl]-N2-cyano-N1-methyl acetamidine	7.1 µg
Clothianidin	1-(2-Chlor-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine	22 ng
Imidacloprid	N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide	18 ng
Nitenpyram	(E)-N-(6-Chloro-3-pyridylmethyl)- N-ethyl-N'-methyl-2-nitrovinylidenediamine	138 ng
Thiacloprid	(2Z)-3-[(6-Chloropyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene}cyanamide	14.6 µg
Thiamethoxam	3-[(2-Chloro-1,3-thiazol-5-yl)methyl]-5-methyl-N-nitro-1,3,5-oxadiazinan-4-imine	30 ng

Figure 1.5 *Structural formulae of neonicotinoids*

Acetamiprid (10)	**Clothianidin** (11)	**Imidacloprid** (12)
		
Nitenpyram (13)	**Thiacloprid** (14)	**Thiamethoxam** (15)
		

2. Materials and Methods

2.1 Neonicotinoids and Diquat Samples

The following table contains information regarding the source of the neonicotinoid samples, and the diquat sample used in the TLC experiments.

Table 2.1 Neonicotinoid and Diquat supplier and purity levels

Substance	**Supplier**	**Purity Level (HPLC)**
Diquat Sample		
Diquat dibromide hydrate	Dr. Ehrenstorfer GmbH	98.0%
Neonicotinoid Samples		
Acetamiprid	Sigma-Aldrich	99.9%, Pestanal ® analytical standard
Clothianidin	Sigma –Aldrich	99.9%, Pestanal ® analytical standard
Imidacloprid	Sigma-Aldrich	99.9%, Pestanal ® analytical standard
Nitenpyram	Sigma-Aldrich	99.9%, Pestanal ® analytical standard
Thiacloprid	Sigma-Aldrich	99.9%, Pestanal ® analytical standard
Thiamethoxam	Sigma-Aldrich	99.7%, Pestanal ® analytical standard

2.2 Preparation of Standards

2.2.1 Individual Standard Samples

Approximately 6 mg each of the six neonicotinoids (i.e. Acetamiprid, Thiacloprid, Imidacloprid, Clothianidin, Thiamethoxam,Nitenpyram) are diluted separately with methanol to 10 mL in volumetric flasks.

Alongside the six neonicotinoids, a sample of diquat is also prepared for the aid of fluorescent reagent determination. Approximately 6 mg of Diquat dibromide hydrate ("diquat") are also diluted to 10 mL with methanol in a volumetric flask.

Table 2.2 Preparation of Standards: Measured mass of neonicotinoids dissolved in 10 mL volumetric flask with methanol

Substance (Abbreviated Form)	**Mass measured in mg**
Acetamiprid (Ac)	6,167
Thiacloprid (Tc)	6,103
Imidacloprid (Im)	6,150
Clothianidin (Cl)	6,015
Thaimethoxam (Tx)	6,140
Nitenpyram (Np)	6,001
Diquat (Dq)	6,175

2.2.2 Mixture of Standards

Mix #1

A mixture of the five standard neonicotinoid solutions was produced by mixing 1 mL of Acetamiprid, Imidacloprid, Clothianidin, Thiamethoxam, and Nitenpyram. Due to the interference caused by Thiacloprid (see 3.3.6), it has been omitted from the quantification tests. Each of the remaining neonicotinoid standards were pipetted into a 5 mL volumetric flask then shaken for 5 mins to ensure a complete mixing of the standards. The concentration of pure substance in this new mixture is 0.12 g/L.

Mix #2

A new mixture from Mix #1 was created with a lower concentration. Once again, 1 mL was pipetted into a 5 mL volumetric flask. However in the case of Mix #2, instead of diluting further with other standard solutions, it is diluted with methanol. The concentration of pure substance in this new mixture is, therefore, 0.024 g/L.

2.3 TLC Plates

2.3.1 HPTLC Kieselgel 60 RP-18 WF254s

The abbreviation “RP-18” stands for the use in reverse phase chromatography with an octadecysilyl C-18 chain. The “W” stands for “water tolerant, wettable layer”. The “F254s” is an indication that a fluorescent indicator with a 254 nm excitation wavelength has also been applied to the plate.

The plates used in the following TLC experiments are the RP-18 WF plates provided from the company Merck.

Two forms of this plate were used. The first form being a glass-backed plate (Serial number: 1.13124) and the second being an aluminium-backed plate (Serial number: 1.05559).

2.3.2 TLC Plate Cutter

For plates which need to be reduced in size, special plate cutters have been produced.

When cutting glass-backed plates, a special diamond-tipped cutter from the company CAMAG was used.

For the aluminium-backed plates, a normal pair of scissors or a paper guillotine was used.

Fig. 2.1 *Paper guillotine* **(21)**

Fig. 2.2 *TLC plate cutter from CAMAG* (22)

2.4 Application of the Standards onto the Plates

2.4.1 Glass Capillary Tubes

The application of samples via glass capillaries, results in small round spots with an application volume according to a defined amount. By varying the length of the capillary, the volume is then also varied. Filling of the tubes is done simply, by using capillary action, and therefore no externally applied suction is required.

Glass capillary tubes are only suitable for applying samples as spots. They should also only be used for a single sample to avoid cross-contamination between samples (3).

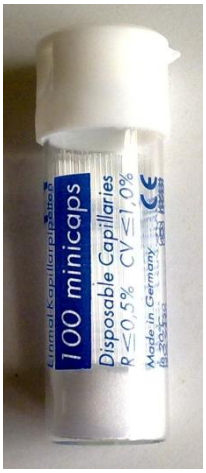


Fig. 2.3 *Minicap glass capillary tubes of 5 µL*

2.4.2 DESAGA AS 30

The DESAGA AS 30 is a device used in the spraying of samples onto plates. Due to its consistency in applying the amount of sample onto a plate, it is much more accurate and more reliable when compared with application via glass capillary tubes. This method of spraying the sample solution onto the plates is more elegant and needs not to touch the plate compared with application via glass capillary tubes. This lessens the risk of damaging the plate surface.

The main disadvantage, though, in using an automatic application device is that TLC loses its status as being a relatively inexpensive separation technique (3).

Fig. 2.4 *DESAGA AS 30 semi-automatic sample application system*

2.5 Separating Chambers for Linear Development

The TLC experiments conducted in this thesis used two types of chambers for the development of a TLC plate. The first type of chamber is known as an N-Chamber, and the second is referred to as an S-Chamber.

2.5.1 N-Chambers ("Trough" Chambers)

The "N" stands for "Normal". As can be seen in Fig. 2.6, a TLC plate is placed vertically into an N-Chamber. Before the plate is placed into the chamber, the solvent is first added in. Approximately 5 mL of solvent is needed to provide a good immersion depth for the plate. The N-chambers used in this thesis was provided by DESAGA.

Fig. 2.5 *Single-trough chamber from DESAGA*

Fig. 2.6 *Front view of a TLC-plate development in a single-trough chamber from DESAGA*

2.5.2 S-Chambers (“Small Chambers”)

The “S” stands for “small”. This is in reference to a vapour space of less than 3 mm. The TLC plate is placed horizontally (faced downwards) in this form of chamber. The solvent is introduced in one of the two chambers (either left or right) and is then left to develop horizontally through the plate. This form of chamber has its advantages against the H-chamber, in that the development time is shorter. This is due to the vertical gravitational component no longer being a factor. This type of chamber also provides more consistent R_f values (3).

It is important to note that in order to know when to stop the plate development, the “End line” (line at which the solvent front is stopped) must be marked on the glass side of the plate (i.e. the side of the plate facing upwards).

The S-chamber used in this thesis was provided by the CAMAG Company.

Fig. 2.7 *Top view of a horizontal S-chamber from CAMAG*

2.6 Detectors

2.6.1 CabUVIS from DESAGA

The CabUVIS device enables the photographic documentation of TLC plates. The chamber of the device is equipped with three light sources of varying wavelengths: daylight, 254 nm, and 366 nm.

After the photographing of the plates is completed, they may then be documented using the program ProViDoc®.

Fig. 2.8 *CabUVIS from DESAGA*

2.6.2 J&M Diode-array Scanner

By using the diode-array scanner, it is possible to record a spectrum directly by scanning a TLC plate. Light scattered from the plate is directed to the detector via optical fibres. The J&M diode-array scanner consists of two parallel light probes and an array of photodiodes. A photodiode is a semi-conductive device with two layers of different conductivity. By using an array of photodiodes, it is possible to simultaneously take measurements at various wavelengths ranging from 190 – 1000 nm.

When scanning a TLC plate, a densitogram is created. Additionally, a spectrum of individual bands is shown. A spectrum is substance-specific and serves in the qualification. It is the intensity of the measuring signal that is used in the quantification of analytes. (16) Measurements are performed by transmittance using the Lambert-Beer law for quantification (3). In order for the equation to work optimally, the correct wavelength at which a maximum absorption is observed must first be selected.

Fig. 2.9 *J&M Diode-array Scanner*

3. Selection of the Mobile Phase Eluent Composition

3.1 Introduction

The mobile phase is the most important factor when performing a TLC experiment (3). What is so challenging in determining the most suitable eluent composition for the mobile phase, is that for many unknown samples, the eluent composition is still unknown. This means that the best method in determining the solvent composition is by trial and error. However there are some clues in finding out the most suitable configuration.

“Like dissolves like”

When it comes to choosing a suitable solvent, the phrase “like dissolves like” is one that plays a very important role. This is due to the fact that samples of known polarity are more able to be dissolved in solvents of similar polarity. Hence, a sample of high polarity is more likely to dissolve in a solvent of equally high polarity. Neonicotinoids are known to have a relatively high polarity (6), and this helps in determining the proper solvent composition.

3.2 Materials and Method

Using the DESAGA AS 30 device, the neonicotinoids were then sprayed onto an RP-18 WF glass-backed plate. Each of the first six tracks on the plate contain 5 µL of neonicotinoid standard sample, the 7^{th} track contains 5 µL of the diquat standard sample. The diquat standard sample was also sprayed onto the plate as a reference when performing the fluorescent reagent tests.

Once all the tracks were sprayed onto the plate, the plates were left in a covered N-chamber containing the solvent solution.

After the front had reached “end line”, the plate was then removed and left to dry for 5 minutes. At the end of the 5 minutes, photos were then taken with the CabUVIS device.

In total 42 solvent composition tests were conducted. In this section, however, only the most suitable solutions will be discussed.

3.3 Example of Layout of Plate

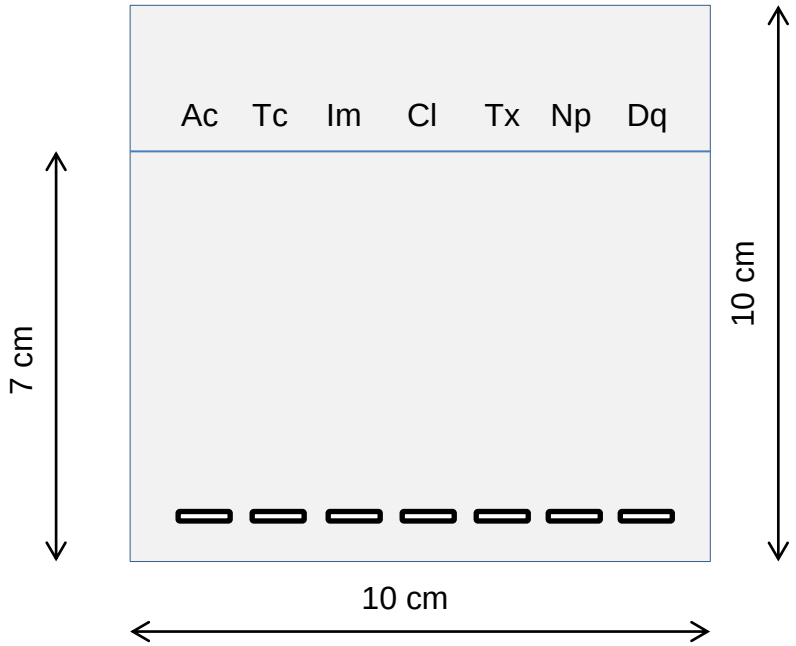


Fig. 3.1 *Typical layout of TLC plate with neonicotinoid standards during solvent composition testing*

3.4 Solvent Compositions

3.4.1 MTBE

Application device:	DESAGA AS 30
Solvent solution:	MTBE
Distance from immersion line to solvent front:	7 cm
Approximate duration of experiment at RTP:	20 mins

It must be noted that as this was one of the first solvents to be tested (Solvent test #4), the diquat sample was not yet included.

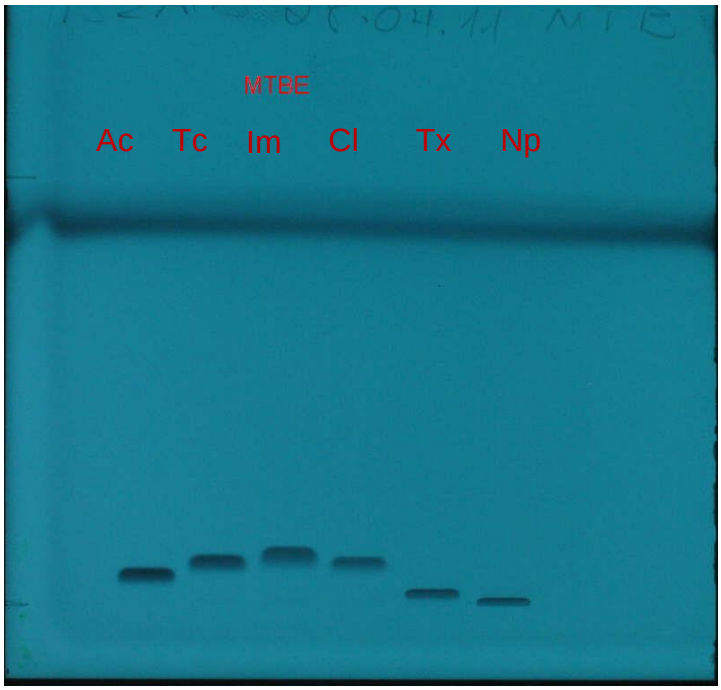


Fig. 3.2 *Application of 5 µL of neonicotinoid per track on RP-18 WF glass plate with MTBE as solvent, illuminated with UV-light at 254 nm*

From the photo, it can be seen that the neonicotinoid samples have been reluctant in leaving the starting line. The reason behind this can be assumed that MTBE is of a relatively lower polarity and, therefore, is unable to increase the distance in which the neonicotinoids travel.

3.4.2 MEK

Application device:	DESAGA AS 30
Solvent solution:	MEK
Distance from immersion line to solvent front:	7 cm
Approximate duration of experiment at RTP:	15 mins

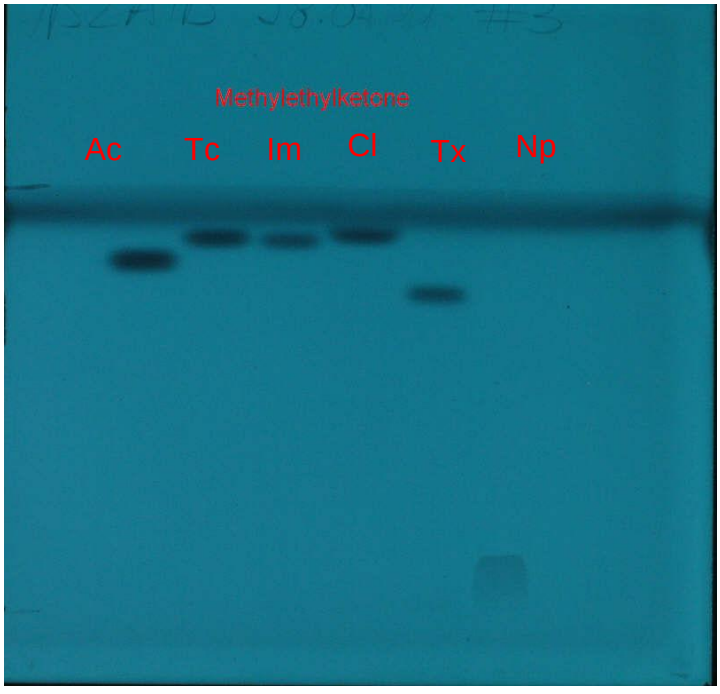


Fig. 3.3 *Application of 5 µL of neonicotinoid per track on RP-18 WF glass plate with MEK as solvent, illuminated with UV-light at 254 nm.*

Due to MEK having a higher polarity as compared to MTBE, the Neonicotinoids have travelled further up the plate. This can be seen when comparing Fig. 3.3 with Fig 3.2. This proves the theory that neonicotinoids will travel further with the solvent front, if the solvent is of a relatively high polarity.

Another point to be noted is that the separation of Thiacloprid, Imidacloprid, and Clothianidin would be difficult by only using MEK, as their R_f values are almost identical. Identification of samples containing these three Neonicotinoids would therefore be very difficult.

Lastly, by looking at the 6th track of Nitenpyram, an effect known as “tailing” can be seen. This occurs when non-absorbed solute migrates through the stationary phase faster than expected (3) and poses a problem in the quantification and qualification of Nitenpyram.

3.4.3 Combination of MTBE and MEK (4 + 1)

Now that two suitable solvents have been found, a combination of the two would perhaps help in preventing the samples from travelling too far up the plate (Fig. 3.3), and help in pushing the samples further up from the starting line (Fig. 3.2) so as to achieve better R_f values.

The following plates also contain a 7th track, which holds 5 µL of the diquat standard.

Application device:	DESAGA AS 30
Solvent solution:	MTBE + MEK
Component Composition:	4 + 1
Distance from immersion line to solvent front:	7 cm
Approximate duration of experiment at RTP:	20 mins

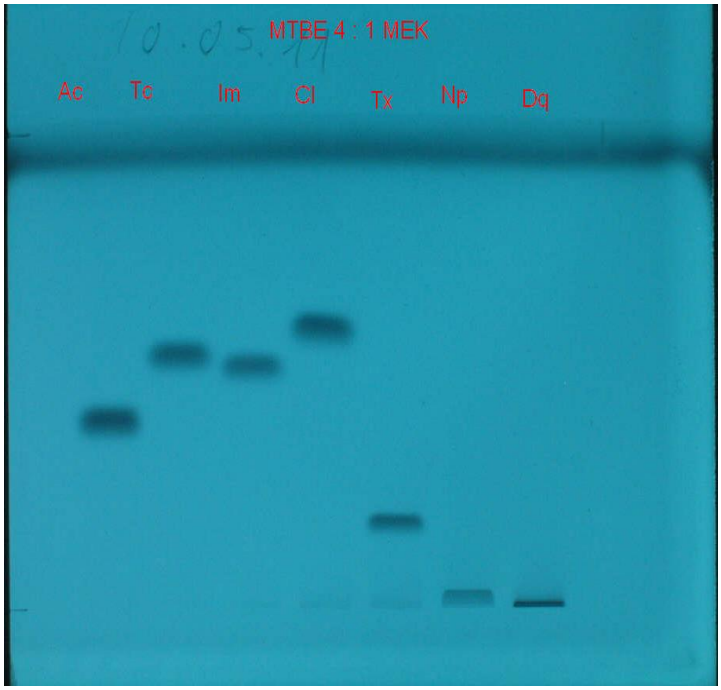


Fig. 3.4 *Application of 5 µL of neonicotinoid per track on RP-18 WF glass plate with MTBE-MEK (4+1) as solvent solution, illuminated with UV-light at 254 nm.*

By observing Fig. 3.4, it can be seen that the theory of preventing the samples from travelling too “far up” the plate has been achieved by this mixture of a relatively polar solvent (MEK) and a relatively non-polar substance (MTBE).

The objective of developing a plate where the substances have an R_f value between 0.1 and 0.85 is also getting nearer. Varying the component composition is the next step in optimizing the R_f values.

One final problem still remaining is the tailing effect experienced by the Nitenpyram sample. The Nitenpyram sample also has a low R_f value and this also needs to be rectified before calibration.

3.4.4 Combination of MEK and MTBE (4 + 2)

Application device:	DESAGA AS 30
Solvent solution:	MTBE + MEK
Component Composition:	4 + 2
Distance from immersion line to solvent front:	7 cm
Approximate duration of experiment at RTP:	18 mins

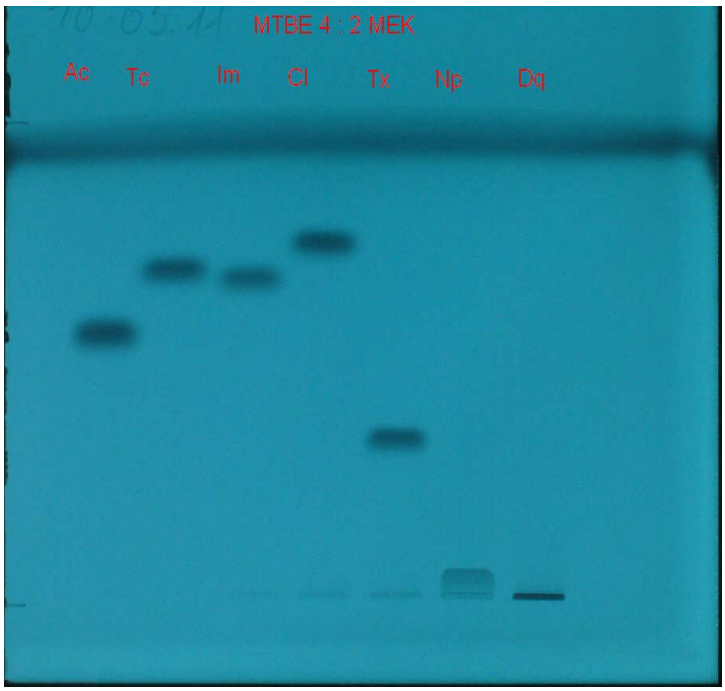


Fig. 3.5 *Application of 5 µL of neonicotinoid per track on RP-18 WF glass plate with MTBE-MEK (4+2) as solvent solution, illuminated with UV-light at 254 nm*

Comparison of Fig. 3.4 and Fig. 3.5

By comparing Fig. 3.4 and Fig. 3.5 we can deduce that the ratio of MTBE to MEK plays a crucial role in achieving an optimal R_f value for the samples. Even though the relative separation from one stain to the next stays approximately constant, the overall distance from the starting line differs greatly. By adding in more MEK relative to the amount of MTBE, the samples travelled further up the plate; and by reducing the amount of MEK, the samples stayed closer to the starting line. This means that by just varying the ratio of MTBE to MEK, not only is a suitable RF-value achievable, but that a separation between the samples is also possible.

The problem of the tailing effect can still be seen in both. This problem is rectified in the following solvent combination.

3.4.5 Solvent Combination of MTBE with MEK and NH_4OH (4 + 2 + 0.1)

As was previously stated above, the problem faced with Nitenpyram was the tailing effect. In order to achieve a more compact stain and remove the tailing effect, ammonium hydroxide was added to a solution of MEK and MTBE.

The “LM” seen in figures 6 and 7 is an acronym for the German word “Laufmittel”, which translates into “eluent”. The number following the letters denotes the test number of the solvent configuration.

Application device:	DESAGA AS 30
Solvent solution:	MTBE+ MEK + NH_4OH
Component Composition:	4 + 2 + 0.1
Distance from immersion line to solvent front:	7 cm
Approximate duration of experiment at RTP:	17 mins

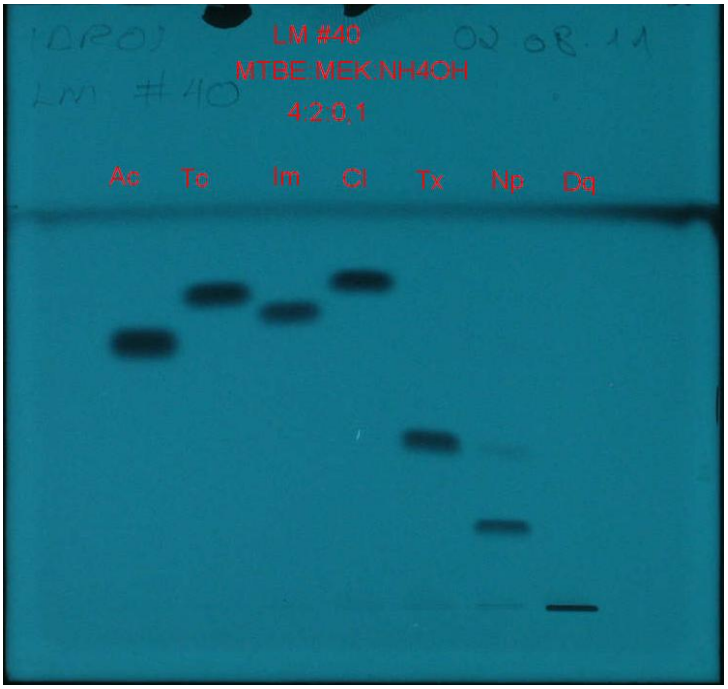


Fig. 3.6 *Application of 5 µL of neonicotinoid per track on RP-18 WF glass plate with MTBE-MEK-NH_4OH (4+2+0.1) as solvent solution, illuminated with UV-light at 254 nm*

Comparison of Fig. 3.5 and Fig. 3.6

The R_f values in Fig 3.5 and Fig. 3.6 are almost identical. The main difference between the two figures, is that the samples seen in Fig. 3.6 appear to be much darker compared to Fig. 3.5. It must also be noted that with the addition of NH_4OH, the tailing effect experienced by Nitenpyram previously described in this section can no longer be seen.

NH_4OH was, therefore, added to all solvent compositions after this point.

3.4.6 Solvent Combination of MTBE with MEK and NH4OH (5 + 2 + 0.1)

Application device:	DESAGA AS 30
Solvent solution:	MTBE + MEK + NH_4OH
Component Composition:	5 + 2 + 0.1
Distance from immersion line to solvent front:	7 cm
Approximate duration of experiment at RTP:	17 mins

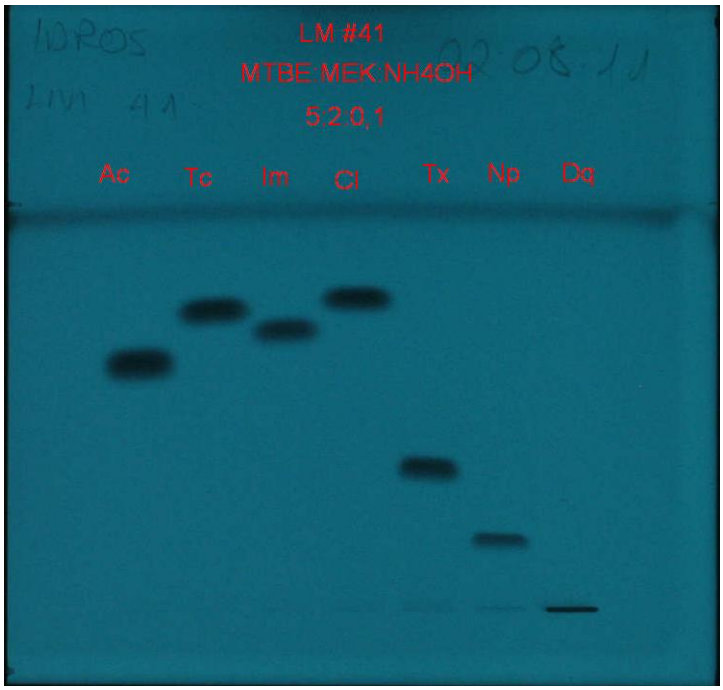


Fig. 3.7 *Application of 5 µL of neonicotinoid per track on RP-18 WF glass plate with MTBE-MEK-NH4OH (5+2+0.1) as solvent solution, illuminated with UV-light at 254 nm.*

Comparison of Fig. 3.6 and Fig. 3.7

When comparing Fig. 3.6 with Fig. 3.7, it can be seen that the solution containing a higher amount of MTBE relative to MEK, produces a more desirable result in terms of R_f value. Therefore, this configuration of MTBE-MEK-NH_4OH (5+2+0.1) has been chosen as the eluent component configuration.

The sole problem remaining with the experiment is that Acetamiprid, Thiacloprid, Imidacloprid, and Clothianidin may not achieve a good separation as their stains are still close to one another. The solution to this problem is the removal of Thiacloprid from further tests to achieve clearer results.

4. Fluorescent Staining Reagents

4.1 Introduction

The advantage of using the RP-18 WF glass-backed plate is that even without a staining reagent, samples can still be seen under UV-light at 254 nm. However, there comes a point when detection of a sample of low concentration requires the aide of a staining reagent to help in the process of quantifying and qualifying. In this section, the effects of staining with fluorescent staining reagents will be discussed. The reagents are: Dichloroacetic acid, and Trichloroacetic acid. The experimental procedure for Dichloroacetic acid and Trichloroacetic acid are the same, and both shall be discussed under the same heading.

4.2 Dichloroacetic Acid and Trichloroacetic Acid Staining Reagents

Trichloroacetic acid stains steroids, alkaloids, digitalis glycosides, vitamin D3, and benzodiazepine-2-one derivatives forming light blue fluorescent zones when illuminated at 365 nm. The reagent can be used with silica gel and cellulose layers (3).

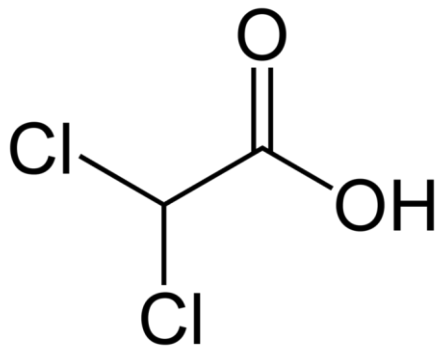


Fig. 4.1 *Dichloroacetic acid* **(17)**

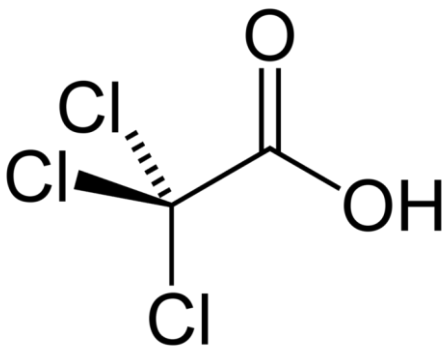


Fig. 4.2 *Trichloroacetic acid* (20)

4.3 Materials and Method

4.3.1 Trichloroacetic acid Experiment

Used plates from the mobile phase combination trials were used in the following experiments.

An hour glass is placed in a glass petri dish of 20 cm diameter. 100 µL of trichloroacetic acid is then pipetted onto the hour glass. After this has been completed, two sticks of magnesia (approximately 5 cm in length) are placed across the hour glass as a means of support for the TLC plate, and allowing the acid vapour to circulate throughout the petri dish. This is then sealed by placing a layer of heat-resistant oven bag over the petri dish and covering it with the petri dish lid.

The petri dish is then placed into an oven. The temperature of the oven and duration in the oven was varied to achieve the best fluorescence (see Table 4.1).

Once the duration in the oven was completed, the petri dish was removed and allowed to cool for 10 minutes at room temperature.

Once cooled, the plate was removed from the petri dish and were then photographed at 366 nm using CabUVIS.

Table 4.1 *Trichloroacetic acid experiment with list of varying temperatures and eluent composition*

Trichloroacetic acid		
Temperature in °C	**Duration in Oven in minutes**	**Eluent Composition**
120	10	MTBE-MEK-NH_4OH (5+2+0.1)
140	10	MTBE-MEK-EtOH-NH_4OH (5+1+0.5+1)
160	10	MTBE-MEK-NH_4OH (5+1+0.1)
180	10	MTBE-MEK-NH_4OH (2+2+1)

4.3.2 Dichloroacetic acid vs. Trichloroacetic acid Experiment

In order to observe and differences in fluorescence between dichloroacetic acid and trichloroacetic acid, and experiment was conducted under similar conditions.

A new RP-18 WF glass-backed plate was sprayed with 6 tracks using the DESAGA AS 30 device. Concentrations of each track are listed in Table (*.*).

The plate is then placed in the horizontal S-chamber, and left to run with the solvent combination described in 3.4.6. Once the front line had reached the end line, the plate was removed from the chamber and left to dry for 5 minutes.

After drying, the plate was then cut in half using the TLC plate cutter from CAMAG.

100 µL of trichloroacetic acid was pipetted onto the hour glass. The experiment was then conducted in the same manner as 4.3.1, with the exception that instead of allowing the plate to cool for 10 minutes after coming out of the oven, it is left to cool for 30 minutes.

The temperature of the oven was 160°C and the duration was for 20 minutes.

After cooling, the plate was then removed from the dish and photographed at 366 nm to observe any fluorescence.

The dish, and hour glass was well rinsed after this experiment. After cleaning, the experiment was then repeated using the same apparatus. The only variance to the first experiment was that dichloroacetic acid was then used, and the oven bag was replaced by a new oven bag.

Table 4.2 *Dichloroacetic acid experiment plate: Tracks and their corresponding pure substance amounts*

Track	1	2	3	4	5	6
Mixture	2	1	1	2	1	1
Amount applied in µL	13	5	10	13	5	10
Amount of pure substance in ng	312	600	1200	312	600	1200

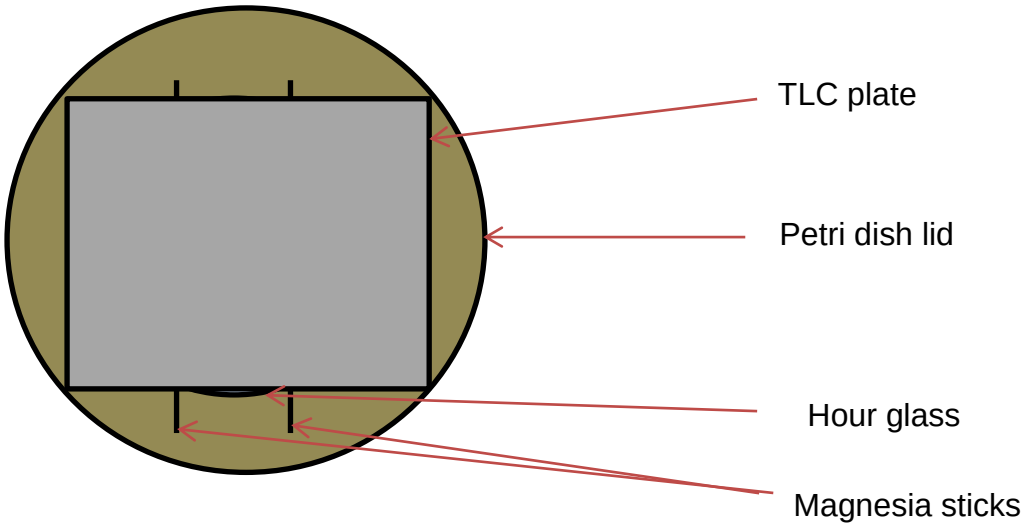


Fig. 4.3 *Top view schematics of trichloroacetic and dichloroacetic acid experiments*

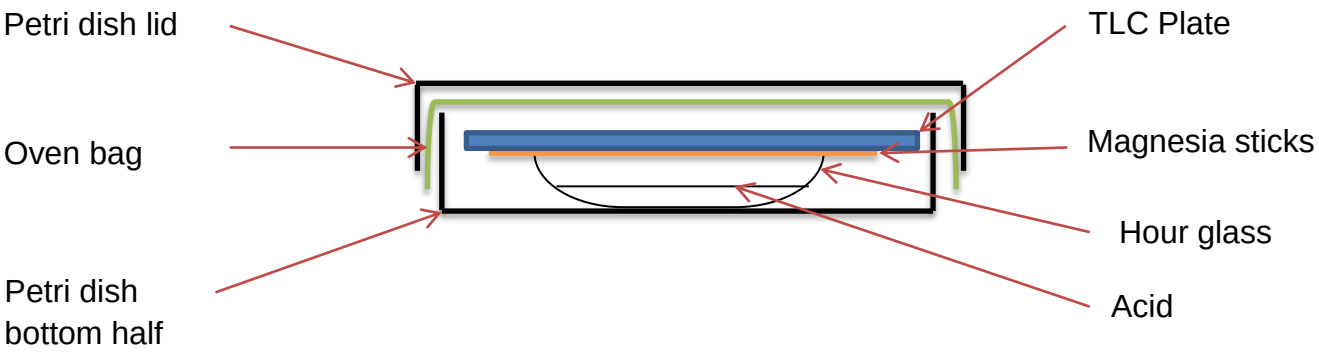


Fig. 4.4 *Cross-section of Trichloroacetic and Dichloroacetic acid experiments*

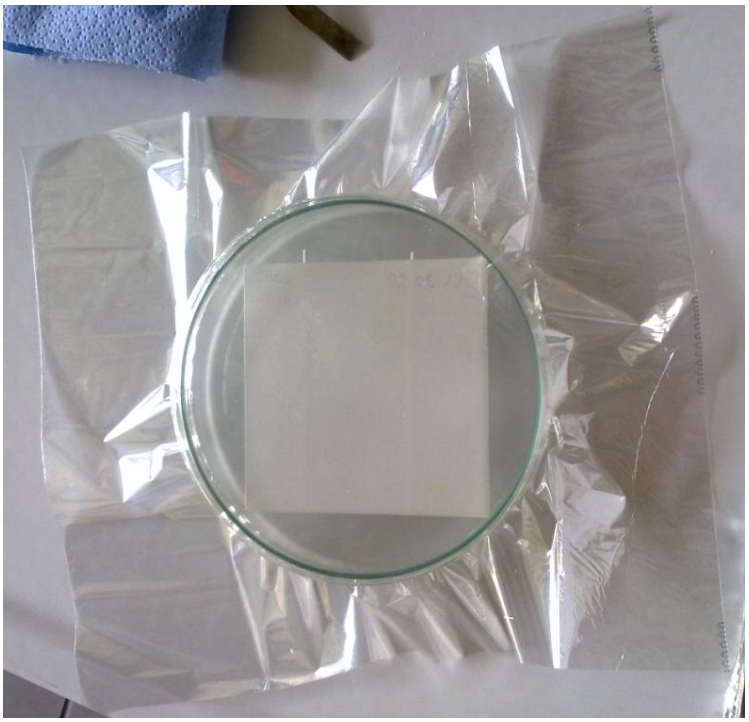


Fig. 4.5 *Top view of actual Trichloroacetic and Dichloroacetic acid experiments*

4.4 Results and Discussion

4.4.1 Trichloroacetic Acid Experiment

Trichloroacetic acid at 120°C for 10 minutes

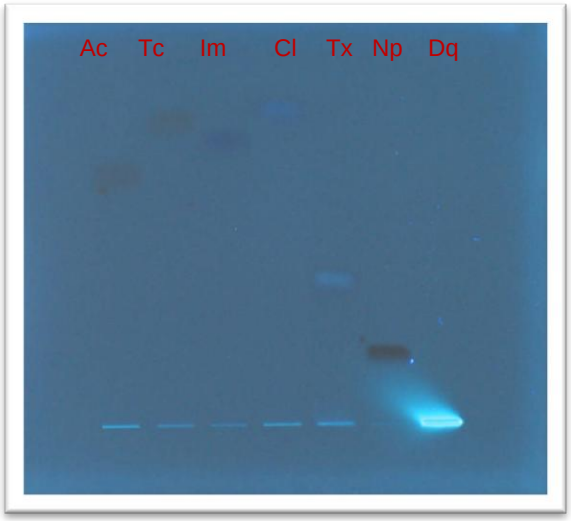


Fig. 4.6 *Trichloroacetic acid test at 120°C for 10 mins, illuminated with UV-light at 366 nm*

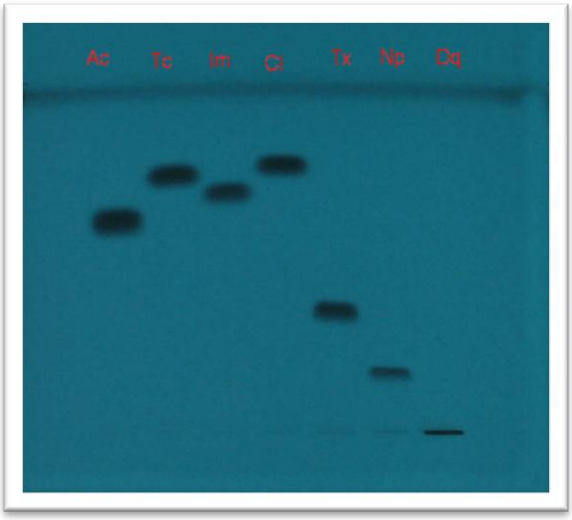


Fig. 4.7 *Trichloroacetic acid test at 120°C for 10 mins, illuminated with UV-light at 254 nm*

As can be seen above, it appears as if only three of the neonicotinoids (i.e. Imidacloprid, Clothianidin, and Thiamethoxam) and the diquat sample have reacted with the trichloroacetic acid.

Trichloroacetic acid at 140°C for 10 minutes

Fig. 4.8 *Trichloroacetic acid test at 140°C for 10 mins, illuminated with UV-light at 366 nm*

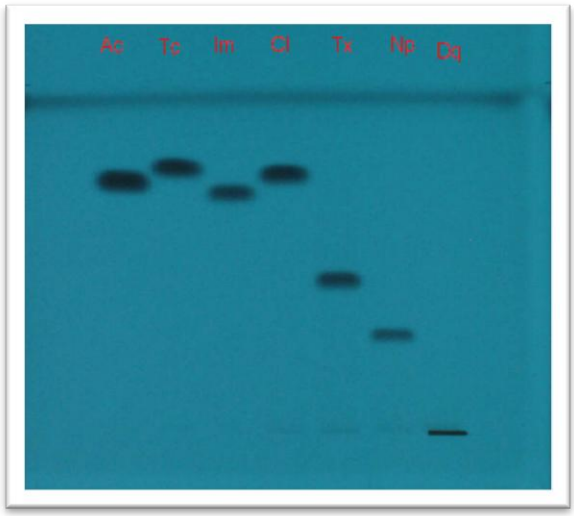


Fig. 4.9 *Trichloroacetic acid test at 140°C for 10 mins, illuminated with UV-light at 254 nm*

The results of this test appear to be the best when compared with the results of the other plates. The acid vapour appears to have completely covered the entire plate, resulting in all of the neonicotinoids producing light blue fluorescent zones.

Trichloroacetic acid at 160°C for 10 minutes

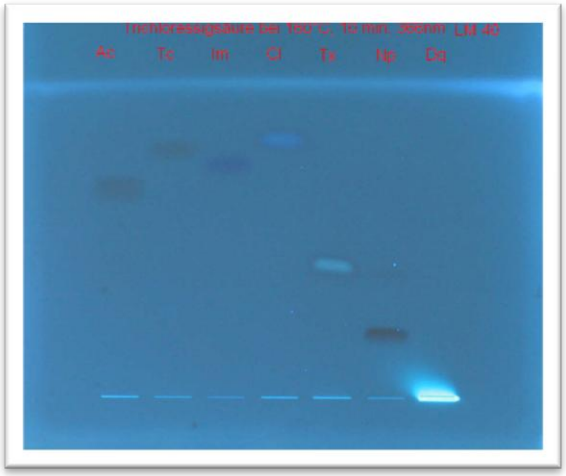


Fig. 4.10 *Trichloroacetic acid test at 160°C for 10 mins, illuminated with UV-light at 366 nm*

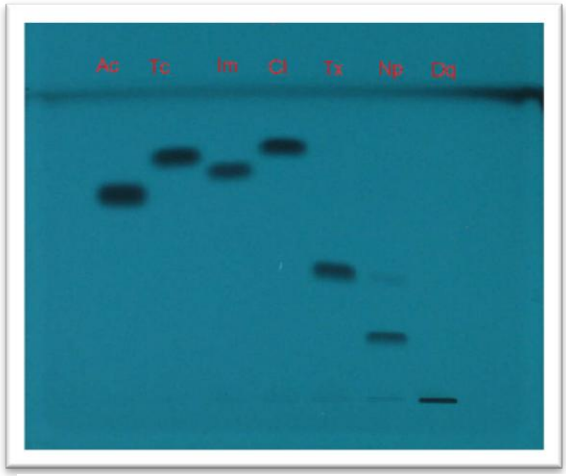


Fig. 4.11 *Trichloroacetic acid test at 160°C for 10 mins, illuminated with UV-light at 254 nm*

The results of the previous experiment could not be repeated in this experiment. As with the experiment completed at 140°C, the only neonicotinoids to show fluorescent blue zones are Imidacloprid, Thiamethoxam and Nitenpyram.

Trichloroacetic acid at 180° for 10 minutes

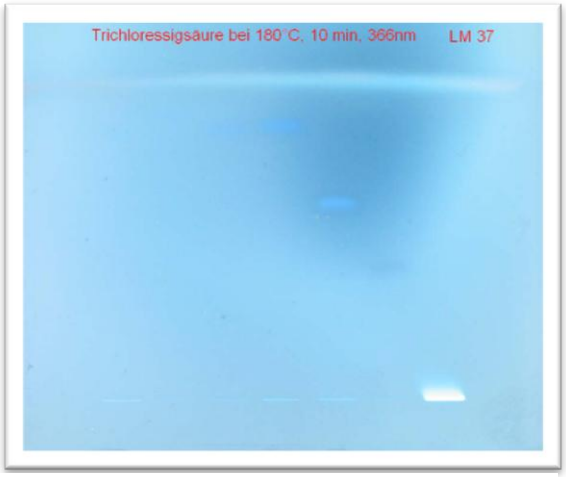


Fig. 4.12 *Trichloroacetic acid test at 180°C for 10 mins, illuminated with UV-light at 366 nm*

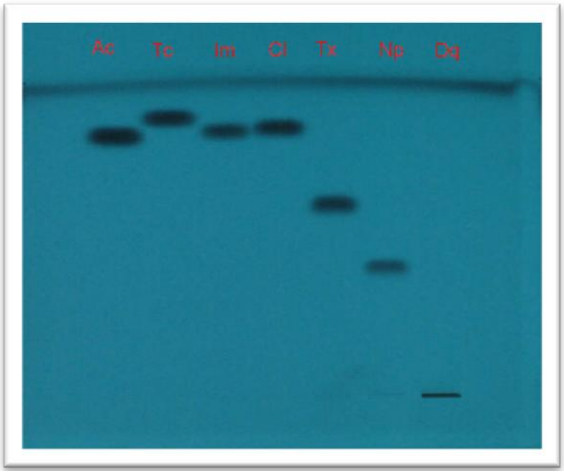


Fig. 4.13 *Trichloroacetic acid test at 180°C for 10 mins, illuminated with UV-light at 254 nm*

Upon observation, it appears as if the entire plate has been covered in acid vapour. However, the light blue parts of Fig. 4.10 are actually the parts of the plate which had been burnt due to the high temperatures of the oven. The only neonicotinoid to appear fluorescing is Thiamethoxam.

180°C was too high a temperature, as the TLC plate had burnt patches and the bottom half of the glass petri dish shattered.

4.4.2 Dichloroacetic Acid vs. Trichloroacetic Acid Experiment

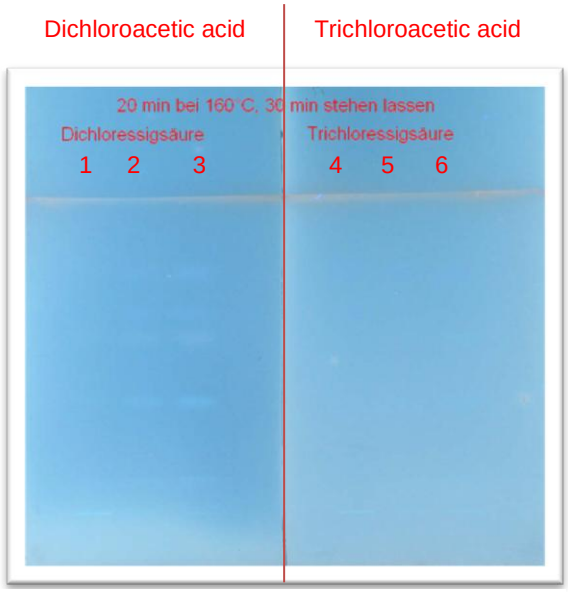


Fig. 4.14 *Trichloroacetic acid and dichloroacetic acid test at 160°C for 20 mins and left to cool for 30 mins, illuminated with UV-light at 366 nm*

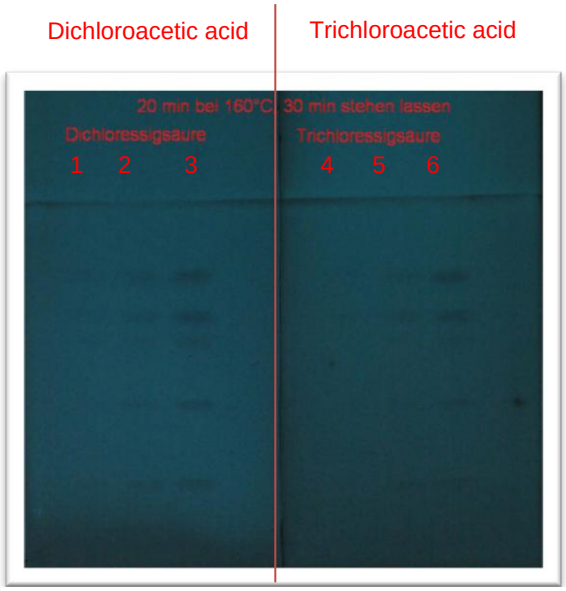


Fig. 4.15 *Trichloroacetic acid and dichloroacetic acid test at 160°C for 20 mins and left to cool for 30 mins, illuminated with UV-light at 254 nm*

By observing Fig 4.14, it can be seen that dichloroacetic acid produces more visible fluorescent blue zones of all 5 neonicotinoids, compared with trichloroacetic acid where no fluorescence can be detected.

For a better comparison, both plates should be scanned with the J&M Scanner, and their peak area values evaluated.

4.4.3 Allantoin

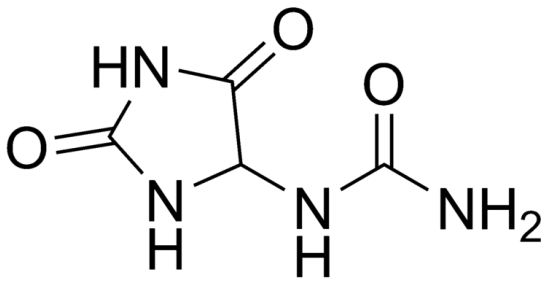


Fig. 4.16 *Structural formula of Allantoin* **(18)**

Allantoin is the compound formed when dichloroacetic acid or trichloroacetic acid reacts with the neonicotinoids to form the fluorescent blue zones. It is this compound that is then detected under UV-light at 366 nm.

Description of Allantoin

Allantoin (IUPAC Name: (2,5-Dioxo-4-imidazolidinyl) urea) is a decomposition product of purin. Allantoin is a white powder, which is odourless and tasteless. Allantoin is also an amphoteric substance. It is produced by the oxidation of uric acid by purine catabolism. It can also be obtained synthetically by the heating of urea and dichloroacetic acid. It is commonly used in the cosmetic industry as a skin-conditioning agent and skin protectant (19).

5. Quantification and Calibration of Standard Samples

5.1 Introduction

The final step in the development of a method to analyse neonicotinoids via TLC is the quantification of the standard samples. By being able to quantify the standards, it enables the quantification of “real world” samples and provides a guide of what to expect when analysing these samples.

Before quantifying any samples, though, a calibration method must first be established. The conversion of the observed signal into absolute amounts is usually achieved though calibration (3). Therefore, after scanning and analysing the densitograms provided by the J&M Scanner, the mean value, variance, and standard deviation must also be calculated for a proper calibration.

Example of calculating the amount of pure substance applied on a TLC plate:

Acetamiprid

$$\text{Pure Substance} = \underbrace{\frac{6\ mg}{10\ mL}}_{1} \cdot \underbrace{\frac{1\ mL}{5\ mL}}_{2} \cdot \underbrace{1\ \mu L}_{3} = 0.120\ \mu g = 120\ ng$$

1: Original concentration of standard

2: Volume of standard pipetted into new mixture divided by total volume of new mixture

3: Volume sprayed onto track

5.2 Measurement of R_f Value

5 µL of the individual standard samples were sprayed using the DESAGA AS 30 device onto an RP-18 WF glass-backed plate (1.13124)

The plate was then left to develop in an N-chamber. Upon completion of development, the plates was then removed from the chamber, and allowed to dry for 5 minutes.

After drying, the plate was then photographed under UV-light at 254 nm using the CabUVIS device.

By using the ProViDoc® program, the R_f values of the standard samples could then be calculated. Results are tabulated below (Table 5.1)

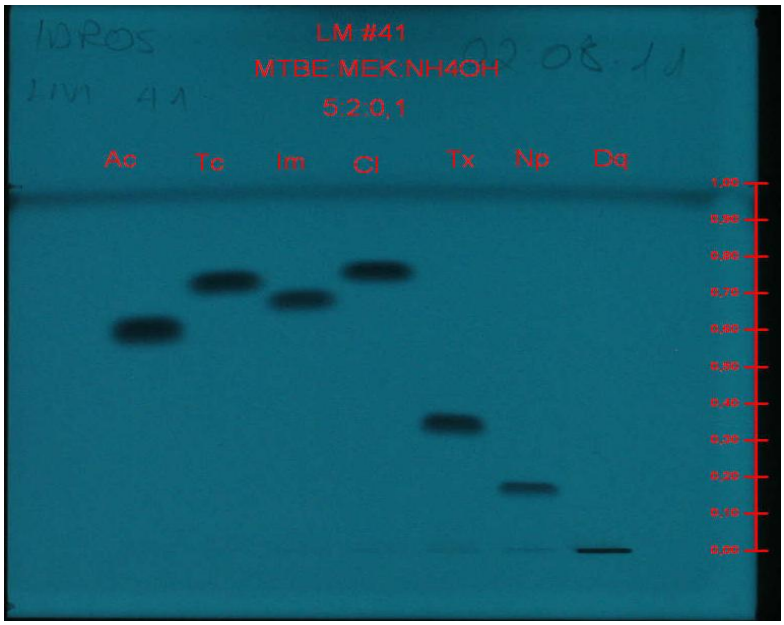


Fig. 5.1 *Measurement of R_f value using ProViDoc® program, illuminated under UV-light at 254 nm*

Table 5.1 *R_f value measurement results in order of increasing value*

Neonicotinoid	**R_f Value**
Nitenpyram	0.18
Thiamethoxam	0.35
Acetamiprid	0.60
Imidacloprid	0.69
Thiacloprid	0.73
Clothianidin	0.76

5.3 Calibration

5.3.1 Calibration Plate 1

Table 5.2 *Plate 1: Tracks and their corresponding pure substance values*

Track	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Mixture	2	2	2	2	2	1	1	1
Amount Applied in µL	1	2	3	4	5	2	3	5
Amount of pure substance in ng	24	48	72	96	120	240	360	600

Application Device: DESAGA AS 30

Solvent: MTBE + MEK + NH_4OH (12.5%)

Component Composition: 5 + 2 + 0.1

TLC Plate: RP-18 WF Glass-backed

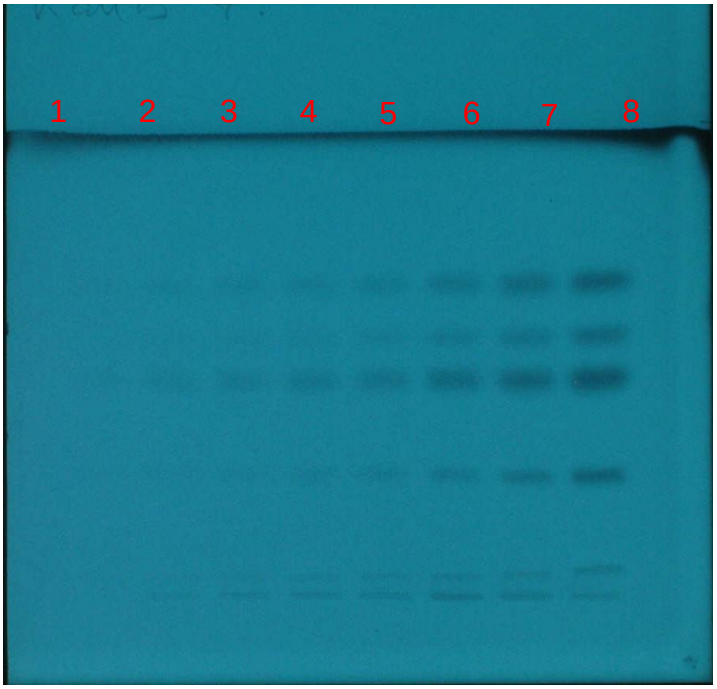


Fig. 5.2 *Calibration Plate 1: 8 Tracks of various amounts of pure substances (24 ng, 48 ng, 72 ng, 96 ng, 120 ng, 240 ng, 360 ng, 600 ng) on RP-18 WF plate with MTBE-MEK-NH_4OH (5+2+0.1) as solvent solution, illuminated at 254 nm*

5.3.2 Calibration Plate 2

By using the DESAGA AS 30 semi-automatic sample application system, 8 tracks of Mix #1 were sprayed onto an RP-18 WF glass-backed plate.

Table 5.3 *Plate 2: Tracks and their corresponding pure substance values*

Track	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Amount Applied in µL	1	1	1	1	1	1	1	1
Amount of pure substance in ng	120	120	120	120	120	120	120	120

Application Device: DESAGA AS 30

Solvent: MTBE + MEK + NH_4OH (12.5%)

Component Composition: 5 + 2 + 0.1

TLC Plate: RP-18 WF Glass-backed

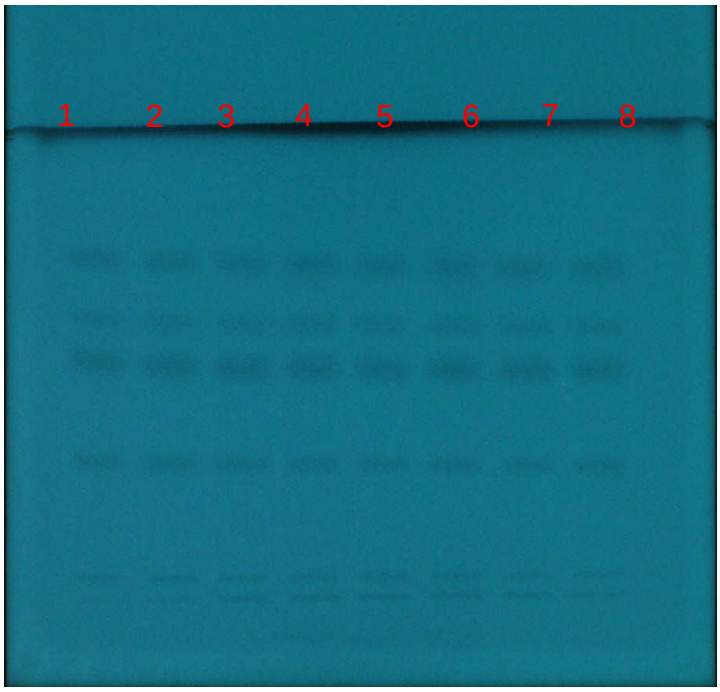


Fig. 5.3 *Calibration Plate 2: 8 tracks of 1 µL of Mix #1 on RP-18 WF plate with MTBE + MEK + NH_4OH (5+2+0.1) as solvent solution, illuminated at 254 nm*

5.4 Results and Discussion

5.4.1 Scan Results and Graphs of Plate 1

Nitenpyram measured at 329 nm with 25 diodes

Table 5.4 *Analysis of Plate 1: Nitenpyram measured at 329 nm*

Amount of pure substance in ng	Area
24	0.12565
48	0.2159
72	0.3321
96	0.5149
120	0.7944
240	1.5986
360	2.1267
600	3.6827

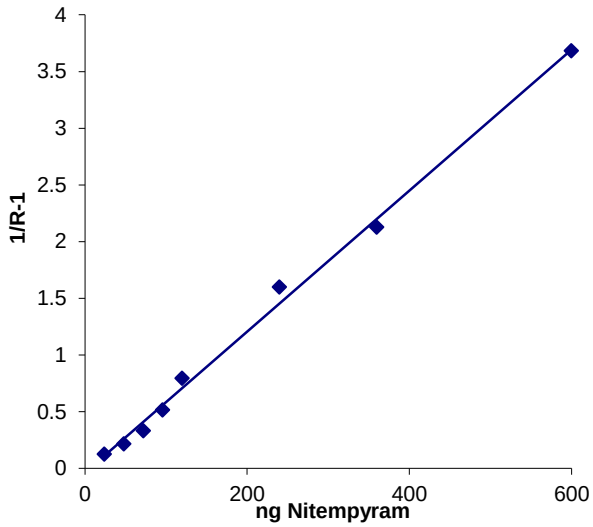


Fig. 5.4 *Analysis of Plate 1: Nitenpyram measured at 329 nm: Graph of peak area/ [1/(R-1]) vs. mass of pure substance in ng*

Acetamiprid measured at 247 nm with 25 diodes

Table 5.5 *Analysis of Plate 1: Acetamiprid measured at 247 nm*

Amount of pure substance in ng	Area
24	0.3324
48	1.0683
72	1.5619
96	1.9926
120	3.006
240	5.7839
360	8.4836
600	13.7335

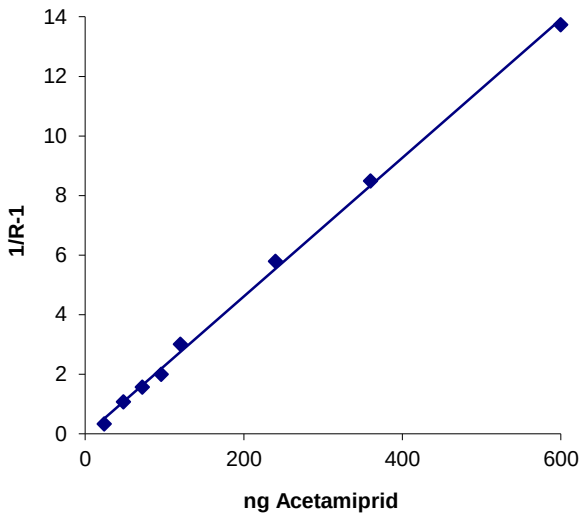


Fig. 5.5 *Analysis of Plate 1: Acetamiprid measured at 247 nm: Graph of peak area/ [1/(R-1])) vs. mass of pure substance in ng*

Nitenpyram measured at 268 nm with 25 diodes

Table 5.6 *Analysis of Plate 1: Nitenpyram measured at 268 nm*

Amount of pure substance in ng	Area
24	0.06
48	0.1469
72	0.2266
96	0.3701
120	0.6109
240	0.9649
360	1.1887
600	1.798

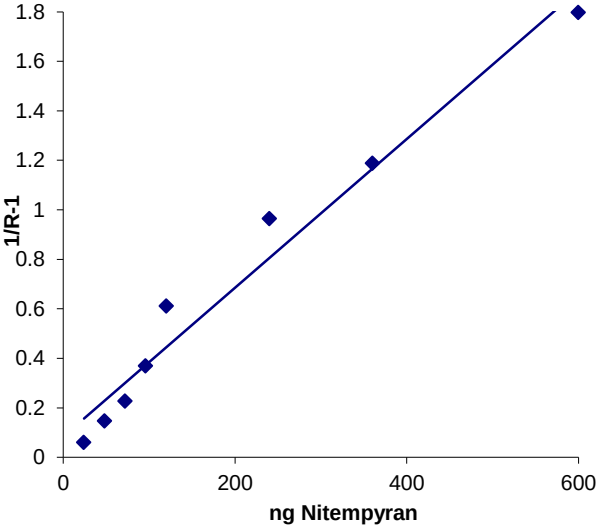


Fig. 5.6 *Analysis of Plate 1: Nitenpyram measured at 268 nm: Graph of peak area/ [1/(R-1])) vs. mass of pure substance in ng*

Thiamethoxam measured at 268 nm with 25 diodes

Table 5.7 *Analysis of Plate 1: Thiamethoxam measured at 268 nm*

Amount of pure substance in ng	Area
24	
48	
72	
96	0.114
120	0.2951
240	0.4226
360	0.9163
600	1.6759

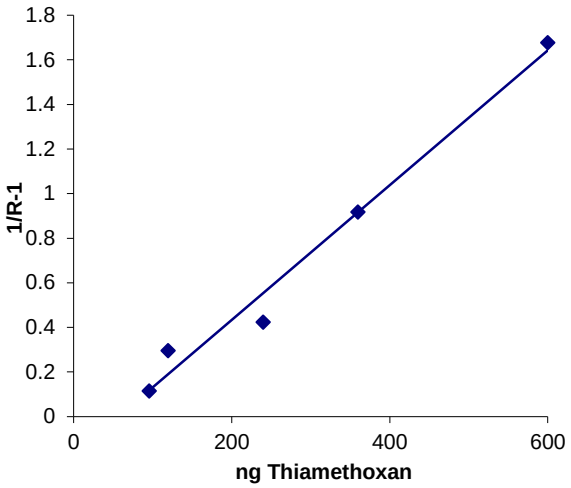


Fig. 5.7 *Analysis of Plate 1: Thiamethoxam measured at 268 nm: Graph of peak area/ [1/(R-1])) vs. mass of pure substance in ng*

Acetamiprid measured at 268 nm with 25 diodes

Table 5.8 *Analysis of Plate 1: Acetamiprid measured at 268 nm*

Amount of pure substance in ng	Area
24	0.177
48	0.53
72	0.9655
96	1.121
120	1.388
240	2.9932
360	4.4051
600	6.9353

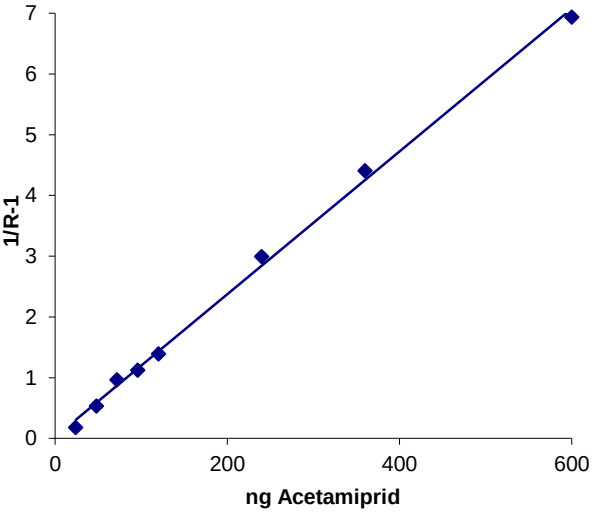


Fig. 5.8 *Analysis of Plate 1: Acetamiprid measured at 268 nm: Graph of peak area/ [1/(R-1])) vs. mass of pure substance in ng*

Imidacloprid measured at 268 nm with 25 diodes

Table 5.9 *Analysis of Plate 1: Imidacloprid measured at 268 nm*

Amount of pure substance in ng	Area
24	0.206
48	0.359
72	0.5173
96	0.7576
120	1.0572
240	2.1096
360	3.5801
600	6.6952

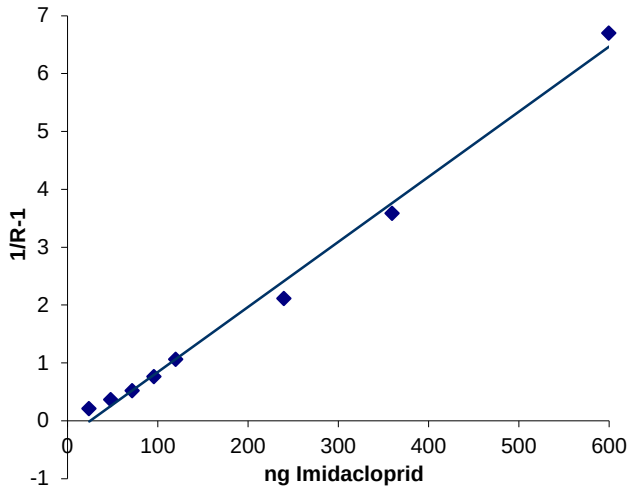


Fig. 5.9 *Analysis of Plate 1: Imidacloprid measured at 268 nm: Graph of peak area/ [1/(R-1]) vs. mass of pure substance in ng*

Clothianidin measured at 268 nm with 25 diodes

Table 5.10 *Analysis of Plate 1: Clothianidin measured at 268 nm*

Amount of pure substance in ng	Area
24	0.1158
48	0.2822
72	0.5021
96	0.7395
120	0.9766
240	1.9356
360	3.7048
600	5.9295

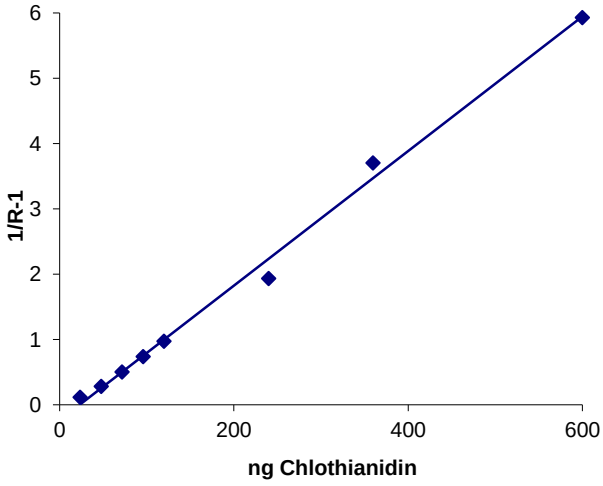


Fig. 5.10 *Analysis of Plate 1: Clothianidin measured at 268 nm: Graph of peak area/ [1/(R-1]) vs. mass of pure substance in ng*

5.4.2 Scan Results of Plate 2

Nitenpyram measured at 328 nm with 25 diodes

Table 5.11 *Analysis of Plate 2: Nitenpyram measured at 328 nm: Track, Peak Area, and Statistical Calculations*

Track, xi	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	1.0474	0.03709625	0.001376132
2	1.00943	-0.00087375	7.63439×10^{-7}
3	0.9727	-0.03760375	0.001414042
4	0.9242	-0.08610375	0.007413856
5	0.9528	-0.05750375	0.003306681
6	1.0527	0.04239625	0.001797442
7	1.0575	0.04719625	0.002227486
8	1.0657	0.05539625	0.003068745

Calculated mean value, $\bar{Y}$: 1.01030375

Calculated σ^2 value: 0.002943592

Calculated standard deviation, σ: 0.05425488

Calculated relative standard deviation in %: 5.370155622

Acetamiprid measured at 247 nm with 25 diodes

Table 5.12 *Analysis of Plate 2: Acetamiprid measured at 247 nm: Track, Peak Area, and Statistical Calculations*

Track, xi	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	2.4904	-0.4501375	0.202623769
2	2.6842	-0.2563375	0.065708914
3	2.7165	-0.2240375	0.050192801
4	2.9179	-0.0226375	0.000512456
5	3.1069	0.1663625	0.027676481
6	3.197	0.2564625	0.065773014
7	3.142	0.2014625	0.040587139
8	3.2694	0.3288625	0.108150544

Calculated mean value, $\bar{Y}$: 2.9405375

Calculated σ^2 value: 0.080175017

Calculated standard deviation, σ: 0.283151933

Calculated relative standard deviation in %: 9.62925767

Nitenpyram measured at 268 nm with 25 diodes

Table 5.13 *Analysis of Plate 2: Nitenpyram measured at 268 nm: Track, Peak Area, and Statistical Calculations*

Track, xi	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	0.66831	0.01825875	0.000333382
2	0.727	0.07694875	0.00592111
3	0.6536	0.00354875	1.25936×10^{-5}
4	0.723	0.07294875	0.00532152
5	0.6817	0.03164875	0.001001643
6	0.6468	-0.00325125	1.05706×10^{-5}
7	0.575	-0.07505125	0.00563269
8	0.525	-0.12505125	0.015637815

Calculated mean value, $\bar{Y}$: 0.65005125

Calculated σ^2 value: 0.00483876

Calculated standard deviation, σ: 0.0695612

Calculated relative standard deviation in %: 10.7008796

Thiamethoxam measured at 268 nm with 25 diodes

Table 5.14 *Analysis of Plate 2: Thiamethoxam measured at 268 nm: Track, Peak Area, and Statistical Calculations*

Track, xi	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	0.1991	-0.0379125	0.03964081
2	0.1992	-0.0378125	0.03968064
3	0.1704	-0.0666125	0.02903616
4	0.2794	0.0423875	0.07806436
5	0.2054	-0.0316125	0.04218916
6	0.292	0.0549875	0.085264
7	0.2775	0.0404875	0.07700625
8	0.2731	0.0360875	0.07458361

Calculated mean value, $\bar{Y}$: 0.2370125

Calculated σ^2 value: 0.066495

Calculated standard deviation, σ: 0.25786624

Calculated relative standard deviation in %: 108.798583

Acetamiprid measured at 268 nm with 25 diodes

Table 5.15 *Analysis of Plate 2: Acetamiprid measured at 268 nm: Track, Peak Area, and Statistical Calculations*

Track, x_i	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	1.3318	-0.1269125	0.016106783
2	1.4393	-0.0194125	0.000376845
3	1.4169	-0.0418125	0.001748285
4	1.4372	-0.0215125	0.000462788
5	1.5832	0.1244875	0.015497138
6	1.5026	0.0438875	0.001926113
7	1.3637	-0.0950125	0.009027375
8	1.595	0.1362875	0.018574283

Calculated mean value, $\bar{Y}$: 1.4587125

Calculated σ^2 value: 0.009102801

Calculated standard deviation, σ: 0.095408602

Calculated relative standard deviation in %: 6.54060355

Imidacloprid measured at 268 nm with 25 diodes

Table 5.16 *Analysis of Plate 2: Imidacloprid measured at 268 nm: Track, Peak Area, and Statistical Calculations*

Track, x_i	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	1.3082	0.12695	0.016116373
2	1.1911	0.00985	9.70225×10^{-5}
3	1.2013	0.02005	0.000402003
4	1.1749	-0.00635	4.03225×10^{-5}
5	1.339	0.15775	0.024885063
6	1.1266	-0.05465	0.002986622
7	1.0527	-0.12855	0.016525103
8	1.0562	-0.12505	0.015637503

Calculated mean value, $\bar{Y}$: 1.18125

Calculated σ^2 value: 0.010955706

Calculated standard deviation, σ: 0.104669507

Calculated relative standard deviation in %: 8.86091065

Clothianidin measured at 268 nm with 25 diodes

Table 5.17 *Analysis of Plate 2: Clothianidin measured at 268 nm: Track, Peak Area, and Statistical Calculations*

Track, x_i	Measured Area of Peak, y_i	$y_i - \bar{Y}$	$(y_i - \bar{Y})^2$
1	1.2986	0.0237625	0.000564656
2	1.3028	0.0279625	0.000781901
3	1.2893	0.0144625	0.000209164
4	1.3337	0.0588625	0.003464794
5	1.3496	0.0747625	0.005589431
6	1.2909	0.0160625	0.000258004
7	1.1963	-0.0785375	0.006168139
8	1.1375	-0.1373375	0.018861589

Calculated mean value, $\bar{Y}$: 1.2748375

Calculated variance, σ^2: 0.00512824

Calculated standard deviation, σ: 0.07161173

Calculated relative standard deviation in %: 5.6173221

6. Conclusion

6.1 The Mobile Phase

The best mobile phase eluent composition found during this thesis, was comprised of MTBE-MEK-NH_4OH with a component composition of 5 + 2 + 0.1.

Whilst the R_f values are considered to be in an optimal range (i.e. between 0.1 and 0.85), much better values must still be sought. In particular, the value of Nitenpyram (R_f Value = 0.18) is very low.

The inclusion of Thiacloprid for future analysis is also important. By comparing plates developed in a horizontal S-chamber and plates developed in an N-chamber, it can be seen that R_f values are not only more consistent, but that a better spacing between the individual neonicotinoids is achieved. This “better spacing” could also leave the necessary room for the development of Thiacloprid in a standard mixture.

New R_f values must be calculated using plates developed in a horizontal S-chamber.

6.2 Fluorescent Reagent

The best fluorescence shown has been observed by using dichloroacetic acid at a temperature of 160°C for 20 minutes, and left to cool in the sealed petri dish for 30 minutes. By using this reagent and method, all five neonicotinoids emit a light blue fluorescence under UV-light at 366 nm.

The limitations of this reagent, though, can still be observed as detection of samples below 300 ng are still difficult to analyse.

6.3 Calibration

Besides Thiamethoxam, all other neonicotinoid standard samples show relatively good %RSD, ranging from approximately 5% to 11%.

After calculation the relative standard deviation for Thiamethoxam, it seems very unlikely that the %RSD could be that large. Recalculations have shown the same percentage of RSD.

By using the statistics calculated in 5.4.1 and 5.4.2, further calibration of the standard samples must be conducted. A calibration of a new plate treated with dichloroacetic acid must also be conducted.

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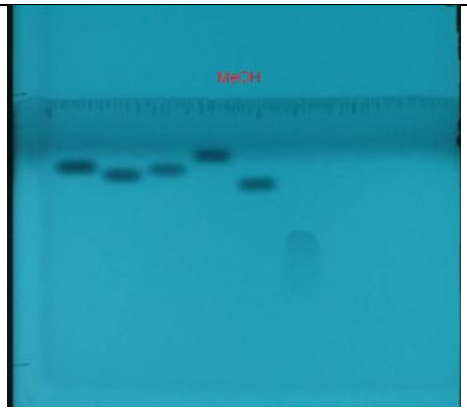
Appendix

I. Complete Listing and Corresponding Photographs of all Eluent Composition Trials.

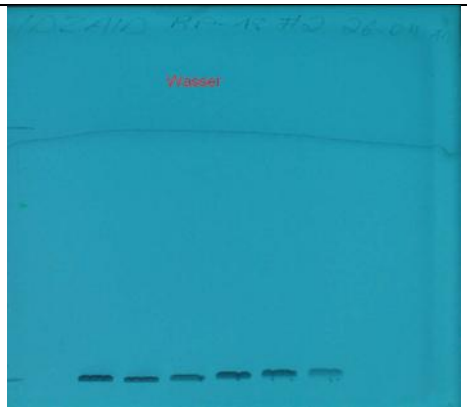
Appendix

Complete Table of All Eluent Compositions and their Corresponding „LM“ Number

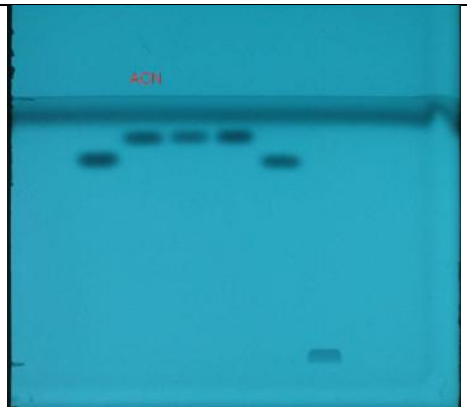
LM Number	Eluent	Component Composition	Duration in minutes
1	MeOH	1	13
2	H_2O dest.	1	18
3	ACN	1	10
4	MTBE	1	20
5	EtOH	1	45
6	MEK	1	15
7	2-Propanol	1	>60
8	THF	1	20
9	MTBE-MeOH	1 + 1	25
10	MTBE-MeOH	5 + 1	23
11	MTBE-MeOH	5 + 1	20
12	MTBE-MEK	4 + 1	20
13	MTBE-MEK	4 + 2	18
14	Aceton	1	12
15	Aceton	1	12
16	Aceton	1	8
17	Aceton	1	10
18	MEK	1	10
19	MTBE	1	17
20	ACN	1	7
21	MeOH	1	14
22	EtOH	1	30
23	THF	1	11
24	MTBE-MeOH	1 + 1	12
25	MTBE-Aceton	4 + 1	13
26	MTBE-MEK-NH_4OH	4 + 2 + 0.5	20
27	MTBE-MEK	4 + 3	27
28	MTBE-MEK-NH_4OH	5 + 1 + 1	20
29	MTBE-MEK-NH_4OH-CH_2Cl_2	4 + 3 + 0.5 + 1	17
30	MTBE-MEK-MeOH-NH_4OH	5 + 1 + 1 + 1	25
31	MTBE-MEK-2 Propanol-NH_4OH	4 + 1 + 1 + 1	27
32	MTBE-MEK-NH_4OH-Cyclohexane	4 + 2 + 1 + 1	18
33	MTBE-Dioxane-NH_4OH	5 + 1 + 1	21
34	MTBE-MEK-NH_4OH	4 + 2 + 1	10
35	MTBE-MEK-NH_4OH	3 + 2 + 1	10
36	MTBE-Dioxane-NH_4OH	5 + 1 + 1	13
37	MTBE-MEK-NH_4OH	2 + 2 + 1	17
38	MTBE-ACN-NH_4OH	5 + 1 + 1	15
39	MTBE-MEK-EtOH-NH_4OH	5 + 1 + 0.5 + 1	20
40	MTBE-MEK-NH_4OH	5 + 1 + 0.1	17
41	MTBE-MEK-NH_4OH	5 + 2 + 0.1	17
42	MTBE-MEK-EtOH-NH_4OH	5 + 1 + 0.5 + 1	20



LM 1



LM 2



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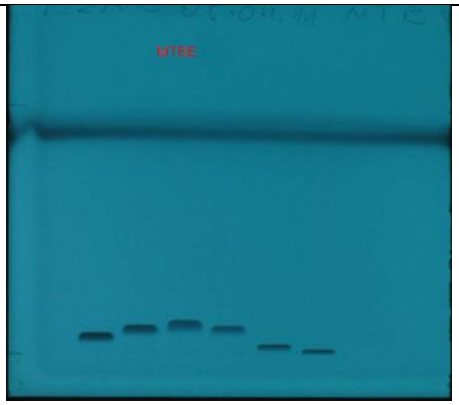

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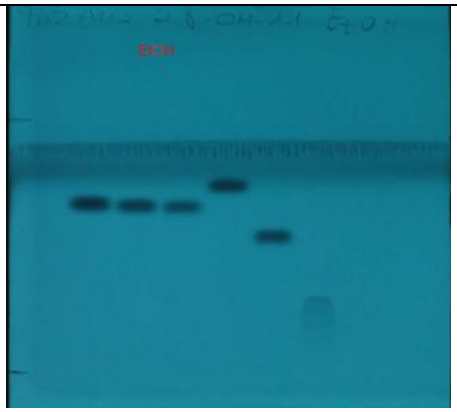

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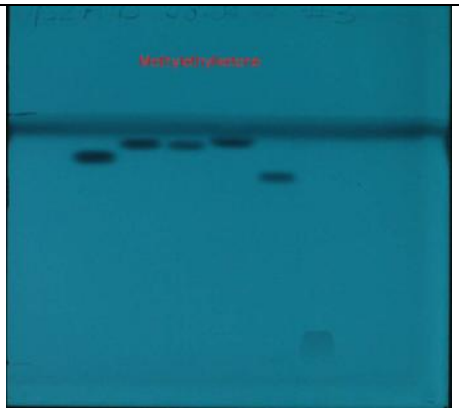

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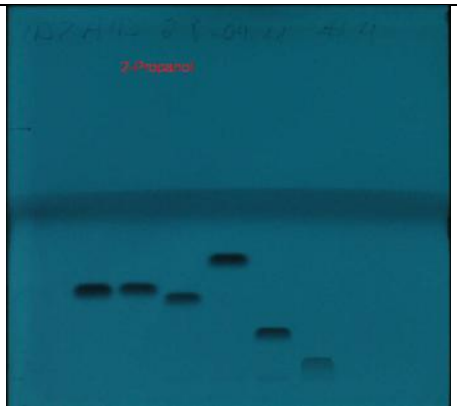

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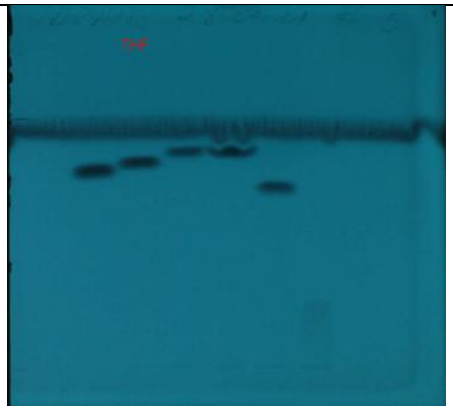

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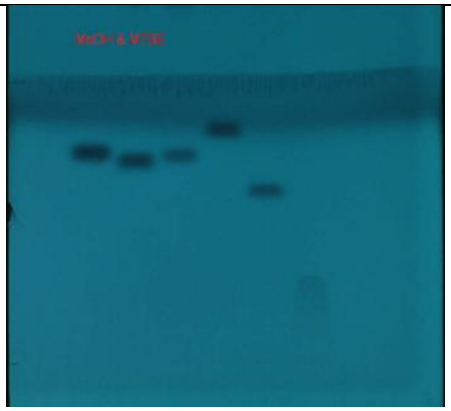

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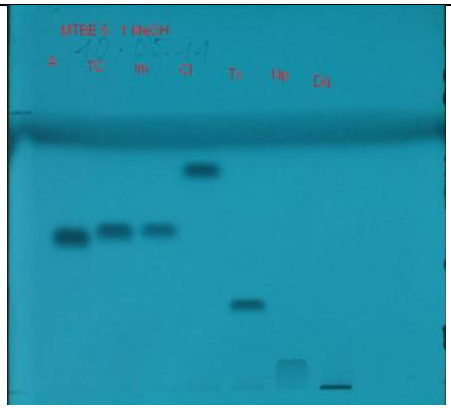

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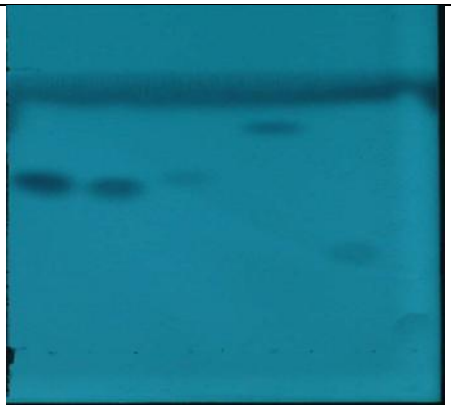

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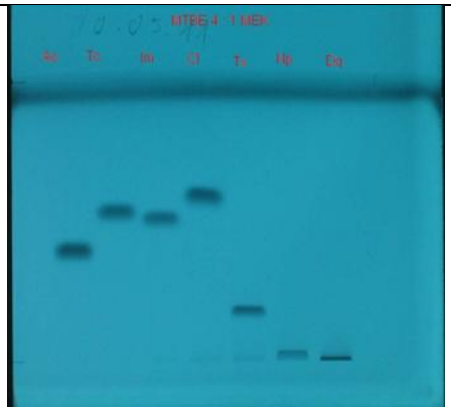

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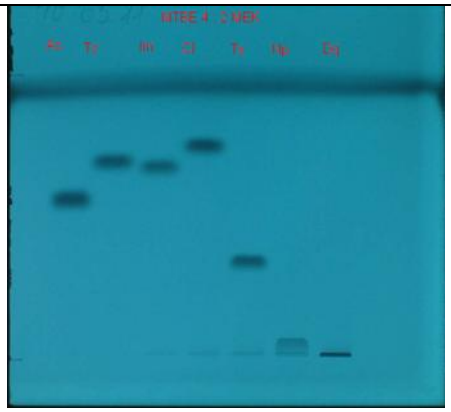

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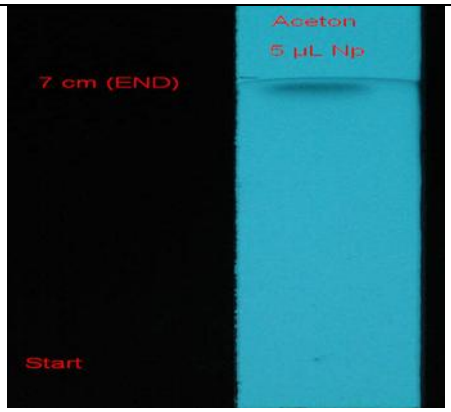

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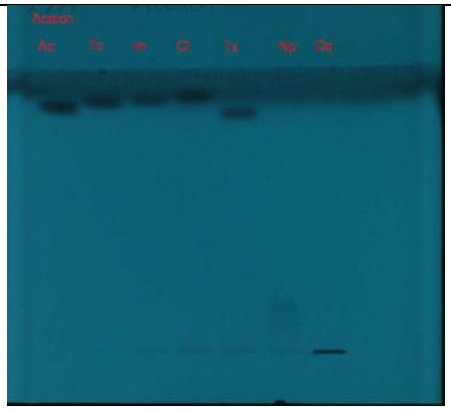

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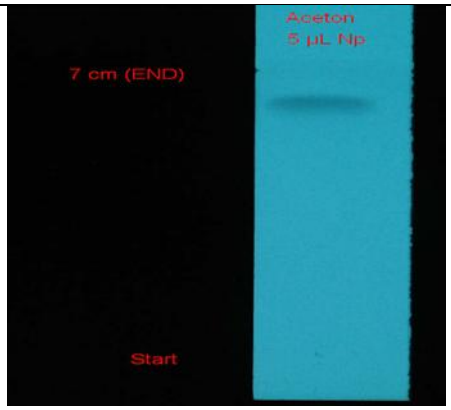

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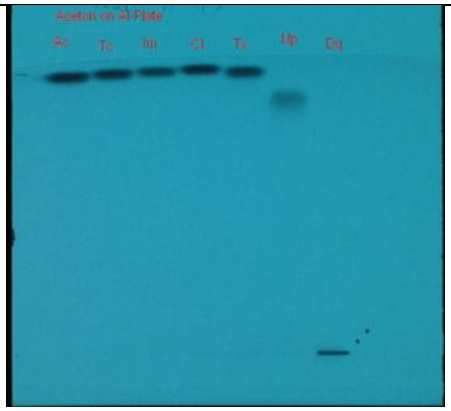

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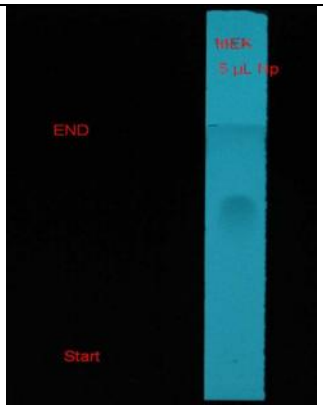

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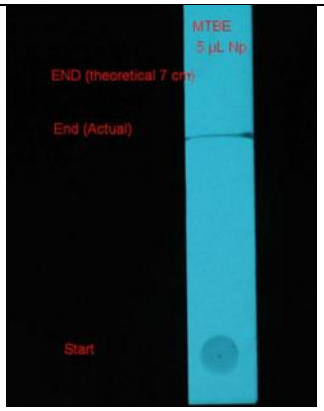

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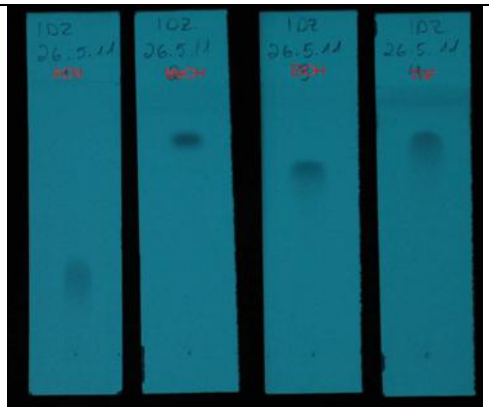

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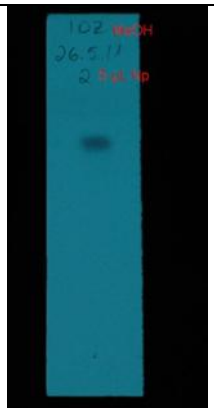

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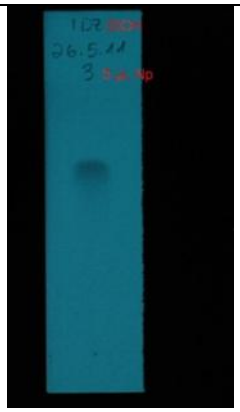

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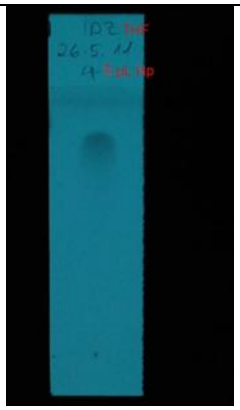

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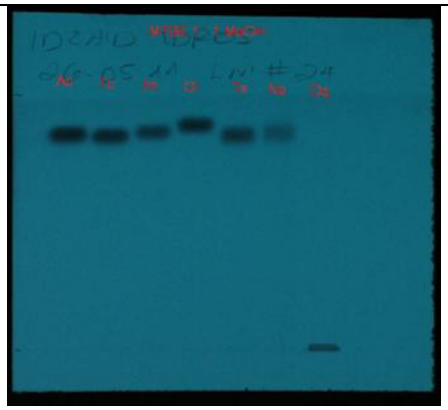

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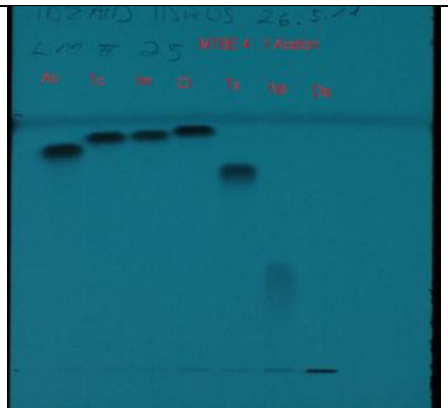

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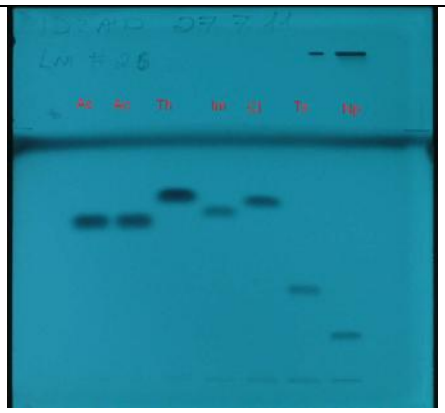

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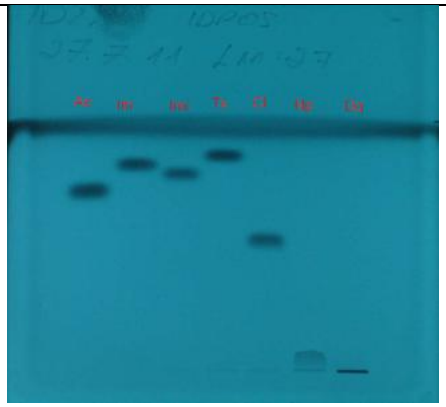

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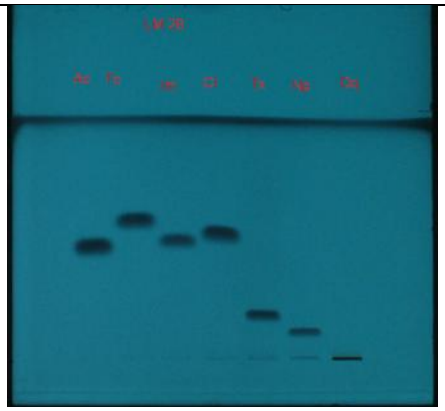

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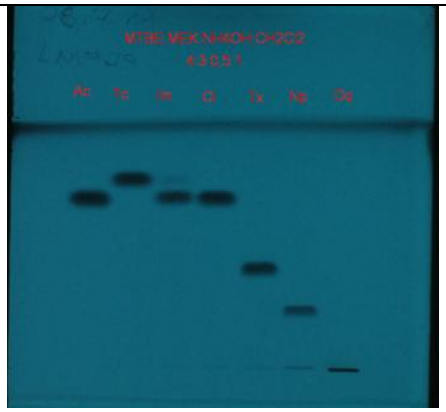

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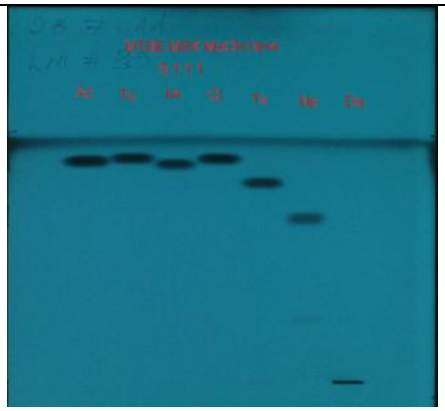

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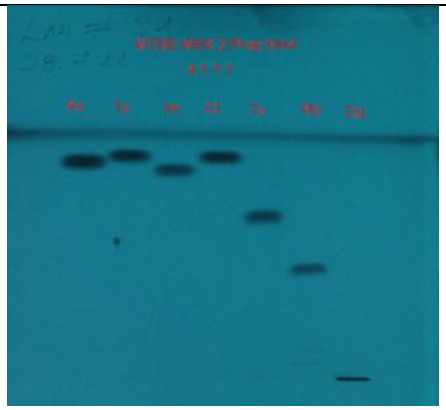

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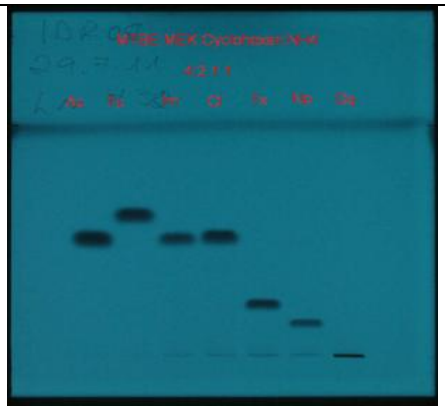

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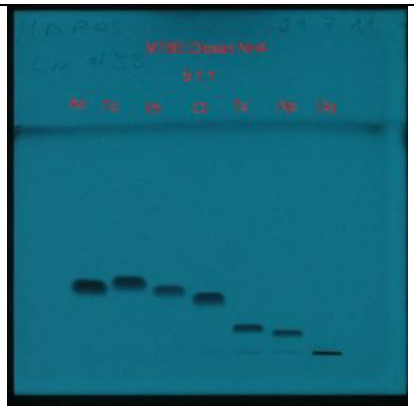

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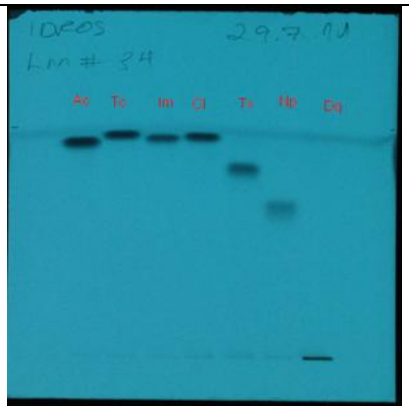

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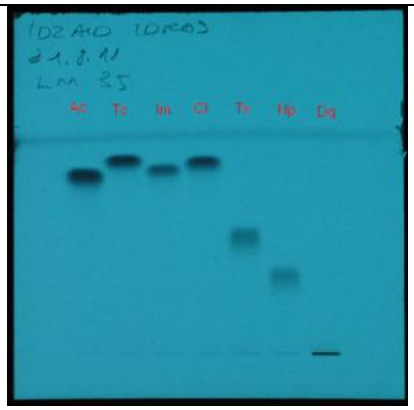

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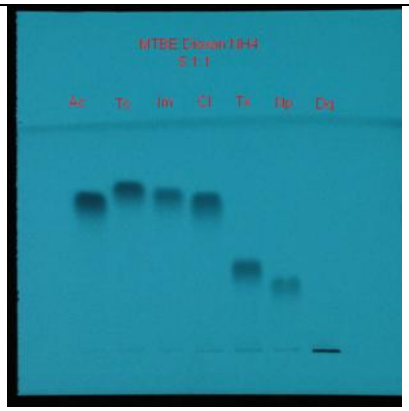

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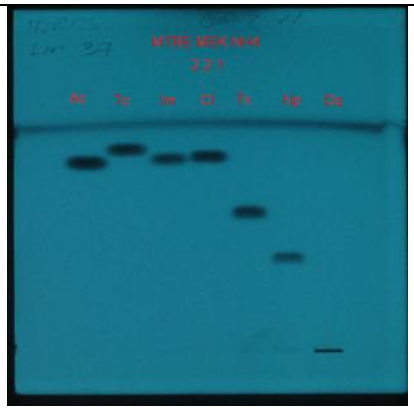

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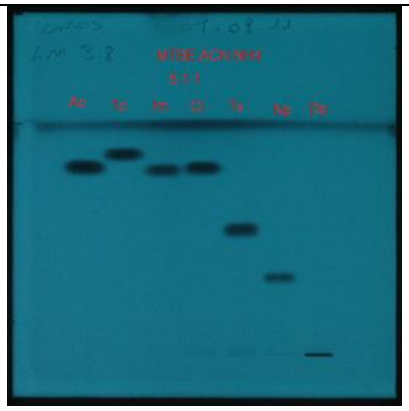

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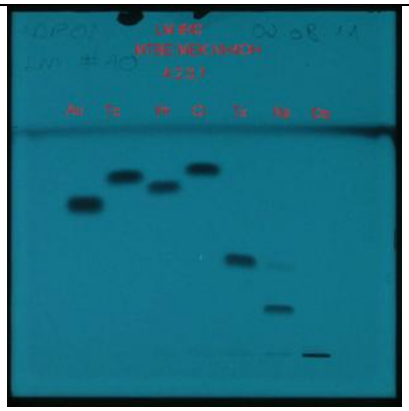

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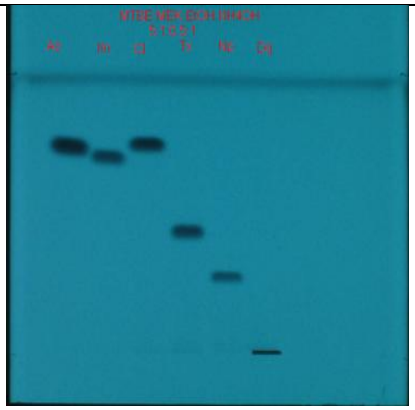

LM 42

Eidesstattliche Versicherung

Hiermit versichere ich eidesstattlich, dass die vorligende Arbeit von mir selbstständig und ohne erlaubte Hilfe angefertigt worden ist, insbesondere, dass ich alle Stellen, die wörtlich oder annähernd wörtlich oder dem Gedanken nach aus Veröffentlichungen, unveröffentlichungen Unterlagen und Gesprächen entnommen worden sind, als solche an den entsprechenden Stellen innerhalb der Arbeit durch Zitate kenntlich gemacht habe.

Ich bin mir bewusst, dass eine falsche Versicherung rechtliche Folgen haben wird.

Ich bin einverstanden mit:

a) der Übertragung des Rechts zur Vervielfältigung der Diplomarbeit für Lehrzweche an der Hochschule (§ 16 UrhG)

b) der Übertragung des Vortrags-, Aufführungs- und Vorführrechts für Lehrzwecke durch Professoren der Hochschule (§ 19 UrhG)

c) der Übertragung des Rechts auf Wiedergabe durch Bild- und Tonträger (§ 21 UrhG)

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(Idzaid Bin Idros)