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Optimization of automated flash chromatography methods for purification of two organic chemistry compounds for production use

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Optimization of automated flash chromatography methods for purification of two organic chemistry compounds for production use

The present study aimed to optimize the purification method of two synthesis intermediates using an automated flash chromatography system, CombiFlash EZPrep. The main parameters to be optimized in the chromatography methods were the solid phase, the mobile phase, its gradient, sample handling, and the loading method. Thin layer chromatography (TLC) was used to optimize the mobile phase for effective compound separation and to verify the separated fractions. Following the optimization of the purification processes, production-scale purifications were conducted using the developed methods.

The objectives were to use less harmful solvents for health and the environment and to minimize the solvent consumption, and waste generated during the purification processes. While the primary objective was to achieve high product purity and yield, the objectives related to the reagent consumption could not be achieved. Nevertheless, working with the equipment reduces health risks and the active working time compared to the manual methods previously used. Furthermore, the data generated by the equipment provided informative data about the separation of compounds, enabling better data storage and traceability of purification runs.

Keywords:Automated flash chromatography, liquid chromatography, thin layer chromatography, optimization

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Ritva Berg

Automaattisen flash-kromatografian menetelmien optimointi kahden orgaanisen kemian yhdisteen puhdistamiseksi tuotantokäyttöön

Tämän opinnäytetyöntyön tavoitteena oli optimoida kahden synteesin välituotteen puhdistusmenetelmä käyttäen automaattista flash-kromatografialaitteistoa CombiFlash EZPrep. Tärkeimmät optimoitavat parametrit kromatografiamenetelmissä olivat kiinteä faasi, liikkuva faasi, sen gradientti, näytteen käsittely ja lataustapa laitteelle. Liikkuvan faasin optimoinnissa ja erotettujen fraktioiden verifioinnissa käytettiin menetelmänä ohutkerroskromatografiaa. Optimointien jälkeen toteutettiin tuotantomittakaavan puhdistukset kehitetyillä menetelmillä.

Menetelmien optimoinnissa pyrittiin vähemmän haitallisten liuotinten käyttöön ja minimoimaan kuluvien liuotinten määrä sekä puhdistusprosessissa syntyvän jätteen määrä. Kun tärkeimpänä tavoitteena oli tuotteen puhtaus ja hyvä saanto, kulutettavien reagenssien määrän ja laadun suhteen ei pystytty pääsemään haluttuihin tavoitteisiin. Verrattuna aikaisemmin käytössä olleisiin manuaalisiin menetelmiin laitteiston käyttö vähensi puhdistuksiin kuluvaa aktiivista työaikaa ja kemikaaleille altistumista sekä paransi työergonomiaa. Lisäksi laitteiston tuottama data toi lisää tietoa yhdisteiden erottumisesta, mahdollisti paremman datan tallennuksen ja jäljitettävyyden puhdistusajoista.

Asiasanat:

Automaattinen flash-kromatografia, nestekromatografia, ohutkerroskromatografia, optimointi

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Glossary and abbreviations

Adsorption:	A surface phenomenon in which molecules or ions adhere to the surface of a solid or liquid (the adsorbent) due to attractive forces.
Chromatography	Chemical method that separates substances by distributing them between a stationary and a mobile phase, allowing the analysis of different compounds.
CV	Column volume
DCM	Dichloromethane
Detector	A device that, upon receiving a signal, generates a measurable response.
EtOAc	Ethyl Acetate
Fraction	Part of a mixture that is separated or separable based on a specific property
Flash chromatography	Method that differs from the conventional column chromatography by using pressurized gas to accelerate solvent flow.
HPLC	High performance liquid chromatography
NPC	Normal phase chromatography. Separation is based on the affinity of substances to the polar stationary phase compared to the non- polar mobile phase.
PE	Petroleum ether
Rf	Retention factor
RM	Reaction mixture

RPC	Reverse phase chromatography. Separation is based on the affinity of substances to the non-polar stationary phase compared to the polar mobile phase.
RTKU	Radiometer Turku Oy
Slope	A certain value based on a Gaussian distribution that must be exceeded for the column effluent to be collected as a peak.
TEA	Triethylamine
TLC	Thin layer chromatography
Threshold	An absorbance value that must be exceeded for the column effluent to be collected as a peak.
t_r	Retention time: The elapsed time for a compound to pass through a chromatographic system.

1 Introduction

1.1 Background of the master’s thesis commission

The present master’s thesis focuses on implementing automated flash chromatography in organic chemistry manufacturing processes. The study has been commissioned by Radiometer Turku Oy. The following paragraphs provide further information about Radiometer Turku Oy including the products it manufactures. Furthermore, it is explained which stage of production is involved in this master's thesis project.

1.2 Radiometer Turku Oy manufactures test and calibration kits for acute point-of-care biomarker testing

Radiometer Oy, established in Denmark in 1935, is a company specializing in the development, manufacturing, and marketing of solutions for processing, analyzing, and monitoring blood samples, as well as monitoring the condition of patients. Radiometer products and solutions are used in hospitals, clinics, and laboratories in over 130 countries. (Kemianteollisuus, 2023.) In 2006, Radiometer expanded its operations to Turku by acquiring Innotrac Diagnostics Oy. The company is a part of the U.S.-based science and technology group Danaher. Specifically, Radiometer Turku Oy focuses on producing test and calibration kits for use in AQT immunoanalyzers.

AQT analyzers are point-of-care (POC) devices utilized in healthcare for immunoassay testing. AQT test kits are capable of testing eight different immunoassay tests such as cardiac, coagulation, and infection biomarkers. In addition, the device can be used to test pregnancy. Biomarker analysis from plasma or whole blood samples takes 11- 21 minutes. (Radiometer, n.d.)

The test and calibration kits of the AQT analyzer incorporate luminescent lanthanide chelates as one of the raw materials. These chelates are synthesized in the organic chemistry laboratory at RTKU.

1.3 Optimizing the purification of two intermediates in luminescent lanthanide chelate synthesis

Luminescent lanthanide chelates are specialized compounds that consist of lanthanide ions, such as europium, bonded to organic molecules known as chelators. These chelates exhibit a unique property called luminescence, which involves emitting light when stimulated by an external energy source. This phenomenon makes them valuable in various applications, including molecular probes for fluorescent labels in diagnostics, and markers in assays. (Hemmilä et al, 1997.)

In the production of RTKU luminescent lanthanide chelates are used as fluorescent labels for immunoassays. The synthesis of lanthanide chelates in an organic chemistry laboratory is a complex process with many steps, including reactions and purifications. This study focuses on the purification of two intermediates involved in chelate synthesis and these two intermediates are referred to as compound 5 and compound 6. Figure 1 shows a part of the chelate synthesis discussed in this study. Compounds 5 and 6 are identified in the figure.

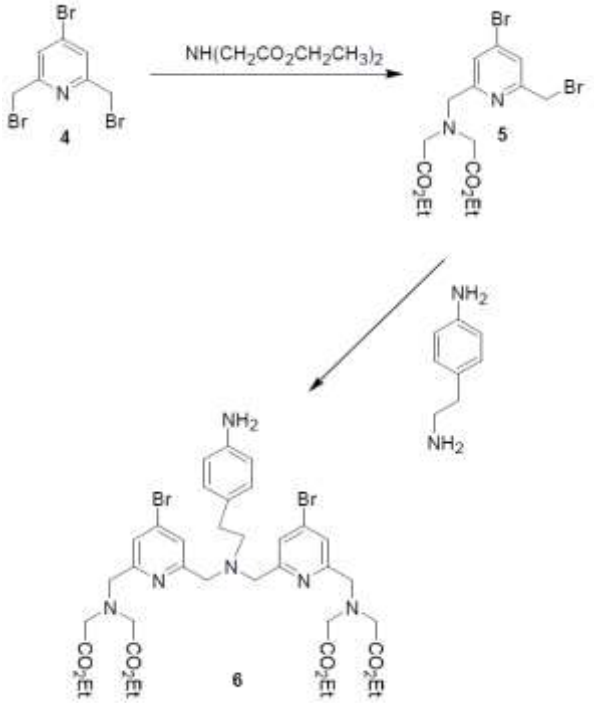

Figure 1. Synthesis scheme of a portion of the chelate synthesis examined in this study.

Until now, the purification steps are performed manually using silica gel chromatography, which is a time-consuming workflow. The procurement process for an automated flash chromatography system has been completed, but the current manual purification processes need to be transferred to an automatic flash chromatography system and optimized for production use. Automated flash chromatography can reduce time consumption and manually work in purification processes. The implementation of automated flash chromatography in organic chemistry manufacturing processes could also allow a reduction in the use of solvents. The aim is to replace solvents that are hazardous to health or the environment with less harmful ones. Introducing the equipment can significantly reduce the manual handling of solvents and other harmful substances, thus minimizing health risks associated with the work. Eliminating manual purification steps can also enhance work ergonomics and tedious task when there is no need for manual collection of fractions.

1.4 Objective of the study

The objective of the master's thesis is to transfer two selected purification steps of lanthanide chelates synthesis from manual purification to an automated flash chromatography process. This study focuses on the optimization of those purification methods and the main targets are product purity and the high yield. The optimization of an automated flash chromatography methods for production use should consider the following aspects:

- **The equipment and its technical characteristics**
  The chromatography system to be implemented for production use is the CombiFlash Ez prep manufactured by Teledyne Isco. The equipment and its specific technical characteristics must be well-known to design an efficient and functional purification process. Columns, solvent delivery systems, fraction collectors, monitoring systems, and control capabilities are factors whose operation affects the performance of the purification process. The automated equipment also generates and stores data on the performed purifications. The chromatogram produced by the equipment provides extensive information about the separation of compounds.

- **Processing of the reaction mixture (RM) before purification by flash chromatography**
  How the product mixture should be prepared before the product is purified by chromatography must be studied, designed, and tested. It is necessary to determine which solvent to dissolve the reaction mixture to be purified in and with which technique to load it into the chromatography system. The state of the mixture to be purified by chromatography can significantly influence the effectiveness of the purification process. (Teledyne Isco, 2017, 5-1-5-6; Teledyne Isco, 2018, 29-36)

- **Stationary phase selection**

  The stationary phase selection depends on the target compounds and their properties. The adsorbent can be either silica, modified silica, or alumina (Sravani, 2018). The silica particle is also available in different shapes and sizes (Teledyne Isco 2018, 27).

- **Mobile phase optimization**

  The mobile phase composition and flow rate are critical factors for efficient purification in flash chromatography. The appropriate solvent or solvent mixture selection should be optimized to achieve the desired purification result. Factors such as compound polarity, solubility, and interaction with the stationary phase must be considered (Sravani, 2018; Teledyne Isco, 2018, 6).

  By using CombiFlash Ezprep, it is possible to use a gradient during the purification run. This means that the run can be designed in a way that allows for the variation of the ratio between more polar to less polar eluent during the separation run, which is not possible with manual methods. (Teledyne Isco, 2018, 18) The gradient is a crucial factor in achieving the best possible parameters for purification. Solvents' effects on workers' health and the environmental impact must also be considered. The aim is to reduce the use of harmful solvents. Using a gradient makes it possible to reduce both the purification run time and the amount of solvent required (Teledyne Isco, 2018, 19.) Gradient optimization is an essential tool for effective separation (Snyder et al, 2010, 406-408.)

- **Scale-up considerations**
  Testing should start on a small scale. Manufacturing processes require larger loading volumes and higher throughputs. It is important to ensure that the system can handle the increased load, and the parameters of the production scale purification runs should be optimized.

In addition, when implementing new methods into production use, the following aspects should be considered. In this case, the work instructions will be updated by the RTKU organic chemistry team. Quality control is essential for ensuring performance of developed purification methods. There will be no changes to the quality control methods; the separated fractions will be verified in the same way as in the existing manual methods.

- **Process integration and implementation**
  Process integration and implementation involve designing the workflow and ensuring compatibility with other process steps. Implementing new equipment and a new workflow for production use requires updating documentation such as process diagrams and work instructions.

- **Quality control**
  Implementing new equipment for production use necessitates appropriate quality control measures. This involves monitoring and analyzing the purity and yield of the collected fractions and ensuring consistent product quality.

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1.5 Methods

Optimizations for CombiFlash Ezprep were performed using a batch of RM 5 (reaction mixture 5) specifically prepared for optimization purposes. RM 5 means crude product mixture from which compound 5 is purified. The separation of compounds was tested using TLC plates with different eluent compositions. Small-scale tests were started with CombiFlash EZPrep, based on the existing manual purification process.

To run several optimization runs without using large amounts of material, the same RM5 was recycled in several optimization runs. After the run, the purified fractions were verified using TLC. The fractions were pooled, and the solvents were evaporated using a rotary evaporator. This allowed reuse of the same material in the purification optimization tests.

Once the purification parameters for compound 5 were optimized, the same process was repeated to optimize the purification of compound 6. After optimizations purification protocols were documented. Following this, a production batch was prepared, and the purification processes for these two stages were performed using the developed methods. The utilization of the new methods in the production batch processing was documented in a planned nonconformity. In this instance performing the purification of the production batch using the CombiFlash EZPrep device instead of manual methods and documenting these purifications in the planned nonconformity replaced the need to perform PQ (performance qualification) for CombiFlash EZPrep. The risks of commissioning the equipment and the required measures, such as updating the work instructions, were considered in the planned nonconformity documentation.

The fractions purified by the CombiFlash EZPrep could be verified using TLC both during the optimization and production batch processing, following the old method and the current working instructions. The process only changed concerning the purification steps, while the other synthesis stages and quality

assurance were performed in accordance with the existing guidelines. Simplified work methods and workflow are presented in Figure 2.

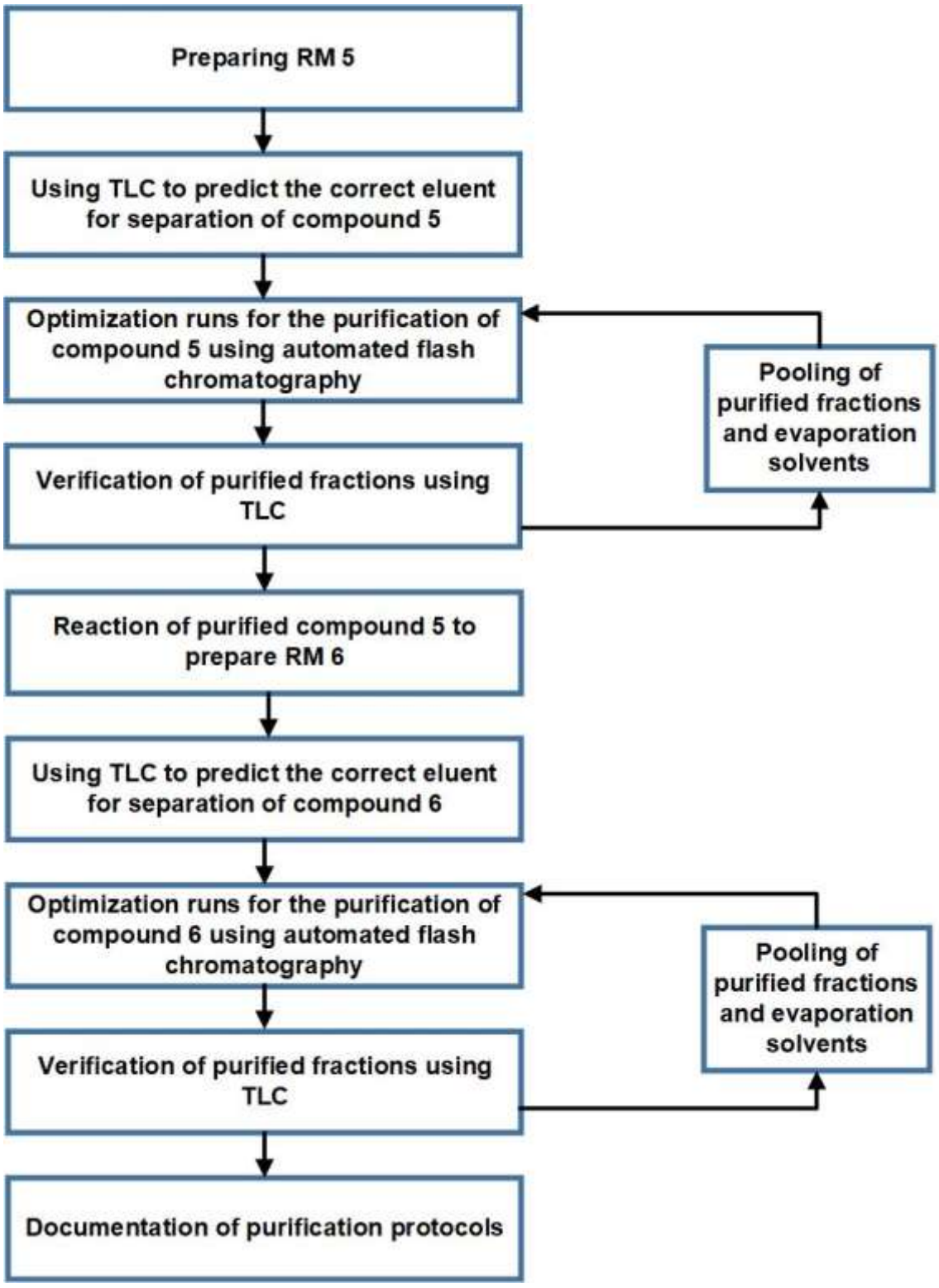

Figure 2. Work methods and workflow.

2 Basic principles of chromatography

Chromatography is a commonly used separation technique in the pharmaceutical and chemical industries. Chromatography can be categorized into two types based on its purpose of use.
Analytical chromatography focuses on characterization and measurement. The objective is to perform the qualitative and quantitative analysis of the different components present in a sample. **Preparative chromatography** is used to purify components within a sample and isolate specific purified compounds for subsequent procedures. (Sravani, 2018.)

In chromatography, separation is based on the distribution of chemical substances between a stationary phase and a mobile phase. Substances move at different rates between these phases, allowing for their separation and analysis. (Snyder et al., 2010, 20-41.)

Liquid chromatographic methods can be categorized based on the separation mechanism such as:

- Adsorption chromatography: Substances adhere to the surface of the stationary phase, forming weak chemical bonds.
- Ion-exchange chromatography: Ionic substances interact with groups containing ions in the stationary phase.
  (Mikkola, 2006, 2; Snyder et al., 2010, 22.)

Chromatographic techniques can also be categorized based on the separation matrix used, including column chromatography and thin layer chromatography (TLC) (Mikkola, 2006, 2.)

Regardless of the separation mechanism or the technique used, the chromatographic principle of chromatographic methods is always the same. Chromatographic separation occurs either on a plate or within a column. Separation is based on the interaction of the sample, stationary phase, and

mobile phase. (Mikkola, 2006, 2.) Chromatographic methods are based on a difference in affinity between a stationary phase, typically silica, and a mobile phase. The mobile phase is the solvent that travels through the stationary phase, referred to as the eluent (Clayden, 2001, 402).

Retention means the adsorption-desorption process that occurs as a compound passes through a stationary phase and **retention time (t_r)** is the time it takes for a compound to pass through a chromatographic system (Mikkola, 2006, 3).

Compounds move with the mobile phase through the stationary phase. The compounds interact with the stationary phase and the strength of the interaction determines retention time within the chromatographic system. The more strongly the compounds interact with the stationary phase, the slower they move. Throughout the retention time, molecules are either bound to the stationary phase or freely moving in the mobile phase. The eluent and the molecules from the sample being separated compete for binding to the stationary phase. (Mikkola, 2006, 2-8.)

Polarity in organic chemistry refers to a property of a molecule or chemical compound stemming from the uneven distribution of its charges or electron density among different parts of the molecule. The atoms of a **polar compound** are positively and negatively charged and usually, the functional group contains an element that is more electronegative than carbon. Such a compound attracts bond electrons and is slightly negatively charged while the carbon atom receives an equally large positive charge. The molecule of a **nonpolar compound** is symmetrical, and the charges cancel out because their centers of mass coincide. (Napari, 2001, 36.)

In RPC (reverse phase chromatography) the stationary phase is non-polar, while the mobile phase is polar. This means that separation is based on the affinity of substances to the non-polar stationary phase compared to the polar mobile phase. (Snyder et al., 2010, 22.)

In NPC (normal phase chromatography) stationary phase, often silica, is a more polar phase while the mobile phase is nonpolar or moderately polar organic solvents (Snyder et al., 2010, 22.). The separation mechanism is adsorption/desorption and compounds are retained based on their adsorptive affinity for the media and are desorbed with increasing solvent polarity. Therefore, low polarity compounds elute earlier while those that are more polar are retained longer on the stationary phase. (Biotage, 2018.) The polarity of the organic solvents used in this study is presented in Table 1.

Hence, the progress of certain compounds in NPC can be accelerated by reducing their binding by using a stronger, more polar, eluent. The interaction difference between the phases and compounds results in the separation of components from one another. (Mikkola, 2006, 2-8.) The compounds in the sample are separated as they move forward into zones that are visible as spots on the TLC plate or as peaks on the chromatogram (Halonen, 2004, 100).

The chromatogram is a graphical representation of the separation process generated by an automated chromatograph, showing the elution of the compounds in the mobile phase as a function of analysis time or column volumes (CV). Separated compounds are detected as signals, peaks. (Mikkola, 2006, 3). Figure 3 outlines the typical characteristics of a chromatogram.

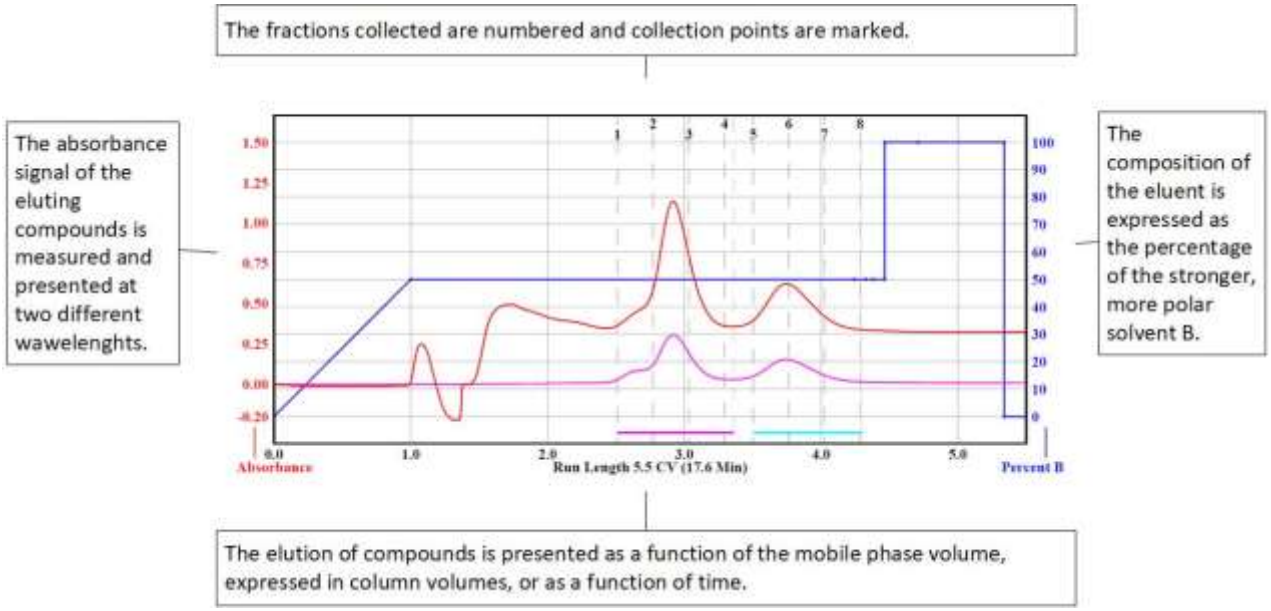

Figure 3. Illustration of a chromatogram.

3 Flash chromatography and thin layer chromatography TLC

3.1 Basic principles of flash chromatography

Flash chromatography is a purification technique that differs from the conventional column chromatography by using slightly smaller silica gel particles and -pressurized gas. Pressurized gas accelerates the solvent flow. This results in faster and more efficient chemical separations when compared to the traditional gravity-based column chromatography method. (Teledyne Isco, 2018.)

In 1978, American organic chemist Clark Still and his colleagues published a paper introducing a purification technique known as flash chromatography. In this technique, the pressure was applied on the top of the column in manual liquid chromatography. (Still et al, 1978) Before this report, column chromatography using silica gel as a stationary phase had already been established as a valuable method for the separation and purification of organic compounds. Elution of the solvent by gravity alone was often a tedious process, requiring several hours and leading to poor separations. Still's innovation led to an increase in the rate of solvent elution and significantly reduced the run time and enabled larger sample volumes to be handled. This allowed for the routine separation of compounds that were previously impossible to separate by column chromatography. Still published detailed instructions on the use of the technique. For example, his publication contains a table correlating column diameter, eluent volume, sample volume, and typical fraction size, providing a useful guide to the application of this technique in the laboratory. (Still et al, 1978; Sravani 2018.)

Manual flash chromatography, which uses a glass silica-packed column and manual separation of fractions in containers, is still used in laboratories for purification steps in organic syntheses. Fractions collected in the manual method are verified by TLC. Modern automated flash chromatography systems

use pre-packaged plastic cartridges and columns. The solvent is pumped through the cartridge and column, and the system incorporates detectors and fraction collectors for automation. The instrument can be programmed to collect only the desired fractions and the waste can be routed directly to a waste container. The integration of gradient pumps has led to faster isolations, less solvent usage, and increased flexibility. (Sandesh et al, 2021.) The automated flash chromatography system is illustrated in Figure 7.

3.2 Using TLC for prediction and verification of separation

Thin layer chromatography (TLC) is a rapid and inexpensive technique to determine optimum stationary phase or eluent compositions for flash chromatography (Sravani, 2018). TLC can also be used to verify fractions purified by flash chromatography.

The principle of separation in TLC is similar to column chromatography. Instead of a column, the stationary phase is attached to the surface of a glass or aluminum foil plate, and the eluent is passed up the plate by capillary action. The analytes are applicated onto a disposable separation plate in small spots, about 10 µl, and the solvent in the sample is allowed to evaporate. The plate is positioned vertically in a container containing a small volume of eluent. As the eluent starts to ascend along the plate's surface due to capillary action, the analytes are transported with it, each based on their characteristic retention behavior. The TLC process is concluded when the eluent has reached approximately 80% of the total plate height. At this point, the sample zones are ready for analysis. (Suomi, 2009, 154-155.)

There are various techniques available for visualizing compounds on a plate. Colored compounds are directly visible and UV-active compounds can be detected by illuminating the plate with UV light, typically at a wavelength of 254 nm. (Mikkola, 2006,19.) Often, the thin-layer stationary phase contains a fluorescent compound. On a thin plate, compounds quench the fluorescence and appear as dark spots. (Jaarinen & Niiranen, 2005, 150.) A known reference

compound must be analyzed on the same plate to identify unknown compounds. Compounds that travel the same distance under the same conditions have similar properties and can be considered the same compound. Retention factors (Rf) can be calculated from the TLC plate for different compounds. (Mikkola, 2006,19.)

The retention factor (Rf) is the value indicating how far the compound has moved relative to the solvent in the chromatographic system. Rf value is calculated by dividing the distance traveled by the compound by the distance traveled by the eluent. The distance traveled by the compound is measured from the top of the spot. (Teledyne Isco, 2018, 6).

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent front}}$$

3.2.1 Using TLC for optimization of purification by automated flash chromatography

TLC can be used to determine the optimal stationary phase or mobile phase composition when performing separation for a specific reaction mixture using automated flash chromatography. According to the equipment manufacturer the optimal Rf for the target compound is Rf=0,25 ± 0,05 when using the CombiFlash EzPrep and the distance to other undesired products should be at least ∆Rf=0,2 for optimal separation. (Teledyne Isco, 2018, 11.)

In flash chromatography, the solvent is pumped through a stationary phase in a column. Instead of time or relative distances, the volume of solvent required to move a compound through the column is determined. This volume is expressed as **column volume (CV).** The number of column volumes required to elute a compound from a flash column is proportional to the reciprocal of the compound's Rf value. In addition, the elution of compounds can be expressed

as a function of time. Flash chromatography provides optimal conditions for compound separation when the target compound elutes in 3-6 CV and the difference with other compounds' ΔCV is greater than 1. The above values can be calculated using the following formulae. (Jandera, 2006; Kręcisz et al, 2022;Teledyne Isco, 2018,9.)

CV=1/Rf Rf= 1/CV

$\Delta CV = CV_b - CV_a$

$\Delta Rf = Rf_b - Rf_a$

An example of the use of TLC as a separation optimization tool is shown in Figure 3. There is separation achieved between the target compound and the impurity. However, with the eluent and stationary phase used, an optimal separation between the target compound and the main impurity of the reaction mixture cannot be achieved by isocratic eluting.

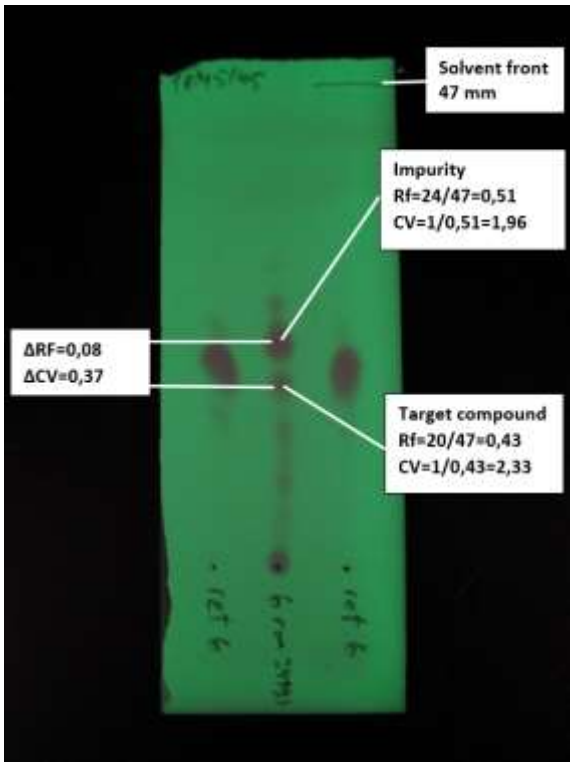

Figure 4. Illustration of TLC plate and its use in separation optimization.

It is practical to experiment with the separation of compounds using different stationary phases with TLC plates. TLC plates are available with the same types of stationary phases as chromatography columns. When determining the appropriate eluent for a separation, it is essential to use a TLC plate with the same stationary phase as the chromatography column (Krecisz et al., 2022).

It should be noted that the theory presented above is directly applicable only in cases of isocratic flash chromatography runs, where the ratio of polar and non-polar components in the eluent remains constant throughout the run. Additionally, the solvent in which the sample is initially dissolved can influence elution times in flash chromatography. Unlike TLC plates, it may not always be feasible to evaporate the sample solvent before conducting a flash chromatography run. (Krecisz et al., 2022; Jandera, 2006.)

TLC is an important tool for verifying separated fractions after flash chromatography separation. Then a known reference compound is applicated to the same plate. In this case, it is irrelevant if a different eluent is used for TLC compared to flash chromatography. The eluent should be capable of effectively separating the desired compounds to a degree where the purity of the fractions is discernible. The crucial factor is that the same plate contains the reference compound for comparison with the compounds separated through flash chromatography.

3.3 Gradient elution in flash chromatography

Normal phase adsorption chromatography NPC is the oldest liquid chromatography mode. The first description of gradient elution theory was presented by American Chemist Dr. Lloyd R. Snyder in 1964. Czech analytical chemist Pavel Jandera developed the Snyder theory and derived a formula for model of the gradient elution (Kręcisz et al., 2022; Jandera 2006).

The mobile phase typically consists of a blend of two solvents: a weaker solvent A, and a stronger, more polar solvent, B. When using TLC or manual column chromatography, only isocratic elution is feasible. In isocratic elution, the ratio of

the two eluents remains the same throughout the run. However, when using chromatography equipment, it becomes possible to modify and regulate the solvent ratio during the procedure, allowing for the design of a more efficient separation. (Doland & Snyder, 2013; Teledyne Isco, 2018, 18).

Mobile phase techniques can be divided into four different techniques:

1. **Isocratic gradients:** The composition of the eluent remains the same for the entire elution period (Sravani, 2018.). Isocratic elution is presented in Figure 5.

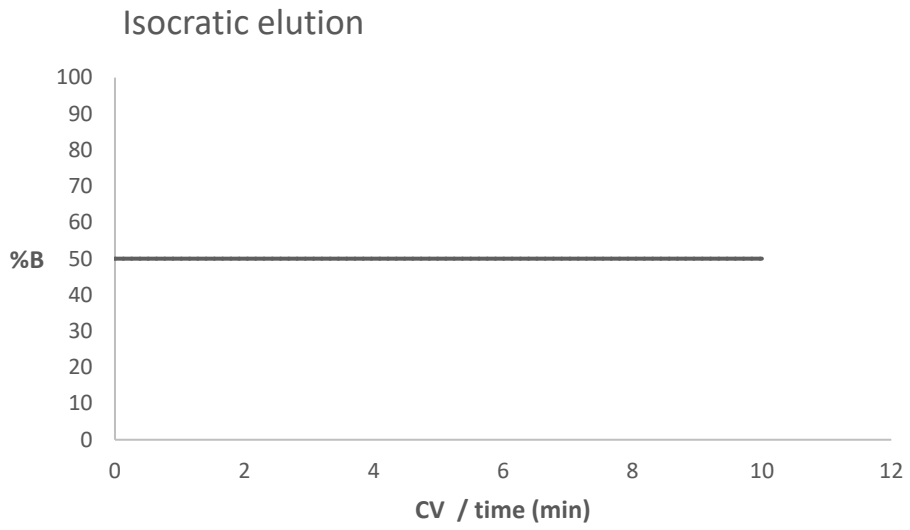

Figure 5. Illustration of isocratic elution.

2. **Linear gradients:** The proportion of stronger solvent is gradually increased over time in the eluent system (Sravani, 2018.) The slope of the linear gradient can be changed to vary the resolution between eluting peaks (Snyder et al, 2010, 408). Linear elution is presented in Figure 6.

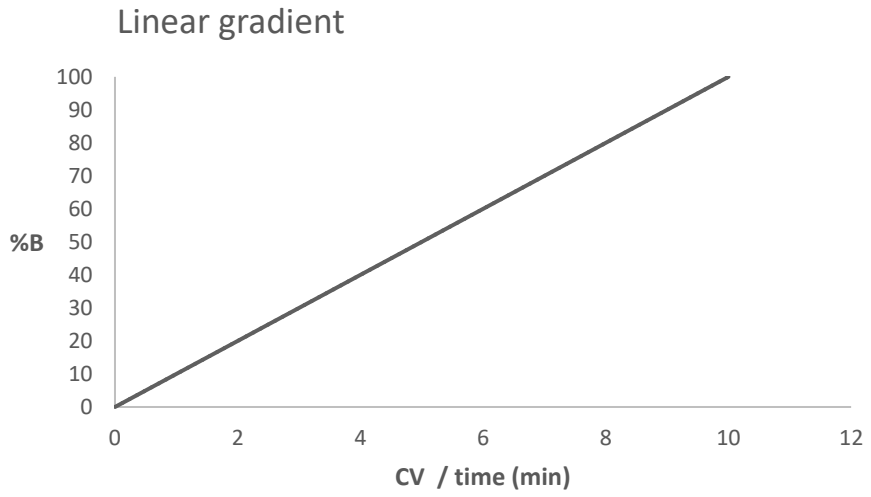

Figure 6. Illustration of linear gradient.

3. **Step gradients:** The strong solvent changes instantaneously to a higher concentration at the defined CV or time intervals (Snyder et al., 2018, 407-408).
4. **Mixed gradients:** These are a combination of linear gradients with isocratic holds or step gradient solvent changes (Teledyne Isco, 2018, 22).

The benefit of isocratic elution is its simplicity. Isocratic separation can be achieved using manual column chromatography without the need for specialized equipment. TLC is an isocratic technique, and the results obtained through TLC can be directly utilized in the development of isocratic separation applications in column chromatography. (Teledyne Isco 2018, 15)

Gradient methods include both stepwise and continuous changes in the solvent blend. A binary gradient is one in which the ratio of two solvents varies during the separation. Ternary (3-solvent) and quaternary (4-solvent) gradients are also used in some cases. The use of gradients allows the separation of closely eluting compounds. In some cases, the retention time of a compound can be reduced so that the desired product can be obtained with a much shorter time and less solvent consumption. The benefits that can be achieved by using a

gradient elution are shorter elution time, higher purity, fewer fractions to deal with, and greater sample loading capacity. (Teledyne Isco, 2018, 18-19).

In step gradient, the solvent strength can be increased after the previous compound has eluted. This is a way of shortening run times and improving column capacity (Teledyne Isco, 2018, 19).

TLC can be used to determine the optimum solvent composition for the separation of compounds in an isocratic elution. TLC is also a useful tool for step gradient design, as the optimal solvent blend for each compound can be investigated. For the optimization of a linear gradient, the results obtained by TLC are more difficult to apply. (Teledyne Isco, 2018,20).

By decreasing the slope of the linear gradient, the separation of the peaks and the broadness of the peaks increase. Finding the optimal linear gradient can be done through trial and error. A good starting point is to begin with a gradient that extends over ten CVs. (Teledyne Isco, 2018, 22).

Several formulas have been developed to calculate the optimal gradient elution (Fornstedt et al., 2017; Kręcisz et al., 2022; Jandera 2006.) Nevertheless, variables related to the available equipment, its specific characteristics, and the way the sample is loaded introduce factors that are not accounted for in these mathematical models.

CombiFlash EzPrep system includes software for gradient optimization. The compound mixture is initially assessed using TLC with two different solvent blends. The retention factors of the compound of interest and the closest impurity are entered into the software. The software then calculates the optimal combination of a linear gradient and isocratic hold before elution, aiming to achieve the most effective separation. (Teledyne Isco, 2018, 23).

However, the software is not able to consider all the properties of the compound mixture or the effect of the solvent used to load the sample. The interactions of compounds between the solid phase and the mobile phase do not always conform to the mathematical model. Furthermore, the compound mixtures and

purification needs are different. Nevertheless, software and mathematical models should be used to optimize the purification method. These tools can offer indicative information and save time and materials spent on experimentation (Fornstedt et al., 2017).

3.4 Automated flash chromatography Combi Flash EzPrep

The CombiFlash EZ Prep system from Teledyne ISCO is a dual function purification system that enables the use of flash and preparative HPLC modes (Teledyne Isco, n.d.). This thesis focuses on the use of the flash function.

Figure 6 shows the main components and functions of the Combi Flash EzPrep.

1. The main components of the system are the tubing, pumps, UV detector and touch screen control, and monitoring panel.
2. A silica-containing sample cartridge or pre-column, into which the reaction mixture to be purified can be absorbed in a suitable solvent before the separation run.
3. A chromatography column containing silica, where the separation of different compounds occurs during the run.
4. The solvents used as eluents and their inlet lines. Eluent A is less polar, while eluent B is more polar. By changing the ratio of these solvents (gradient), the retention times of different compounds in the column can be influenced. Lines C and D can be used, for example, for column storage solvent or for rinsing the lines with the solvent used after the operation.
5. The fraction collector can be set to collect all fractions or only separate compound peaks detected at the chosen wavelength by the detector, separating fractions into glass tubes. The eluent that is not collected is diverted to the waste container.

Figure 7. Combi Flash EzPrep equipment.

4 Used Eluents, their hazards, and hazard mitigation

The selection of the eluent solvents used in this work was made based on previous experiments. A Bachelor's thesis on optimizing the same equipment has been done previously by Rene Julin, 2018. According to Julin's testing, the chosen eluent works effectively and is not among the most hazardous options from a health and environmental perspective. As the non-polar solvent, heptane is utilized, while the polar solvent of choice is ethyl acetate. Julin’s experiments were related to different intermediate products of the chelate synthesis. Consequently, eluent compositions could not be directly applied to the purification of compounds 5 and 6.

In the manual method, petroleum ether is used as the polar solvent, which may have carcinogenic properties. Dichloromethane is used for sample dissolution before the purification process. In addition, the purification of compound 6 required the use of triethylamine (TEA) as a mobile phase modifier. The following paragraphs discuss the health and environmental hazards of substances used in chromatography and the possibilities for reducing these hazards.

4.1 Health hazards of organic solvents and how to reduce them

Organic solvents are liquid compounds, and most of them are highly volatile at room temperature. Solvents are readily absorbed into the body because they are fat-soluble. Exposure to solvents primarily occurs through the inhalation of solvent vapors, but they can also be readily absorbed through intact, healthy skin. Additionally, many solvents are flammable and combustible liquids, which can, under certain conditions, pose a fire and explosion hazard. (Työterveyslaitos.)

Solvents can have both immediate and long-term health effects. Solvent vapors can irritate the nasal and throat mucous membranes, respiratory tract, and eye conjunctiva. Rapidly occurring neurological symptoms from inhaling vapors

include dizziness, headaches, nausea, fatigue, and a feeling of intoxication. In addition, splashes can damage the eyes, and handling liquids can dry and irritate the skin. Sudden, usually accidental, exposure to extremely high solvent vapor concentrations can even lead to unconsciousness and life-threatening situations. Typically, short-term exposure symptoms are transient. Long-term exposure can result in irreversible conditions such as cancer. (Työterveyslaitos.)

Solvents must be minimized using all available means Primary efforts should focus on avoiding the use of solvents and other harmful substances whenever possible. If the use of these substances cannot be avoided, efforts should be made to utilize substances that pose the least harm to health. Whenever possible, modern techniques should be adopted to reduce the use of harmful substances or transition to work practices that entail less risk. When working with solvents, attention should be given to ventilation, and local exhaust systems, such as fume hoods, should be used. The handling of substances should involve the use of appropriate gloves and safety goggles. When transferring chemicals from original containers to others, the new container should be clearly labeled with the chemical's name and hazard warnings. In addition, safety data sheets must always be available. Proper and fire-safe storage of solvents should be given special attention (Työterveyslaitos.)

4.2 The information and properties of chemicals used for the purification of compounds 5 and 6

The American Chemical Abstracts Service (CAS) maintains a registry that compiles chemical identifications of substances. Each identification included in the registry is assigned a CAS Registry Name and a CAS Registry Number. CAS Registry Numbers are used worldwide. In addition, hazard statements in chemical safety data sheets are harmonized and recognized internationally. However, hazard statements may vary for different chemicals, due to the legislation of different countries. As more information on the harmfulness of

chemicals becomes available, this information will be updated. (Sosiaali- ja terveysministeriö, 2020.)

Table 1 compiles information on the solvents and other reagents used in the purification of compounds 5 and 6. Petroleum ether is not used in purifications performed with CombiFlash EzPrep but is utilized in purifications conducted using the manual method. Solvent and reagent properties are sourced from current safety data sheets and polarity information is from Biotage, 2018. Table 2 summarizes the relevant hazard statements.

Table 1. The properties of the solvents and other reagents used in the purification of compounds 5 and 6.

Solvent / Reagent CAS-number	**Structure Polarity**	**Molecular weight g/mol**	**Boiling point (°C)**	**Hazard statements**	**Carcinogenicity**
Dichloromethane 75-09-2	CH_2Cl_2 0,32	84,93	39,8	H315 H319 H351 H336	Suspected of causing cancer. H351
Ethyl Acetate EtOAc 141-78-6	$C_4H_8O_2$ 0,43	88.11	77,1	H225 H319 H336	N/A
Heptane 142-82-5	$H_3C(CH_2)_5CH_3$ 0,01	100,2	98,4	H225 H304 H315 H336 H410	N/A
Triethylamine TEA 121-44-8	$(C_2H_5)_3N$ N/A	101,19	88,8	H225 H302 H311 H314 H318 H331 H335	N/A
Petroleum ether PE* 8032-32-4	N/A	N/A	30-60	H225 H315 H336 H304 H411	May cause genetic damage. May cause cancer.

*PE is a mixture of liquid aliphatic hydrocarbons. It does not have a known chemical formula, molecular weight, or precise boiling point. In the safety data sheet, PE did not include any hazard statements indicating carcinogenicity. However, in the 'Toxicological Information' section, it is noted that it “May cause genetic damage” and “May cause cancer”.

Table 2. Essential hazard statements in accordance with the CLP Regulation (EU No. 1272/2008).

H225	Highly flammable liquid and vapour
H302	Harmful if swallowed
H304	May be fatal if swallowed and enters airways
H311	Toxic in contact with skin
H314	Causes severe skin burns and eye damage
H315	Causes skin irritation
H318	Causes serious eye damage
H319	Causes serious eye irritation
H331	Toxic if inhaled
H335	May cause respiratory irritation
H336	May cause drowsiness or dizziness
H350	May cause cancer
H350i	May cause cancer by inhalation
H351	Suspected of causing cancer
H410	Very toxic to aquatic life with long lasting effects
H411	Toxic to aquatic life with long lasting effects

4.3 Environmental footprint of flash chromatography operations and how to reduce it

Liquid chromatography is a method widely used in science and the pharmaceutical and diagnostic industry for analyzing and separating compounds. The method has a relatively large environmental footprint due to the solvents and reagents used and the waste generated from them. (McClain et al.,2016.)

In flash chromatography, the solvents and the silica used as the solid phase typically become waste after a single use. This practice exemplifies the adage that 'to make a small amount of something pure, we often generate a large

amount of something else as waste.' Typically, using liters of solvents and kilograms of silica yields only milligrams of the desired compound. (McClain et al.,2016.)

When aiming to reduce the harmful environmental impacts of flash chromatography, a key focus is to use solvents and reagents that are less harmful. Processes should be optimized to minimize the consumption of solvents and other harmful reagents and at the same time as little waste as possible is generated. Proper disposal of waste is essential. (Kannaiah et al., 2021.)

5 Optimization of purification methods using automated flash chromatography

5.1 Optimization of compound 5 purification

For optimizing the purification of Compound 5, two batches of RM5 (24190 and 24258) were prepared, which were manufactured for research purposes only. After optimizations, the production batch (24483) was synthesized, and the purification of Compounds 5 and 6 during the chelate synthesis process was performed using the newly optimized methods established in this work, utilizing CombiFlash EzPrep instead of manual techniques.

The separation of the RM 5 compounds using different eluents was assessed through TLC plates. Based on prior research, the selected eluents were defined as heptane and ethyl acetate, with dichloromethane used for sample preparation (Julin, 2020, 19). Consequently, testing was primarily focused on determining the optimal concentration of the more polar eluent for achieving the best separation on the TLC plate. As a point of comparison, PE was tested in place of heptane. PE is used in the manual purification method. However, when PE was used, the compounds migrated further on the TLC plate, resulting in no significant improvement in separation. The best separation on the TLC plate was achieved with a 20:80 EtOAc/Heptane ratio.

Optimizing the solid phase was unnecessary for purifying this compound because it was known in advance that there was no justified alternative to the manufacturer's normal phase silica Redisep Gold column, which has a particle size of 20-40 µm. In the manual method, a silica with a particle size of 40-63 µm was used. Generally, a smaller particle size allows for better separation, as it provides more surface area for compounds to adhere to. However, a smaller particle size also generates more backpressure compared to a larger particle size. Backpressure induced by smaller particle sizes is easier to control in purifications performed with the equipment than in the manual method. (Teledyne Isco, 2018, 28-29.)

The optimization of purification using the CombiFlash EzPrep system commenced with a small quantity of approximately 1,2 g of RM 5 and a 40-gram column. RediSep prepacked disposable sample load cartridges were used for sample loading, into which the dissolved sample was adsorbed. No other alternative methods for sample loading were necessary to be tested because this was a practical way to load sample and previous experiments had obtained the best results with this sample loading method (Julin, 2020, 22-24). In the first runs, a 12g silica containing sample cartridge was used and the sample was dissolved in approximately 5 ml of DCM. Following sample absorption, dichloromethane was evaporated from the sample cartridge under a nitrogen flow for a minimum of 30 minutes. Subsequently, the impact of dichloromethane on compound separation was examined, and the evaporation step of dichloromethane was omitted. ‘

Optimizing the gradient was conducted through five runs, using a 40 g column and approximately 1.2 g of the sample. An isocratic gradient of 20:80 EtOAc: Heptane was employed, with a linear increase in EtOAc from 0% to 20% within 1 minute. The EtOAc proportion was raised to 100% at the end of the run to ensure complete elution from the column. This gradient successfully separated the starting material, product, and impurity.

Subsequently, a larger-scale experiment was conducted, purifying approximately 6 g of the sample using a 25 g silica sample cartridge and a 120 g column. Initially, dissolving the 6 g of the sample required 25 ml of DCM, but the 25 g sample cartridge couldn't accommodate this volume, leading to the use of a 65 g silica sample cartridge. An attempt to dissolve the sample in EtOAc was made, but this polar solvent negatively impacted compound separation.

Following this, scaling up the purification to production scale was attempted. However, as the sample size increased, unreacted starting material began to precipitate during purification, causing elevated pressure in the CombiFlash Ezprep and potential clogging. To mitigate the precipitation, the polar solvent ratio in the isocratic phase was increased to 30:70 EtOAC: Heptane. Despite this adjustment, the risk of system clogging remained, requiring the division of

crude, unpurified production batches into two parts and separate purification runs to manage the sample size. Changing the flow rate from 85 ml/min to 100 ml/min did not impact compound separation or starting material precipitation.

After optimization, the CombiFlash Ezprep was used for purifying Compound 5 in the production batch (24483). For this process, a planned nonconformity was prepared to describe the equipment's usage instead of the manual method. Compound 5 could not be efficiently separated as planned by splitting the batch into two portions and conducting two purification runs. Although the RM5 had undergone acetonitrile removal by adding DCM and evaporating it, the presence of acetonitrile resulted in inadequacy separation. Additionally, a higher quantity of compounds to be separated resulted in broader peaks with partial overlap.

Purification runs were performed for both portions of the batch. Due to inadequate separation in these runs, fractions containing compound 5 were collected, and subjected to re-purification. In the re-purification step, it was possible to use an isocratic ratio of 20:80 EtOAc: Heptane, which improved the separation significantly. There was only a small amount of unreacted starting material present, and there were no issues with material precipitation or equipment clogging.

The optimization process for purifying compound 5 is presented in Figure 8. The results of the optimization are presented in section 4.

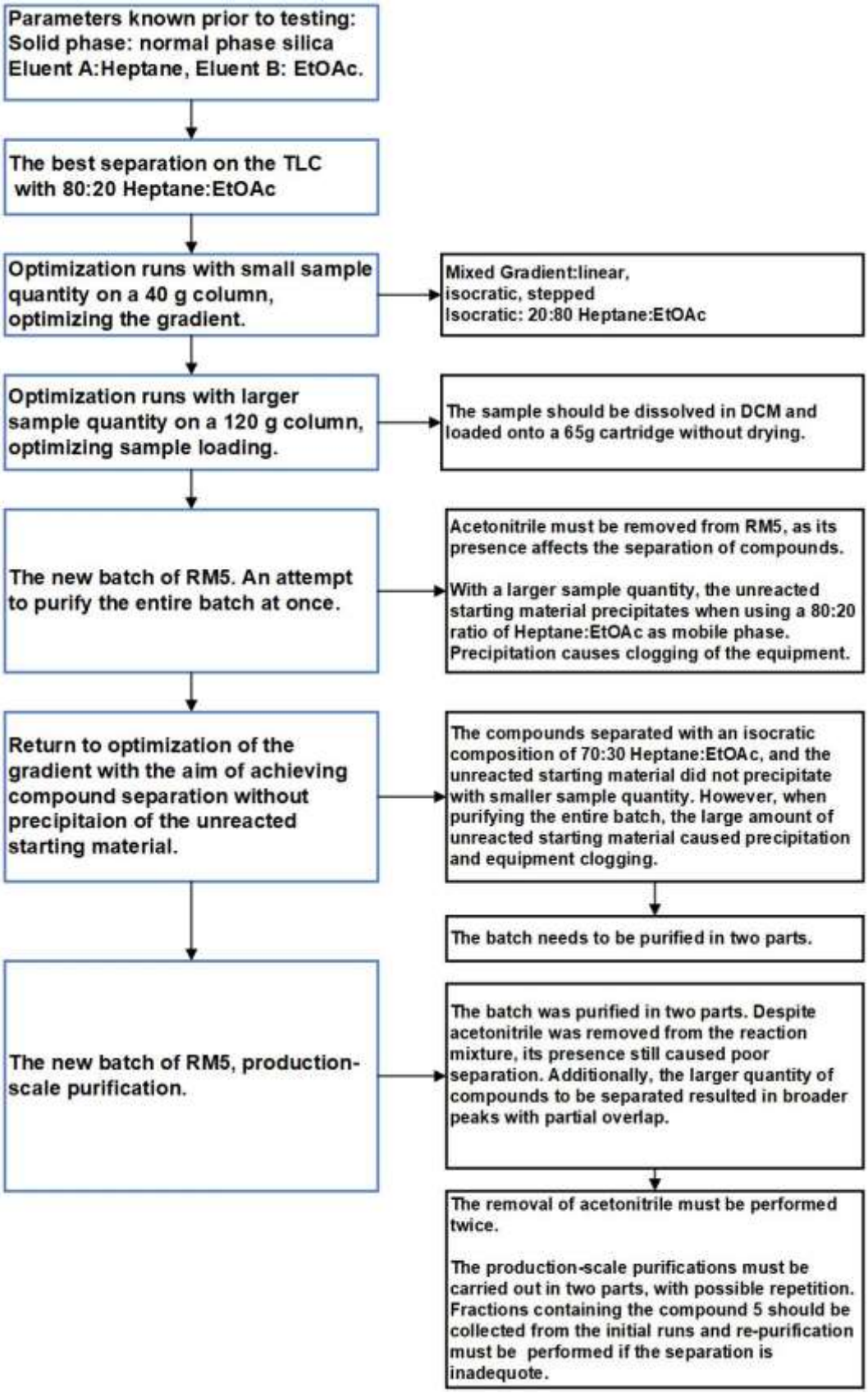

Figure 8. Optimization of compound 5 purification.

5.2 Optimization of compound 6 purification

In the optimizations, batches 24190 and 24258 of RM 6 were used. The purification of compound 6 was more challenging due to the presence of free amino groups in the compound. These amino groups tend to adsorb to NPC (normal phase chromatography) silica strongly. In the manual purification method, NPC silica was used as the solid phase, and the eluent required the addition of 20% TEA as a mobile phase modifier to deactivate the silanol groups on the solid phase. TEA is a potent base that prevents basic compounds' binding to silanol groups on silica. However, TEA is toxic and harmful to health, therefore the optimization of the purification process aimed at avoiding its use.

5.2.1 Experiments using amino-functionalized silica as the solid phase

The aim was to optimize the purification of compound 6 using amine functionalized silica as the solid phase. Amine functionalized silica features amine groups covalently bonded to its surface, effectively eliminating the need for TEA as a mobile phase modifier, which is typically required when dealing with acidic silanol groups on regular silica (Teledyne Isco, 2018, 19). Compound 6 is an organic amine, and when using normal-phase silica, the separation process involves the presence of a competing amine in the solvent system. Using amine functionalized silica eliminates or at least improves the solute-sorbent mass transfer kinetics and removes the need to add a competing amine (Biotage, 2018).

The optimization began by experimenting with the separation of RM6 compounds using different eluents on a TLC plate with amine functionalized silica as a solid phase. The focus of eluent testing was to find the optimal ratio between EtOAc and heptane to achieve the best separation of RM6 compounds. Although proper separation of compound 6 and impurities was not observed on the TLC plate, the optimization continued with CombiFlash EzPrep, as it was possible that separation could be achieved using a gradient.

The Redisep Gold Amine 30g column manufactured by Teledyne Isco with a particle size of 20-40 µm was used. A prepacked cartridge was not available, so the empty cartridge was packed manually with amine-functionalised silica with a particle size of 40-75 µm. Experiments were performed with a small sample size, less than 300 mg. A minimal amount of dichloromethane was used to dissolve the sample. Loading of the sample was tested with and without the evaporation of dichloromethane from the sample cartridge. The presence of dichloromethane did not impact the separation of compounds. To optimize the purification of compound 6 with amino-functionalized silica, eight test runs were performed, testing different gradients. Regardless of adjustments to the conditions, sufficient separation was not achieved. It was also attempted to collect fractions containing compound 6 from the initial run and repeated the purification process for these fractions However, even using this method did not result in a sufficient separation between compound 6 and impurity.

5.2.2 Experiments using normal phase silica as the solid phase

When purifying compound 6 using amino-functionalized silica was determined to be unfeasible, optimization was continued using normal phase silica. The optimization process began by conducting experiments on TLC plates, testing various combinations of EtOAc and heptane as eluents, as well as different concentrations of TEA as a mobile phase modifier. Although optimal separation was not achieved on TLC plates, some separation between impurities and the product was achieved using 10% TEA in the ratio of 1:1 Heptane: EtOAc as mobile phase. Based on this result, optimization runs were conducted on the CombiFlash EzPrep using different gradients.

The sample materials remained in batches 24190 and 24258, which were recycled from one optimization run to another by combining fractions and evaporating the solvents. The mobile phase containing 10% TEA was used in 14 test runs, optimizing the gradient. The sample was dissolved in the minimum amount of DCM, and prepacked sample cartridges with normal phase silica were used. The evaporation of dichloromethane from the sample cartridge was

found to be unnecessary in this case as well. Additionally, an attempt was made to reduce the TEA concentration to 5%, resulting in even poorer separation.

Finally, it was decided to experiment with increasing the amount of TEA to 15% and then to 20%. The separation between compound 6 and impurities significantly improved with the increase in the concentration of TEA.

After optimization, the CombiFlash Ezprep was used for purifying compound 6 in the production batch. For this process, a planned nonconformity was prepared to describe the equipment's usage instead of the manual method. The purification runs proceeded as planned. It was known that the first purification run would not achieve adequate separation and would require repetition. Fractions containing compound 6 were collected from the first purification run and subjected to a second purification, achieving pure compound 6 with a high yield.

The optimization process for purifying compound 6 is presented in Figure 9. The results of the optimization are presented in section 4.

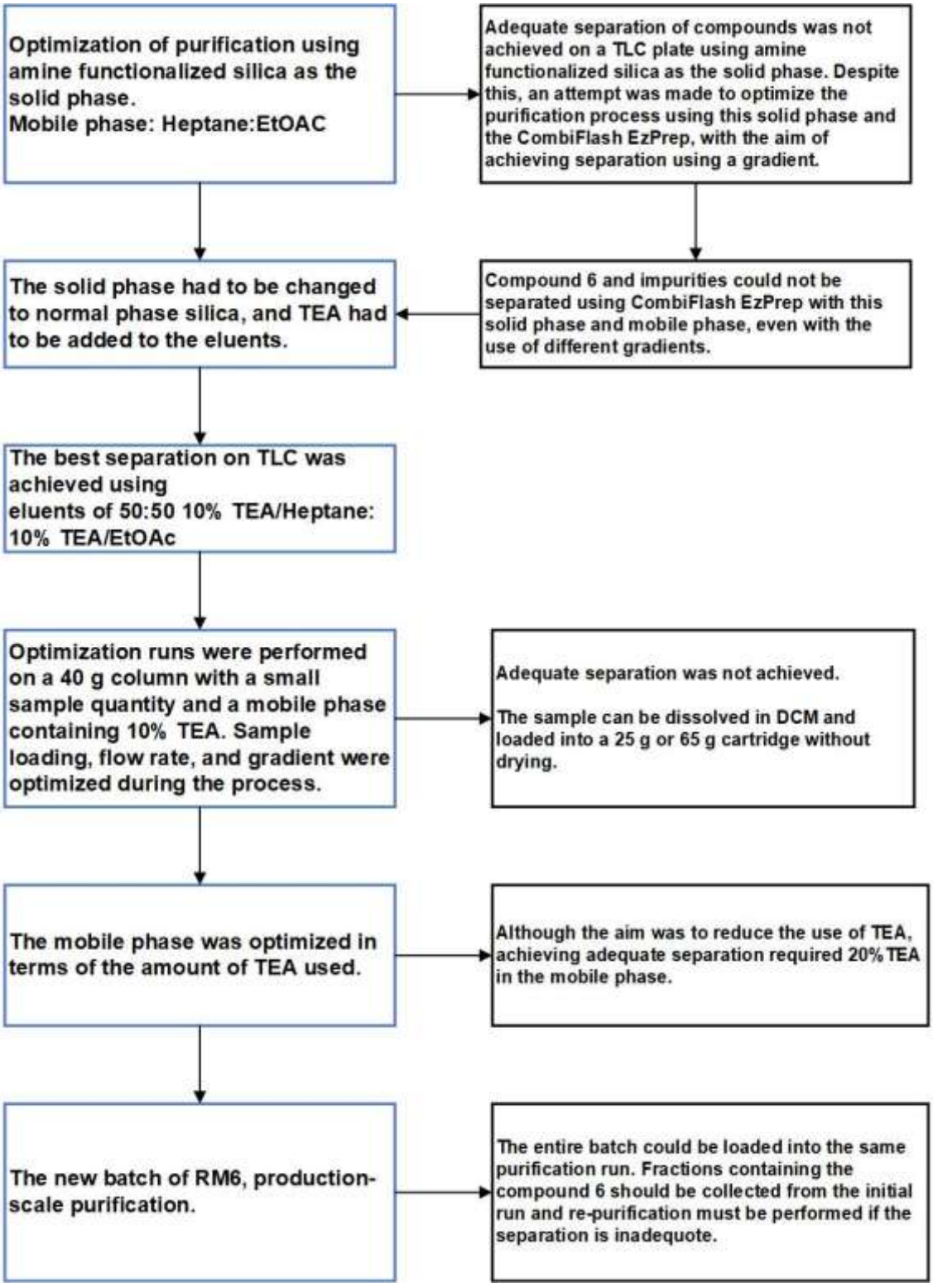

Figure 9. Optimization of compound 6 purification.

6 Results

6.1 Optimized Parameters for Purifying Compounds 5 and 6 with CombiFlash EzPrep

The optimized parameters for purifying compounds 5 and 6 are presented in Table 3. The specific details of the gradients used can be found in the chromatograms, figures 11, 13, 15, 18, and 20.

According to the manufacturer's information, NPC silica columns are disposable. However, during optimization, it was observed that the same column could be used in multiple purification runs without any problems. In production, the column can be used for purifications of the same compound during the same day.

Table 3. The optimized purification parameters for compounds 5 and 6.

Object of optimization	**Result in the purification of compound 5**	**Result in the purification of compound 6**
Sample cartridge	RediSep prepacked disposable sample load cartridge 65 g	RediSep prepacked disposable sample load cartridge 25 g or 65g
Loading amount	Half of the batch (< 8 g crude material)	The entire batch (~3-5g crude material)
Sample dissolving	~ 25 ml DCM	~ 14 ml DCM
Sample drying	No drying	No drying
Stationary phase Column	Normal Phase Silica Particle size: 20-40 µm spherical RediSep Gold 120g The same column can be used for the same purification phase during the same day.	Normal Phase Silica Particle size: 20-40 µm spherical RediSep Gold 120g The same column can be used for the same purification phase during the same day.
Column equilibration	With 100% eluent A: 3 CV if the column is dry and unused, and 1,5CV for the second run.	With 100% eluent A: 3 CV if the column is dry and unused, and 1,5CV for the second run.
Mobile phase Eluents	A: Heptane B: EtOAc	A: 20% TEA, 80% Heptane B:20% TEA, 80% EtOAc
Gradient	Mixed: linear, isocratic, stepped	Mixed: linear, isocratic stepped
Flow rate	100 ml/min	85 ml/min
Detection wavelengths	254 nm (red *) and 280nm (purple*) to detect the fractions to be collected	280 nm (purple*) to detect the fractions to be collected, 254nm (red*) for monitoring *Colors in chromtograms
Maximum fraction size	50 ml	50 ml

6.2 Results of Compound 5 production-scale purification with optimized parameters

Figure 10 shows a TLC plate used to predict RM 5 compounds separation. The stationary phase was normal phase silica, and the mobile phase was 70:30 heptane: EtOAc. The result on the TLC plate predicts that this combination of solid phase and mobile phase can effectively separate these compounds using the CombiFlash Ezprep with the same conditions.

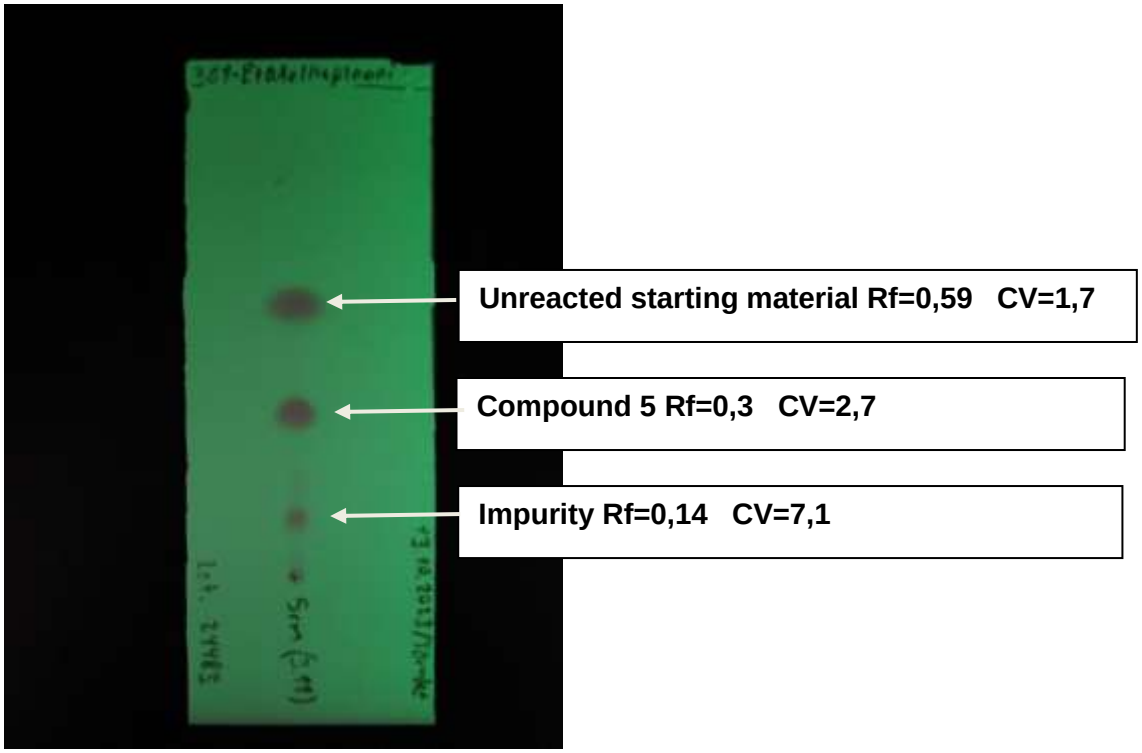

Figure 10. TLC for predicting compound 5 purification

When comparing the CV values calculated from the TLC plate in Figure 10 to the peak elution CV values in Figures 11 and 13, it becomes apparent that the tests conducted on the TLC plate provide only a rough estimate of compound separation. It should be highlighted that the gradients in the runs performed with the CombiFlash Ezprep are not entirely isocratic, as in the TLC plate separation, but rather start with 100% eluent A Heptane. There are several factors affect the elution of compounds on the CombiFlash Ezprep, such as the sample size, the use of sample cartridge for sample loading, the choice of

solvent for dissolving the sample, and the quantity of silica that compounds must pass in the sample cartridge before entering the column.

The purification of compound 5 was performed in three purification runs. Initially, the batch was split in half, and the halves were purified in runs A and B. All fractions containing the product were collected from these runs and subjected to re-purification run C.

Figure 11 displays the chromatogram from the first production run. The gradient used is indicated by the blue line, showing the percentage of eluent B EtOAc. However, the compounds did not separate as planned. A small amount of acetonitrile was left in the reaction mixture, affecting compound separation. Consequently, fractions containing compound 5 needed to undergo re-purification.

Figure 12 shows the TLC plates used for verifying the fractions obtained in run A. Fraction verification was conducted following the same guidelines as in the manual method. The references are located on both edges of the TLC plates The fractions are numbered on the upper edge of Figure 10's chromatogram and Figure 11's TLC plates. Fractions 3-6 contain pure unreacted starting material. Fractions 7-15 contain impure compound 5 and were chosen for the re-purification process.

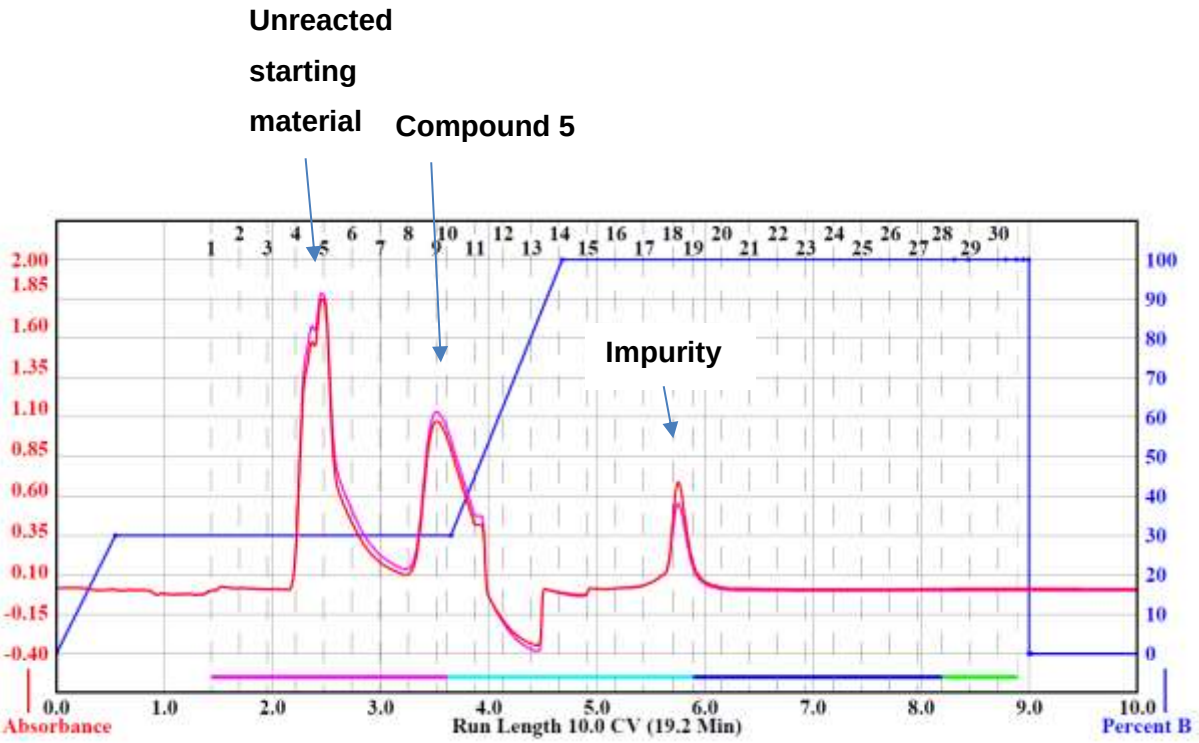

Figure 11. Chromatogram: Compound 5 production run A (First portion of the batch, single acetonitrile removal).

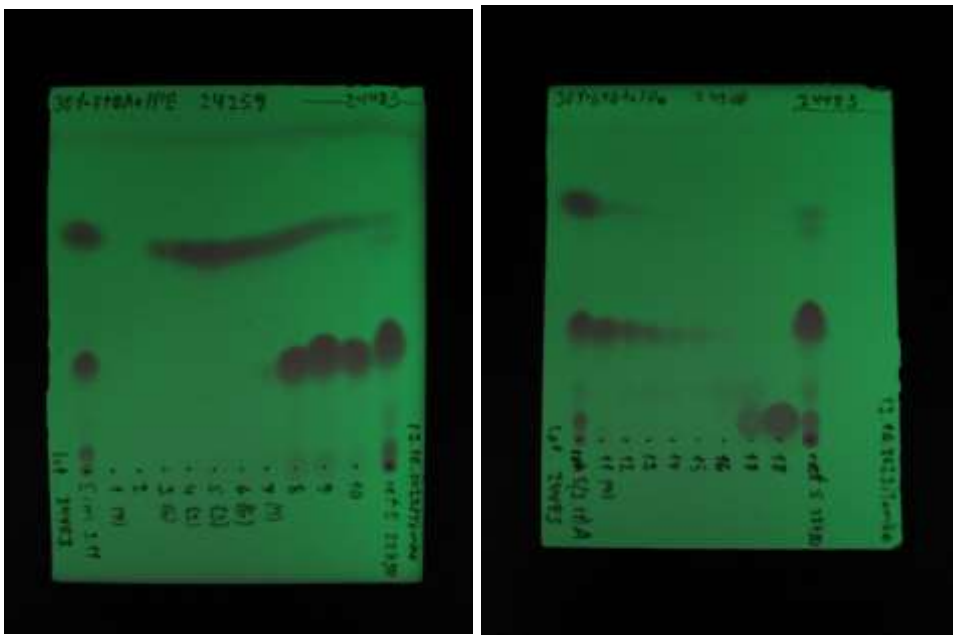

Figure 12. TLC verification of fractions from run A.

Figure 13 displays the results of the second production run. A double acetonitrile removal process was applied to the second half of the production batch, resulting in a slightly improved separation. However, optimal separation was not achieved between the unreacted starting material and compound 5. The peaks are broad and partly overlapping.

Figure 14 shows the TLC plates used for verifying the fractions obtained in run B. Fractions 1-3 contain pure unreacted starting material. Fractions 4-13 contain impure compound 5 and were chosen for the re-purification process.

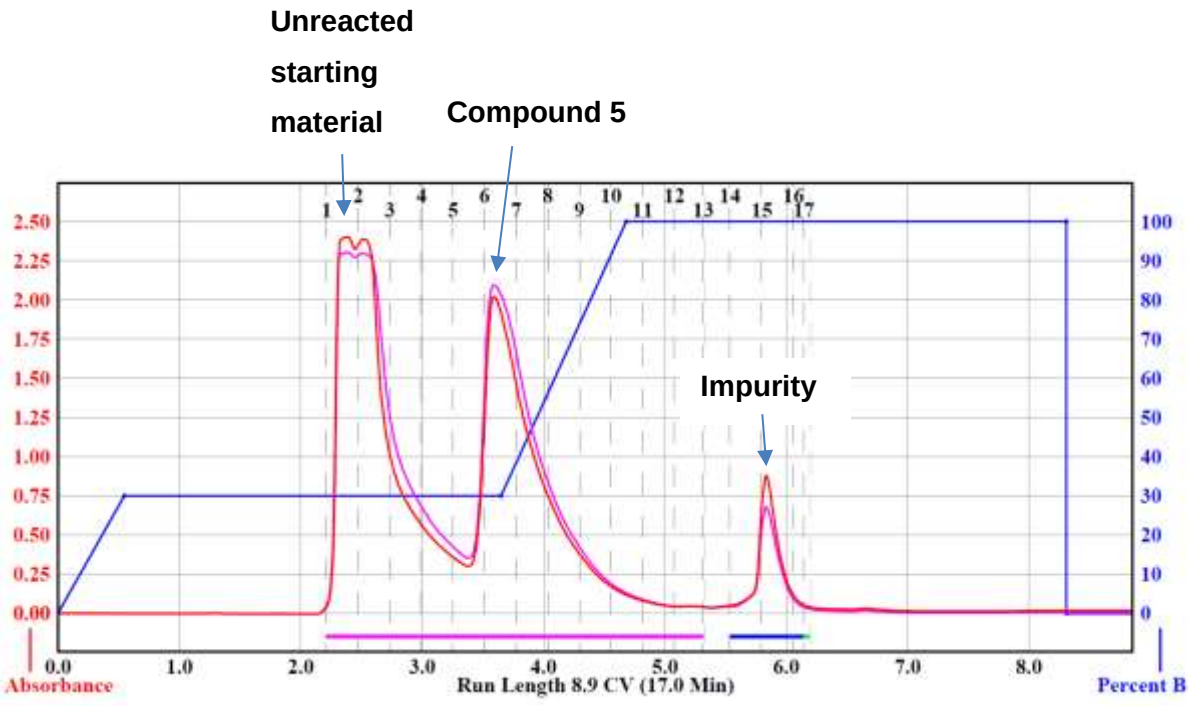

Figure 13. Chromatogram: Compound 5 production run B (second portion of the batch, double acetonitrile removal).

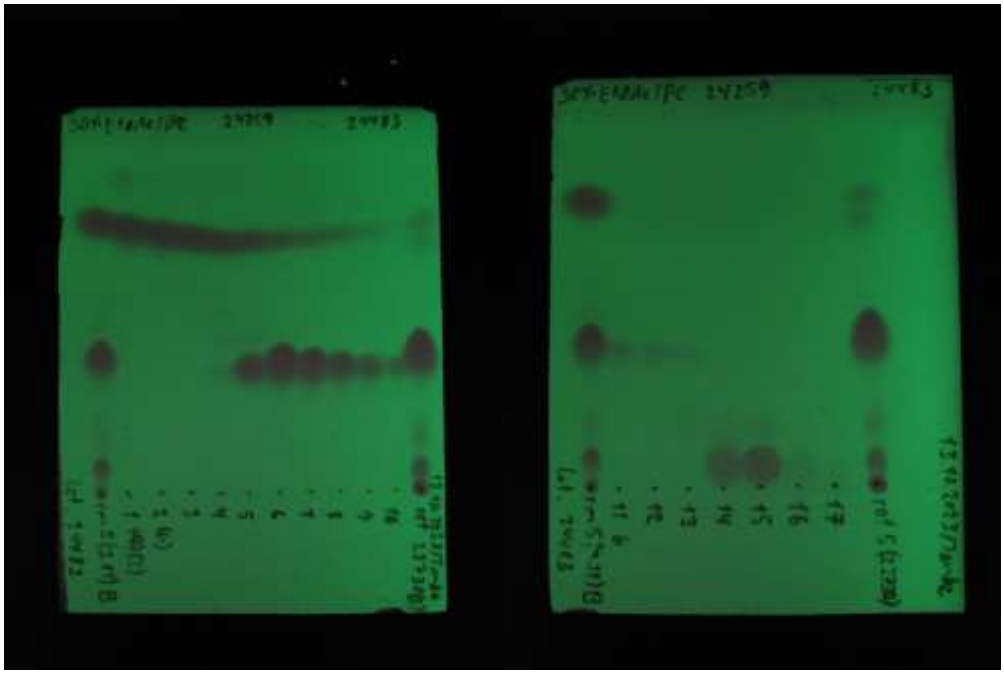

Figure 14. TLC verification of fractions from run B.

Figure 15 shows the chromatogram for run C. For this run, fractions from runs A and B containing compound 5 were combined. Since there was only a small amount of easily precipitable unreacted starting material in this run, the EtOAc ratio in the isocratic portion was reduced to 20%. This adjustment achieved optimal separation between the unreacted starting material and compound 5.

Figure 16 displays the TLC plate used for verifying the fractions obtained in run C. Fractions 1-5 contain pure unreacted starting material, and fractions 6-7 contain pure compound 5.

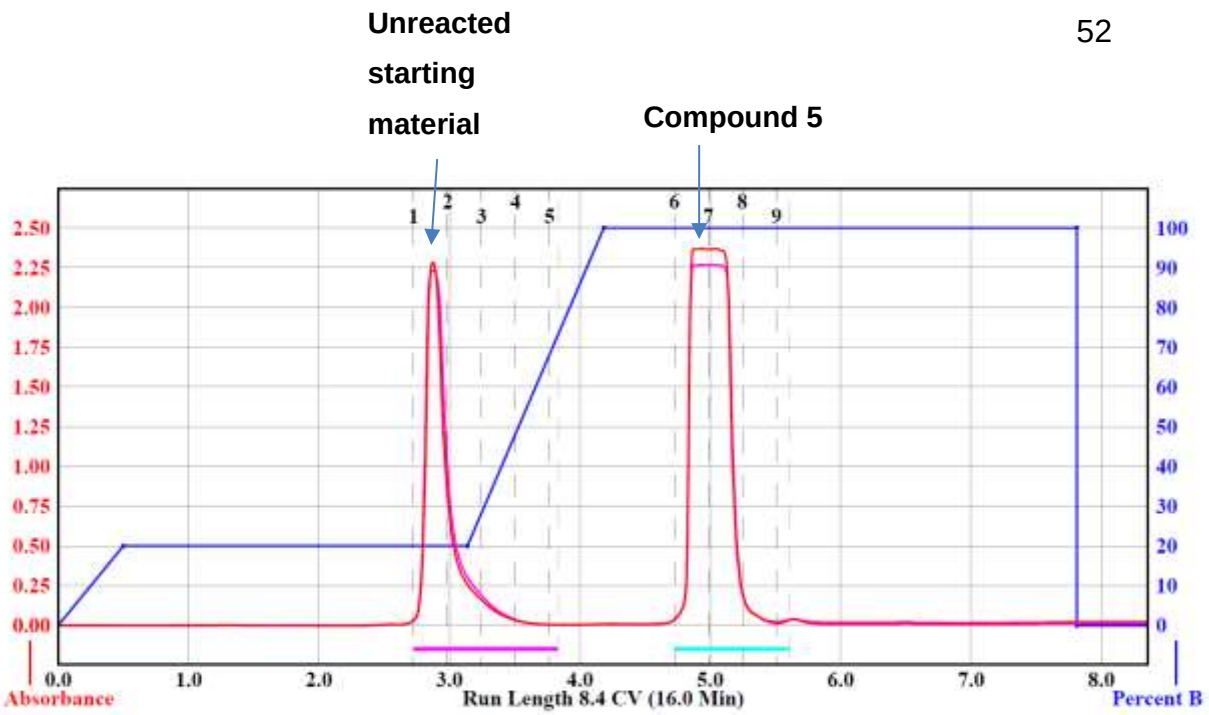

Figure 15. Chromatogram: Compound 5 production run C, re-purification.

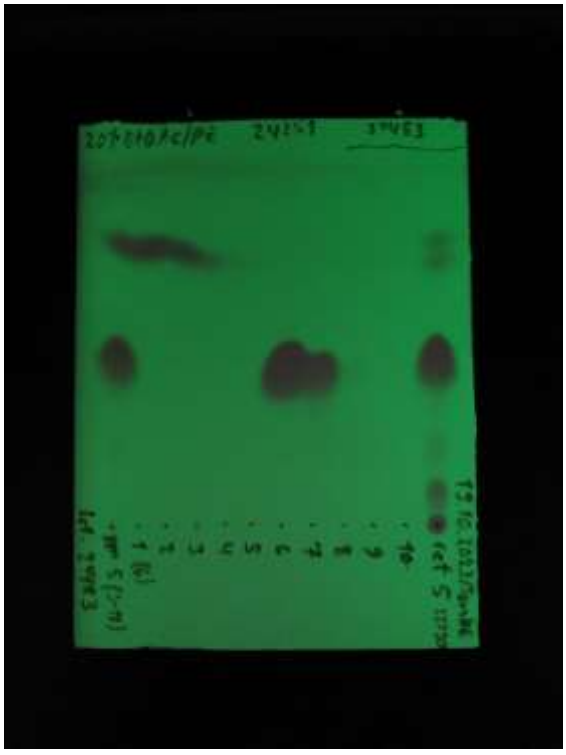

Figure 16. TLC verification of fractions from run C.

6.3 Results of Compound 6 production-scale purification with optimized parameters

Figure 17 shows a TLC plate used to predict the separation of RM6 compounds. There is normal phase silica as a solid phase, and 20% TEA in a 1:1 ratio of Heptane and EtOAc as the mobile phase. From the compound migration on the plate, it can be inferred that this solid phase and mobile phase combination allows for some separation between compound 6 and the impurity. However, the Rf value of compound 6 is significantly distant from the manufacturer's recommended optimal Rf value of 0.25 ± 5. Additionally, the difference between compound 6 and the impurity is not sufficient for optimal separation. This presents a challenging separation.

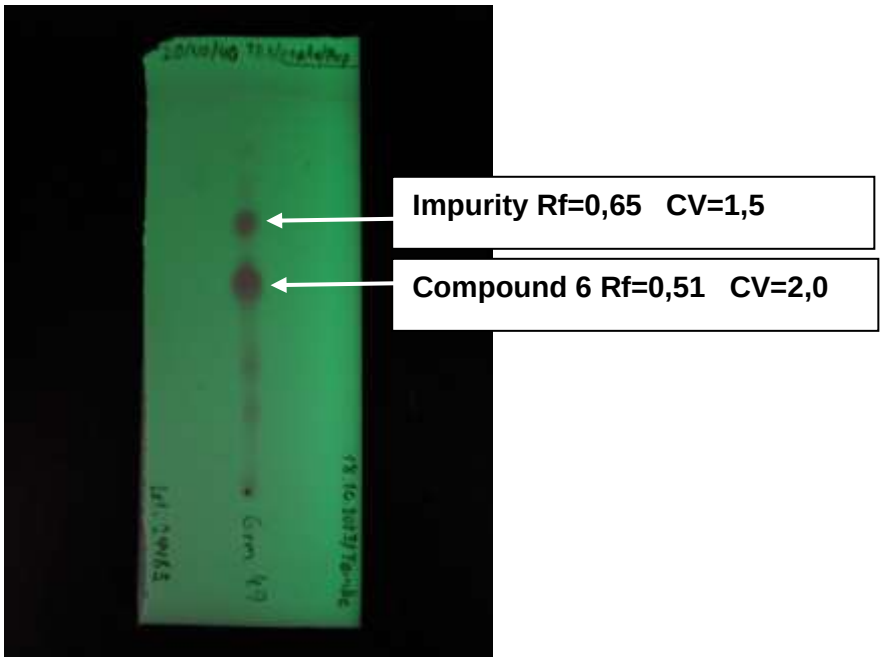

Figure 17. TLC for predicting compound 6 purification.

Figure 18 shows the chromatogram of the first production run. Based on the chromatogram, it seems that there was no separation between the impurity and compound 6. Figure 19 displays the TLC plate used for verifying the fractions. Upon verifying the fractions using TLC, it was found that fractions 7 and 8

contained pure compound 6. The impure fractions containing compound 6, fractions 6 and 9, were collected for re-purification.

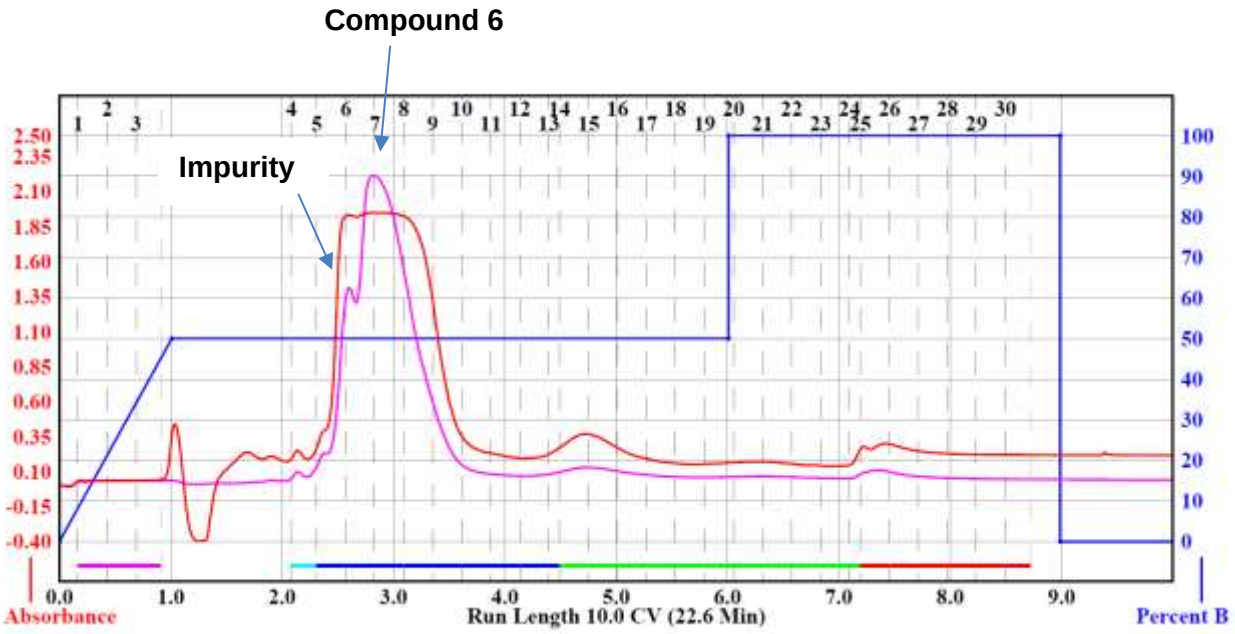

Figure 18. Chromatogram: Compound 6, the first production run which includes the entire batch.

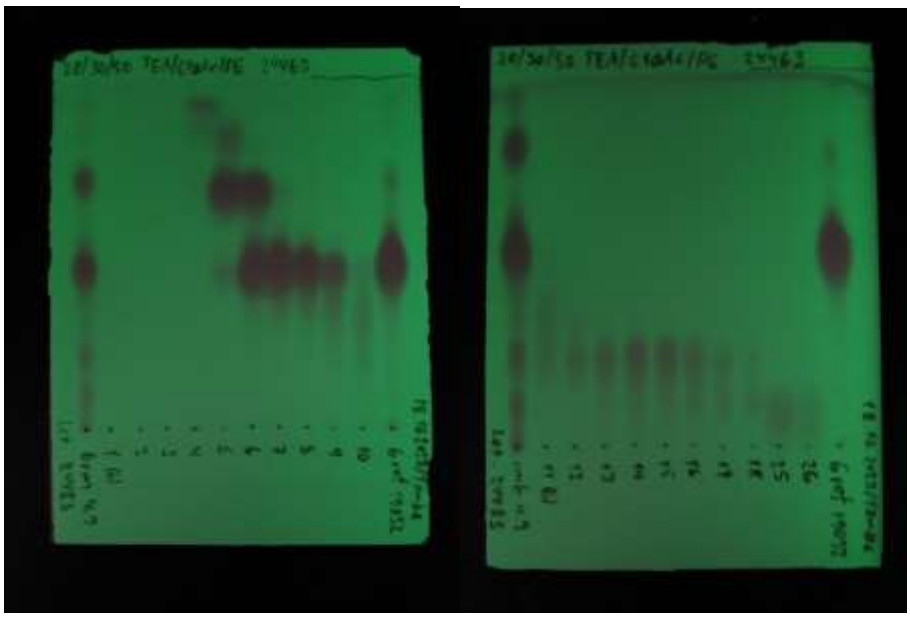

Figure 19. TLC verification of fractions from the first run.

Figure 20 presents the chromatogram of the second production run. In this run, impure fractions containing compound 6 were collected from the first run. Based on the chromatogram, it seems that optimal separation between the impurity and compound 6 was not achieved. A TLC analysis in Figure 21 reveals that fractions 4-6 contain pure compound 6. This purification method allowed for a high yield of pure compound 6. The yield % for different purification methods is presented in Table 4.

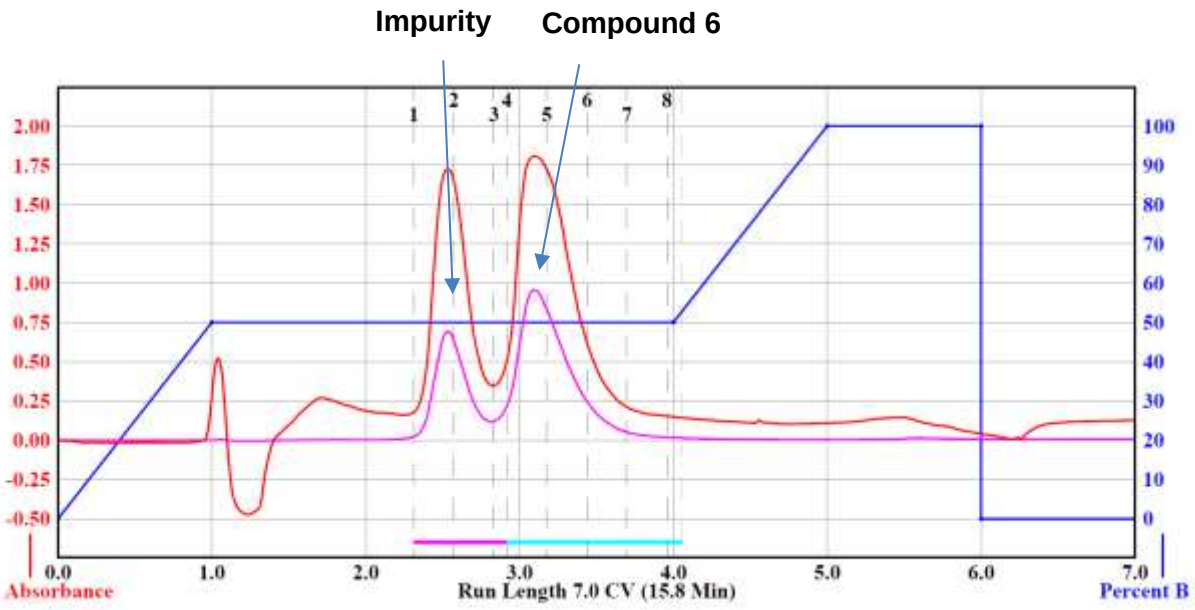

Figure 20. Chromatogram: Compound 6, second production run, re-purification.

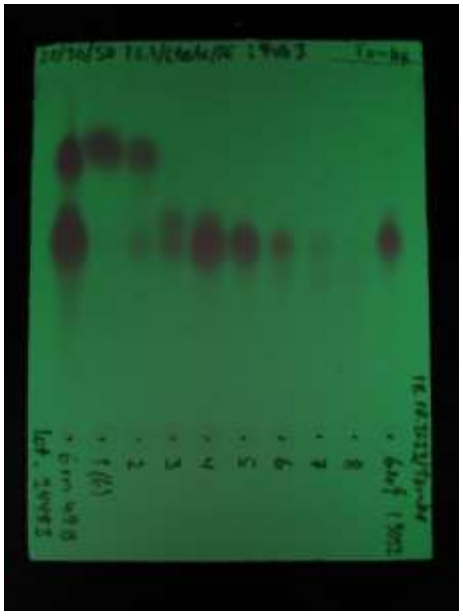

Figure 21. TLC verification of fractions from the second run.

6.4 Results concerning material consumption, environmental impact, health effects, working time, and yield

Table 4 presents a comparison of purification between the manual method and CombiFlash EzPrep in terms of the materials used, working time consumption, and yield achieved. The material consumption values presented in Table 4 are average values for the manual methods, calculated as the mean consumption of three different batches. Material consumption for purifications performed using CombiFlash Ezprep was calculated based on a batch purified for production use. Purifying compound 5 required three purification runs, while purifying compound 6 required two purification runs.

Table 4. Comparison of purification methods: Manual vs. CombiFlash Ezprep-materials, working time and yield %.

	Compound 5 Manual method	**Compound 5 CombiFlash Ezprep**	**Compound 6 Manual method**	**Compound 6 CombiFlash Ezprep**
Consumption of DCM (ml)	2000	80	-	30
Consumption of EtOAc (ml)	670	3000	1300	1600
Consumption of heptane (ml)	-	3000	-	2400
Consumption of PE (ml)	2700	-	2100	-
Consumption of TEA (ml)	-	-	870	1000
Consumption of solid phase, silica (g)	480	120 g column 3x 65 g cartridge	480	120 g column 2x 25 g cartridge
Total consumption of solvents and TEA (ml)	5370	6080	4270	5030
Yield % *	59 %	66 %	69 %	71 %
Working time (h)	7,5-15	4-6	7,5-15	3-5

*Yield % is calculated by dividing the measured yield by the theoretical yield, which is determined based on the quantities of the starting material and added reagents. The theoretical yield is calculated using the molecular weights of these substances to determine how much of the desired compound could theoretically be formed in the reaction. This calculation ensures that the yield % can be compared even when the amount of starting material varies in different batches. The yield % for manual methods presented in the table are based on the average of 16 batch yield % values, while the yield % achieved using CombiFlash Ezprep presented in the table are the results of production purifications that were performed as part of this optimization work.

The amount of solvents used could not be reduced by using CombiFlash Ezprep, but it was possible to eliminate the use of potentially carcinogenic PE. Most of the solvents used in the new methods are heptane and EtOAc. DCM is only used to dissolve the sample. Despite attempts, the use of TEA could not be eliminated. In the purification of compound 6 using the new method, TEA is consumed even slightly more than in the old manual method. Unfortunately, within the scope of this study, it was not possible to address the environmental impacts of the purifications. The amount of materials used and the resulting waste are the indicators of environmental impact in this work. To reduce the environmental impact, further optimization is required for these purifications.

However, the new methods performed with CombiFlash EzPrep offer improvements in terms of the health effects of solvents and reagents. Operators of the new methods are much less exposed to harmful substances during the purification process with the equipment than when performing the work manually. The equipment-based purification eliminates the need for manually filling a large glass column with silica or pouring solvents into the column. New methods eliminate the need for hours of monitoring eluting liquids and manually collecting fractions into containers. These factors are also of great importance for work ergonomics. When working with the CombiFlash Ezprep, there are fewer fractions to deal with. The equipment can be set to collect only the fractions containing the target compounds. In contrast, when using the manual method, all manually collected fractions must be verified using TLC.

Both the manual method and the use of the equipment are conducted in a fume hood, which reduces the risk of inhaling volatile solvents. However, the manual method involves many steps that involve handling hazardous substances. In the method using CombiFlash Ezprep, the eluents pass from closed bottles into the equipment and are collected directly in a closed waste container or through an automatic fraction collector. The use of CombiFlash Ezprep significantly reduces the active work time required for the process. With both the manual method and the method using CombiFlash Ezprep, it may be necessary to repeat the purification process to achieve sufficient purity. However, with the

equipment method, re-purifications can be completed within the same workday, while with the manual method, re-purification would require an additional day.

The new purification methods achieved at least equivalent yield % than the manual method, which is an important result for productivity. Concluding the consistency of purifications performed with the CombiFlash Ezprep based on a single batch purification is impossible. The information on yield % and purity becomes more precise after purifying several batches. Nevertheless, it can be assumed that purifications performed using the equipment will produce a more uniform outcome compared to the manual method.

7 Conclusions and discussion

Automated chromatography equipment has been available on the market for decades. Automated systems reduce the need for manual steps, improve efficiency, and decrease exposure to chemicals in chromatographic processes. Processes in the pharmaceutical and diagnostic industries are precisely defined and documented. Making changes to processes is laborious and time-consuming. There must be a convincing case for changing processes. Acquiring suitable equipment, optimizing methods, and implementing the equipment for production use require understanding and time, often necessitating separate resources.

In this study, a total of 29 optimization runs were conducted to enhance the purification process for compound 5 before the production runs. Similarly, 25 optimization runs were performed for the purification of compound 6 before the production phase. In addition, various eluent compositions were experimented using TLC. During these test runs, the solid phase, mobile phase and its gradient, sample handling and the loading method were optimized for the purification of these target compounds. Scaling to production scale was performed, and associated issues, such as precipitation problems with compound 5, were resolved. However, the desired reduction in solvent consumption could not be achieved as multiple purification runs were necessary.

It was clear that there are mathematical formulas available for gradient optimization, and the equipment itself has software to calculate a functional gradient based on TLC values. However, in practice, TLC only provided approximate information about the separation of compounds within the column. Gradient optimization had to be performed on the equipment through trial and error.

In conclusion, this study provided valuable insights into the equipment operation and efficient working practices. These findings can be applied when transitioning the purification of other intermediate products in chelate synthesis

from manual methods to the use of the CombiFlash EZPrep. In addition, experience was gained in documenting the change of purification method and the transfer of the new process to production use, which will facilitate the transition to new purification methods in the future.

The new methods successfully reduced active working time and manual handling, thus reducing health risks associated with solvents and reagents. Furthermore, the developed methods seemed to improve the yield compared with the manual methods. More precise information about the yield and product purity will be obtained when the equipment has been used to purify more batches. However, the objective of reducing environmental impact was not achieved. The consumption of solvents and other reagents, and consequently the generated waste, could not be reduced.

The synthesis of the labeling reagent in the organic chemistry laboratory involves several purification steps. The knowledge gained in this study forms a strong basis for transferring other purification steps from manual methods to the CombiFlash EZPrep. Future research may focus on optimizing and implementing additional purification steps using CombiFlash EZPrep.

The organic chemistry laboratory at RTKU has extensive experience in verifying compounds of chelate synthesis using TLC. Due to the type of the process and this expertise, TLC serves as a sufficient method for quality control of compounds and separations. If the precise structure of purified compounds is required, it could be analyzed using mass spectrometry. However, at the time of conducting the study, RTKU did not have access to mass spectrometry. If necessary to confirm the exact structure of the compounds, it is possible to analyze them using mass spectrometry in the future.

In contrast to the manual method, the equipment detects eluting compound peaks. The chromatogram obtained from the equipment provides important information about eluting compounds, eliminating the need to verify all fractions using TLC. Instead, it is sufficient to analyze compound peaks using TLC. If the separation method performed by the equipment results in distinct compound

peaks, and the method consistently operates, routine verification of compound peaks using TLC may be unnecessary. In challenging separations, where only partial separation is achieved, TLC remains an important tool for fraction verification.

While introducing an automated flash chromatography equipment for production use involves a considerable amount of effort and costs, using the equipment for purifications in the long term is more cost-effective than manual methods. Finally, the benefits achieved using automated system compared to manual methods are summarized as follows:

- High yield and purity. Additionally, the consistency of purifications is expected to improve. This could positively impact on the quality of the final product.
- The time required for the purification process could be halved. Re-purifications are possible to perform within the same day.
- Fewer fractions to handle. Significantly less need for TLC analyses.
- Less monotonous tasks when the need for manual collection of fractions is eliminated.
- Less handling of solvents and other chemicals and lower exposure risks.
- Better work ergonomics.
- The chromatograms produced by the equipment provide informative data about the separation of compounds. Improved data storage and traceability of purification steps.

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