The development of simple HPLC methods to separate methylene blue and its metabolites

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“The final aim of the dissertation should be none other than the glory of God”

~ Johann Sebastian Bach

J.J.

PREFACE

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ABSTRACT

Keywords: Methylene blue; Azure B; Alzheimer's disease; method development; High performance liquid chromatography; Validation.

The possible treatment and prevention of Alzheimer's disease (AD) is one of many clinical applications of methylene blue (MB) that has recently attracted much interest. Due to its ability to interact with various targets, MB exhibits multiple mechanisms by which the progression of neurodegenerative diseases may be decreased. Recently, it has been reported that azure B (AB), the major metabolite of MB, possesses superior effects at various pharmacological targets compared to MB. This finding raises the question that much of the documented pharmacological effects of MB observed in previous studies may in fact be due to the actions of AB.

The structural similarities between MB and its metabolites have made the analysis of these compounds very challenging. The published analytical methods for MB and its metabolites have significant disadvantages such as low sensitivity, uneconomically high costs, the need of professionally trained personnel and very expensive, high technology apparatus. These disadvantages delays and limits the research into MB as a drug for the treatment of AD and other neurodegenerative disorders, and also hampers investigations into the pharmacology of MB. In this study, simple and cost effective analytical methods were developed to analyse and separate MB and its metabolites. An accurate, sensitive and reliable high performance liquid chromatography (HPLC) method with which MB and its metabolites were successfully separated, was developed and fully validated. A Synergi Polar-RP column (150 x 4.6 mm, 4 µ, 80 Å) and a mobile phase composed of two parts: ammoniumacetate that is dissolved in a mixture of water and methanol (part A) and a mixture of acetonitrile and methanol (part B). The analysis were done on a Hitachi Chromaster chromatographic system. Also, successful normal phase and reverse phase thin layer chromatography (TLC) methods were developed as a crude method for accessing the purity of MB.

UITTREKSEL

Sleutelwoorde: Metileenblou; Azure B; Alzheimer se siekte; metode ontwikkeling; Hoë werksverrigitings vloeistofchromatografie; Validering.

Die moontlike behandeling en voorkoming van Alzheimer se siekte (AD) is een van baie kliniese toepassings van metileenblou (MB) en het onlangs baie belangstelling ontlok. MB werk deur verskeie meganismes om die verloop neurodegeneratiewe siektes te vertraag. In hierdie opsig ondergaan MB interaksie met verskeie reseptore en ensieme wat relevant is vir die behandeling van AD. Daar is onlangs getoon dat azure B, die hoofmetaboliet van MB, ook soortgelyke interaksies kan ondergaan het in sommige gevalle hoër potensie as MB getoon. Hierdie ontdekkings impliseer dat sommige farmakologiese effekte wat voorheen aan MB toeskryf is, moontlik eerder die werking van azure B is.

Omdat die strukture van MB en sy metaboliete nou verwant is, is die analise daarvan 'n uitdaging. Die analitiese metodes wat wel ontwikkel en gepubliseer is vir die analise van MB en sy metaboliete het onoorkombare nadele soos lae sensitiwiteit en hoë kostes, asook die noodsaaklikheid van professioneel opgeleide personeel en gesofistikeerde analitiese toerusting. Híerdie nadele vertraag en beperk verdere ontwikkeling van MB as 'n potensiële geneesmiddel vir die behandeling van AD en ander neurodegeneratiiwe siektes. Verder verhinder dit ook in-diepte ondersoek van die farmakologiese effekte van MB en sy metaboliete. Tydens hierdie studie is eenvoudige en koste-effektiewe analitiese metodes ontwikkel vir die ontleding en skeiding van MB en sy metaboliete. 'n Akkurate, sensitiewe en betroubare hoë-prestasie vloeistofchromatografie (HPVC) metode waarmee MB en sy metaboliete suksesvol geskei is, is ontwikkel en ten volle gevalideer. 'n Synergi Polar-RP kolom (150 x 4.6 mm, 4 µm, 80 Å) en 'n mobiele fase wat bestaan uit ammoniumasetaat wat opgelos is in 'n mengsel van water en metanol (deel A) en 'n mengsel van asetonitrile en metanol (deel B) is tydens die studie gebruik. Die analises is uitgevoer met 'n Hitachi Chromaster chromatograaf. Metodes vir die suksesvolle normale-fase en omgekeerde-fase dunlaag chromatografie (DLC) van MB en sy metaboliete is ook ontwikkel as 'n onverfynde metode vir die bepaling van die suiwerheid van MB.

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This dissertation is dedicated to Jesus Christ, my Lord and Saviour.

I would like to thank Him for giving me the opportunity to pursue a MSc degree and for gifting me with the necessary intelligence, logic and determination to see it through. I owe all I am and have accomplished to Him alone.

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LIST OF ABBREVIATIONS AND ACRONYMS

5-HT	Serotonin
3xTg-AD	Triple transgenic Alzheimer's disease mouse model
A	
AA	Azure A
AB	Azure B
AC	Azure C
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AOAC	Association of Official Analytical Communities International
API	Active pharmaceutical ingredient
APOE-ε4	Apolipoprotein E- ε4
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
B	
BBB	Blood brain barrier
BuChE	Butyrylcholinesterase
BACE	Beta-secretase
C	
CE	Capillary electrophoresis
ChAT	Choline acetyltransferase
CoA	Co-factor A
cGMP	Current Good Manufacturing Practice
cGMP	Cyclic guanosine monophosphate
D	
DP	Drug product

DS	Drug substance
E	
EDRF	Endothelial-Derived Relaxing Factor
EMEA	European medical agency
ESI	Electrospray ionization
F	
FADH_2	Flavin adenine dinucleotide
FDA	Food and Drug Administration
G	
G6PD	Glucose-6-phosphate dehydrogenase
GLP	Good Laboratory Practice
H	
H_2O	Water
H_2O_2	Hydrogen peroxide
HC	Health Canada
HCl	Hydrogen chloride
HPLC	High pressure liquid chromatography
I	
ICH	International Conference on Harmonization
IEC	International Electrotechnical Commission
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
L	
LOD	Limit of detection
LOQ	Limit of quantification
LeucoMB	Leucomethylene blue
Log P	Partition coefficient
M	

MAO	Monoamine oxidase
MAO-A	Monoamine oxidase type A
MAO-B	Monoamine oxidase type B
MAOI	Monoamine oxidase inhibitor
MB	Methylene blue
MP	Mobile phase
MS/MS	Tandem mass spectrometry
N	
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NFT	Neurofibrillary tangles
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
NO-cGMP	Nitric oxide cyclic guanosine monophosphate
NOS	Nitric oxide synthases
NOS-NO-cGMP	Nitric oxide synthases- nitric oxide-cyclic guanosine monophosphate
NPC	Normal phase chromatography
NSAID	Nonsteroidal anti-inflammatory drug
O	
OH	Hydroxide
P	
pH	Potential of hydrogen
pKa	Acid dissociation constant
R	
Rf	Retardation factor
RFR	Relative response factor
ROS	Reactive oxygen species

RPC	Reverse phase chromatography
Rs	Chromatographic resolution
RSD	Relative standard deviation
S	
SAP	Serum amyloid P component
SP	Stationary phase
T	
TLC	Thin layer chromatography
U	
UHPLC	Ultra high pressure liquid chromatography
UNIDO	United Nations Industrial Development Organisation
UNODC	United Nations Office on Drugs and Crime
USP	United States Pharmacopoeia
UV	Ultra violet
W	
WHO	World Health Organization

LIST OF SYMBOLS

B	Beta
µ	Micro
π	Pi
Å	Angstrom
ºC	Degrees Celsius
Y	Gamma
H	Efficiency
N	Plate number
α	Selectivity

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CHAPTER 1 - INTRODUCTION

1.1. Problem statement

Alzheimer's disease (AD) is the most common cause of the majority of dementia cases in elderly people (Barten & Albright, 2008; Gura, 2008; Swaab, 2014). AD is among the most traumatic of all mental degenerative diseases and affects the family and caregivers of the patient extremely. Despite the availability of a few drugs that slows the progression of AD, the treatment still remains ineffective, especially in severe stages of AD (Scarpini *et al*., 2003; Wischik *et al*., 2008). The most common and currently first line treatment, is based on the inhibition of acetylcholine esterase (AChE). Other drugs that have been researched have very high toxicity profiles and can therefore not be used as treatment. (Scarpini *et al*., 2003). The interest in methylene blue (MB) as a possible treatment for AD, have recently attracted much attention (Gura, 2008; Sullivan, 2008; Wischik *et al*., 2008). Other than traditional AD treatment, MB exhibits a variety of mechanisms by which it combats AD due to its pleiotropism.

A summary of the effects of MB on AD:

- MB inhibits Aβ peptides and tau protein aggregation (Akoury *et al*., 2013; Cawein *et al*., 1964; Louters *et al*., 2006; Luna-Muñoz *et al*., 2008; Necula *et al*., 2007; Paban *et al*., 2014; Schirmer *et al*., 2011; Sullivan, 2008; Wischik *et al*., 2008).
- MB inhibits AChE (Petzer *et al*., 2014; Pfaffendorf *et al*., 1997; Wischik *et al*., 2008).
- MB inhibits Aβ42 oligomerization and Aβ42 fibrilisation (Akoury *et al*., 2013; Necula *et al*., 2007; Oz *et al*., 2009; Taniguchi *et al*., 2005; Wischik *et al*., 1996).
- MB acts as a neuroprotective agent against neurodegeneration (Schirmer *et al*., 2011).

MB has very few side effects and is relatively safe in humans (Küpfer *et al.,* 1994; Oz *et al.,* 2009, 2011; Riha *et al.,* 2005; Wainwright *et al.,* 2007; Wainwright & McLean, 2017). MB is readily available and can be purchased commercially (DiSanto & Wagner, 1972; Ramsay *et al.,* 2007; Wagner *et al.,* 1998).

The metabolisation of MB yields N-demethylated metabolites. One of these metabolites, azure B, have shown superior effects to MB to most of its indications (Buchholz *et al.,* 2008; Petzer *et al.,* 2012, 2014; Taniguchi *et al.,* 2005; Wischik *et al.,* 1996). However, the existence and effects of

azure B, has only recently been discovered (Shirmer *et al.,* 2011; Warth *et al.,* 2009). Due to the lack of knowledge, the effects seen in earlier studies done on MB, was associated with MB alone, which led to misleading conclusions (Bruchey & Gonzales-Lima, 2008; Shirmer *et al.,* 2011). Although it has been demonstrated that azure B has superior neuroprotective and memory enhancing abilities in comparison to MB, it is not clear which compound mediates the effects with the administration of commercially available MB, since a batch of MB is contaminated with a great amount of azure B (Shirmer *et al.,* 2011). Also, the influence of these metabolites on each other still remain unknown.

The chemical structure of MB when compared to its metabolites, are very similar, making the separation and isolation of these compounds extremely difficult (Kim *et al.,* 2014; Schirmer *et al.,* 2011). The few methods published for the quantification of MB and/or its metabolites suffer from great disadvantages, thus making the analysation process of MB very expensive (Kim *et al.,* 2014; Warth *et al.,* 2009). Therefore, despite of the great effects that MB and/or its metabolites exhibits, the lack of available analytical methods by which these compounds can be analysed, delayed further research. For accurate and reliable data of the mechanisms and effects of azure B (and the other metabolites), as well as for the continual research and development of MB as a possible treatment for AD and many other diseases, an analytical separation method that is simple, sensitive and cost effective needs to be developed.

1.2. Aims and objectives

- To develop an effective thin layer chromatography method for the analysis of MB and its metabolites.
- To develop a simple, cost effective and reliable high performance liquid chromatography (HPLC) method for the analysis of MB and its metabolites.
- To separate and identify MB and each of its metabolites on the generated chromatograms.
- To validate the newly developed HPLC method.

1.3. Hypothesis

It is postulated that methylene blue and its metabolites can be separated with a simple, rapid and sensitive HPLC method. This hypothesis is based on a study done by Boehme & Strobel (1998), who managed to successfully analyse and separate chlorpromazine and its metabolites. Boehme & Strobel (1998), have developed sensitive HPLC methods for the resolution and quantification of chlorpromazine during their study. The surprising similarities between the chemical structure

of MB, its metabolites and phenothiazine compounds such as chlorpromazine (figure 1.1), justifies the hypothesis of this study. The analysation of MB and its metabolites can possibly be done if compounds with similar chemical structures were successfully analysed in previous studies.

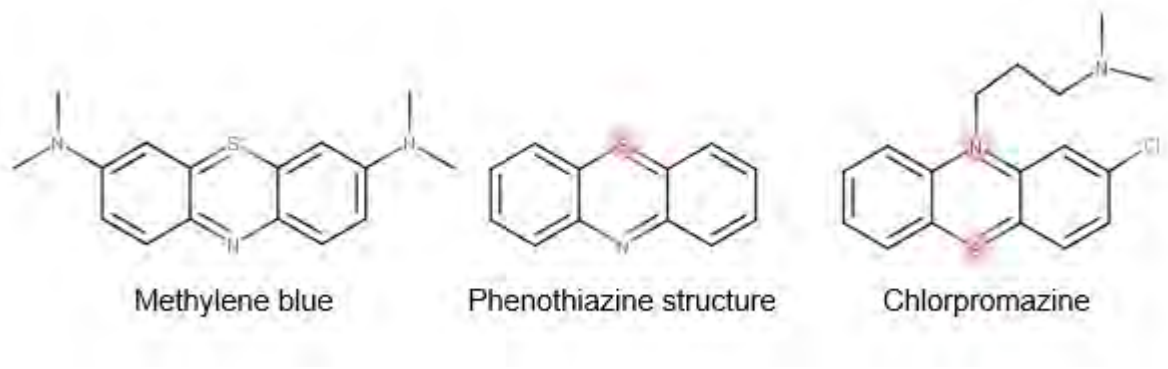


Figure 1.1: Comparison between the chemical structure of methylene blue, chlorpromazine and the phenothiazine functional group.

1.4. Study layout

As initial process of elimination, normal phase and reversed phase TLC plates will be used to attempt the separation of the substances. Reversed phase TLC plates acts on the same principle as the HPLC that will be used in this study, the only difference is that the TLC plates have flat surfaces where the HPLC has a column (MIT, 2004). It is thus possible to evaluate the chemical interactions on the reversed phase TLC plates before analysing it on the HPLC (MIT, 2004).

The primary method for the separation/analysis of methylene blue will be HPLC. Different mobile phases, column types and wavelengths will be used for the development of a HPLC method for the separation of methylene blue and its metabolites. A Hitachi Chromaster chromatographic system will be used to evaluate and develop HPLC methods. The system consists of a 5410 UV detector, an auto-sampler (5260) with a sample temperature controller and a solvent delivery module (5160).

CHAPTER 2 - LITERATURE STUDY

2.1. Methylene blue

2.1.1. History and general background

The first drug to be fully synthesised was methylene blue (MB) (Kim *et al.,* 2014; Lo *et al.,* 2014). MB was synthesised in 1876 for the purpose of cotton dying by Heinrich Caro (Kim *et al.,* 2014; Lo *et al.,* 2014; Wainwright & Crossley, 2002; Wainwright & McLean, 2017; Watts *et al*., 2013). The ability of MB to stain certain pathogens, led to the argument that MB may possibly have a harmful effect on a targeted pathogen. This led to the conclusion that MB may successfully treat certain diseases (Lo *et al.,* 2014; Wainwright & McLean, 2017). Modern drug research and further investigation of the therapeutic potential of MB, was based on the staining qualities that MB possessed (Barcia, 2007; Fleischer, 2004; Oz *et al.,* 2009, 2011; Schirmer *et al*., 2003; Wainwright *et al.,* 2007; Wainwright & McLean, 2017). During a study done by Robert Koch and Paul Ehrlich, the discovery was made that MB did not only stain certain pathogen cells, but also deactivated them. MB became the first synthetic compound used as a clinical antiseptic and the first antiseptic dye to be used therapeutically, specifically for malaria (Coulibaly *et al*., 2009; Lo *et al.,* 2014; Oz *et al.,* 2009, 2011; Peter *et al.,* 2000; Vennerstrom *et al*., 1995; Wainwright *et al.,* 2007). Also, MB was indicated for the treatment of certain cancers and illnesses which involves abnormal cell growth before the discovery of penicillin and sulphonamides (Peter *et al.,* 2000; Wainwright & Crossley, 2002; Wainwright & McLean, 2017; Wainwright, 2003). The MB-based treatment of malaria was already common in the 1890's, and recently this indication has been re-evaluated and re-implemented (Akoachere *et al*., 2005; Lo *et al.,* 2014; Schirmer *et al.,* 2003, 2011; Walter-Sack *et al.,* 2009). Due to its significant staining potential, MB is included as an ingredient of the Giemsa solution which is used for staining and characterising red blood cells and malaria parasites (Barcia, 2007; Fleischer, 2004; Watts *et al*., 2013). Further studies on the unique biochemical properties of MB led to many other scientific breakthroughs such as identification of the microscopic pathogen that causes tuberculosis; *Mycobacterium tuberculosis* (Ehrlich, 1886; Garcia-Lopez *et al*., 2007). The knowledge gathered on the biochemical properties of MB also allowed scientists to stain nerve tissue and discover its structural organisation. In 1886, MB was referred to as the "magic bullet" after Paul Ehrlich discovered that MB is absorbed by nervous tissue selectively (Coulibaly *et al*., 2009; Rojas *et al*., 2012; Wainwright *et al.,* 2007; Wainwright & Crossley, 2002). Ehrlich's discovery led to studies that proved and supported not only the antioxidative activity of MB (Ohlow & Moosmann, 2011; Peter *et al.,* 2000; Rojas & Gonzalez-Lima, 2010; Watts *et al*., 2013), but also its ability to protect the brain against neurodegenerative

processes and memory loss due to its high selectivity for nervous tissue (Artuch *et al*., 2004; Lensman *et al*., 2006; Rainer *et al*., 2000; Teichert *et al*., 2003; Watts *et al*., 2013). Over the last 120 years, the number of indications that MB could possibly be used for has increased enormously (Clark *et al*., 1925; Watts *et al*., 2013).

During studies on the chemical structure and properties of MB, the similarity it had with drugs known as phenothiazines was surprising (Oz *et al.,* 2009; Paban *et al.,* 2014; Watts *et al*., 2013). Phenothiazine drugs are known for their antihistamine and neuroleptic effects, all of which are accomplished by therapeutic action in the brain (Watts *et al*., 2013). The similarities between the chemical structures of MB and the phenothiazines gave scientists reason to postulate that MB may potentially have a positive effect on mental and psychotic disorders (Clifton & Leikin, 2003; Deutsch *et al*., 1997; Harvey *et al*., 2010; Kelner *et al*., 1988b; Lo *et al.,* 2014; Miclescu *et al*., 2006, 2007; Naylor *et al*., 1986; Paban *et al.,* 2014; Pelgrims *et al*., 2000; Sharma *et al*., 2011; Wainwright & Crossley, 2002). MB have been implemented as additional add-on therapy for psychosis to determine patient compliance by analysing the urine of a patient for the typical blue discolouration (Lo *et al.,* 2014; Schirmer *et al.,* 2011). It was observed that the patients who received the add-on MB treatment, showed an improvement regarding their general mental status, proving that MB, like phenothiazines, also results in positive psychotropic and antidepressant effects (Dhir & Kulkarni, 2011; Eroglu & Caglayan,1997; Harvey *et al*., 1990, 1996; Lo *et al.,* 2014; Schirmer *et al.,* 2011; Wainwright & Crossley, 2002; Watts *et al*., 2013). Modern nervous system altering drugs such as chlorpromazine and tricyclic antidepressants, as well as drugs against cancer and infectious diseases, were discovered during studies where MB were used as the lead compound (Ohlow & Moosmann, 2011; Wainwright & Crossley, 2002).

2.1.2. Physicochemical properties of MB

MB, which is also known as tetramethylthionin chloride (figure 2.1), is classified as a member of the drugs that belongs to the phenothiazine group. As for its chemical properties, MB is classified as an aromatic, tri-heterocyclic cationic dye (Akoury *et al.,* 2013; Lo *et al.,* 2014; Necula *et al.,* 2007a; Ohlow & Moosmann, 2011; Oz *et al.,* 2009, 2011; Rojas *et al.,* 2012; Wainwright & Amaral, 2005; Wainwright & Crossley, 2002; Watts *et al*., 2013). MB is commercially available and can be purchased in the form of a dark green powder, however, at room temperature MB normally exists in the form of odourless, dark blue crystals which are highly soluble in water due to its hydrophilic nature (DiSanto & Wagner, 1972; Lo *et al.,* 2014; Necula *et al.,* 2007a; Ramsay *et al*., 2007; Wagner *et al*., 1998). The phenothiazine molecules which are present in the nucleus of MB absorbs light at a wavelength of 609 nm and 668 nm, thus yielding a bright blue solution when

dissolved in a water-based liquid (Ohlow & Moosmann, 2011; Oz *et al.,* 2009, 2011; Ramsay *et al*., 2007; Wagner *et al*., 1998; Wainwright & McLean, 2017).

An interesting discovery on the chemical structure of MB, which is the foundation of its unique abilities, is the delocalised positive charge it carries at neutral pH. MB is therefore also classified as a cationic redox compound with the ability to autoxidise without stoichiometric reduction changes (Akoury *et al.,* 2013; Atamna & Kumar, 2010; Oz *et al.,* 2011). The great reduction potential that MB possesses is caused by the thiazine ring system (Wainwright & Crossley, 2002). MB therefor acts as a redox cycling agent *in vitro* and *in vivo.* Despite the positive charge it possesses, MB is a highly stable compound due to the presence of the imine group (Moosmann *et al.,* 2001).

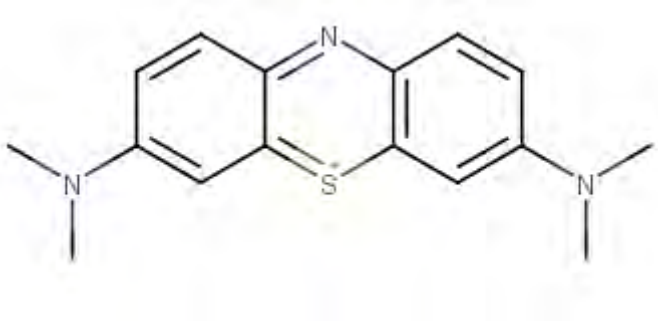


Figure 2.1: Chemical structure of methylene blue (Oz *et al.,* 2009).

The reduction of MB yields a compound named Leuco-methylene blue (LeucoMB) (Atamna & Kumar, 2010; Buchholz *et al.,* 2008; Peter *et al.,* 2000; Schirmer *et al*., 2011; Wainwright *et al.,* 2007). This compound is colourless because of its inability to absorb light in the visible region (Buchholz *et al.,* 2008; Peter *et al.,* 2000; Oz *et al.,* 2011; Ramsay *et al*., 2007). LeucoMB has no charge at a normal pH, and is up to 20 times more lipophilic than MB (Harris & Peters, 1953; Müller, 1998, 2000).

When the hydrophilicity of MB is considered, the normal conclusion would be that MB is unable to penetrate lipid bilayers and the blood brain barrier (BBB) due to the positive charge it possesses (Sweet & Standiford, 2007). However, isobolic potential curves (figure 2.2) indicates that this positive charge which is located on the sulphur and nitrogen atoms, is distributed evenly throughout the whole compound, allowing it to still penetrate membranes (Artuch *et al*., 2004; Lensman *et al*., 2006; Necula *et al.,* 2007a; Peter *et al.,* 2000; Rainer *et al*., 2000; Sweet & Standiford, 2007; Teichert *et al*., 2003; Wagner *et al*., 1998).

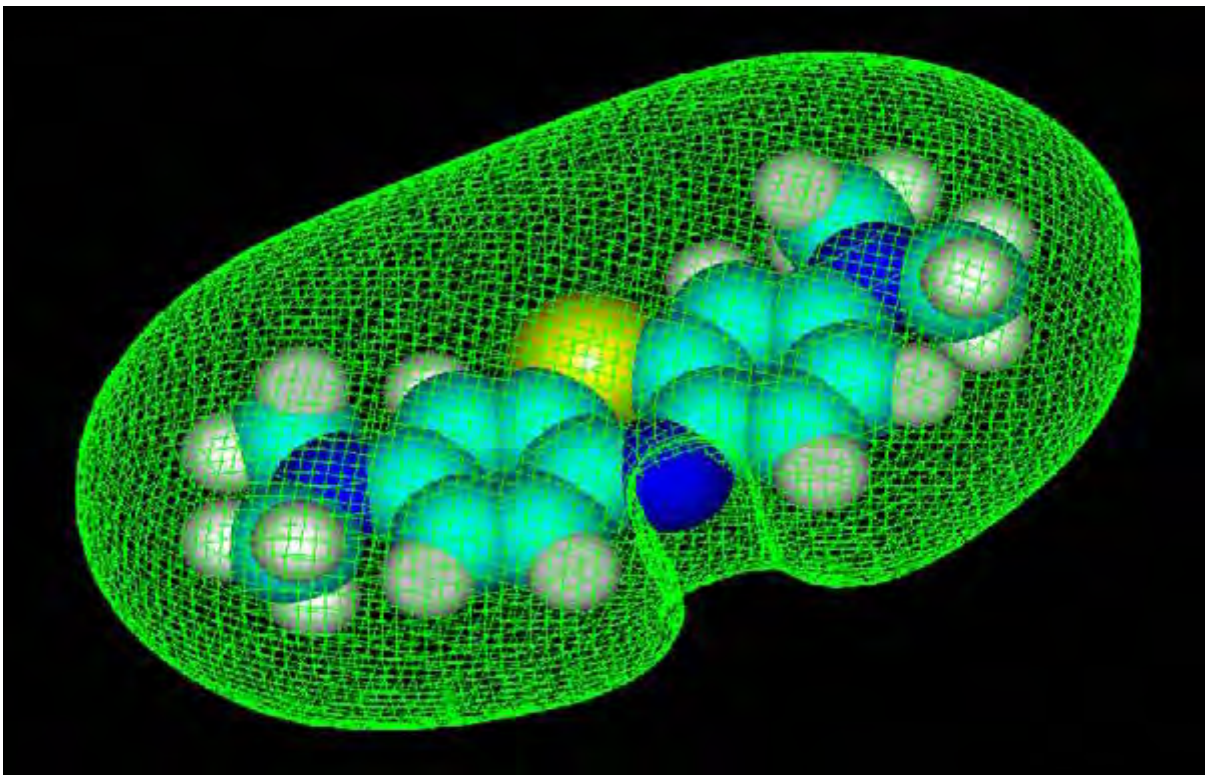


Figure 2.2: The isopotential surface surrounding the methylene blue structure in 3D space as described by Oz *et al.* (2009).

When MB and LeucoMB is compared in terms of ionisation state and lipophilicity, the penetration of the BBB can easily be explained (Clifton & Leikin, 2003; DiSanto & Wagner, 1972; Lorke *et al*., 2008; McCord & Fridovich, 1969; Oz *et al.,* 2011):

- MB is reduced to LeucoMB in peripheral tissue.
- Because of its lipophilicity, LeucoMB can cross lipid membranes and enter cells by means of diffusion.
- Once in the cell, the unstable LeucoMB is re-oxidised to cationic MB (Clifton & Leikin, 2003; Locke *et al*., 2008; McCord & Fridovich, 1969; Müller, 1998, 2000; Oz *et al.,* 2011).

A reversible oxidation/reduction system is formed by MB and LeucoMB and exists as an electron donor-acceptor couple in equilibrium (figure 2.3). After a study done on rats where MB has been injected into the heart of rats, efficient levels of MB have been observed to penetrate the BBB, however, both MB and LeucoMB were present in the brain. Because of the significant differences between the chemical structure of these two compounds, major differences in their biological activities can be expected. However, it is still not clear which form of MB mediates the biological and therapeutic activities observed during MB therapy. A study has shown that MB has an absolute bioavailability of 72.3% after oral administration (Oz *et al.,* 2009; Walter-Sack *et al*., 2009).

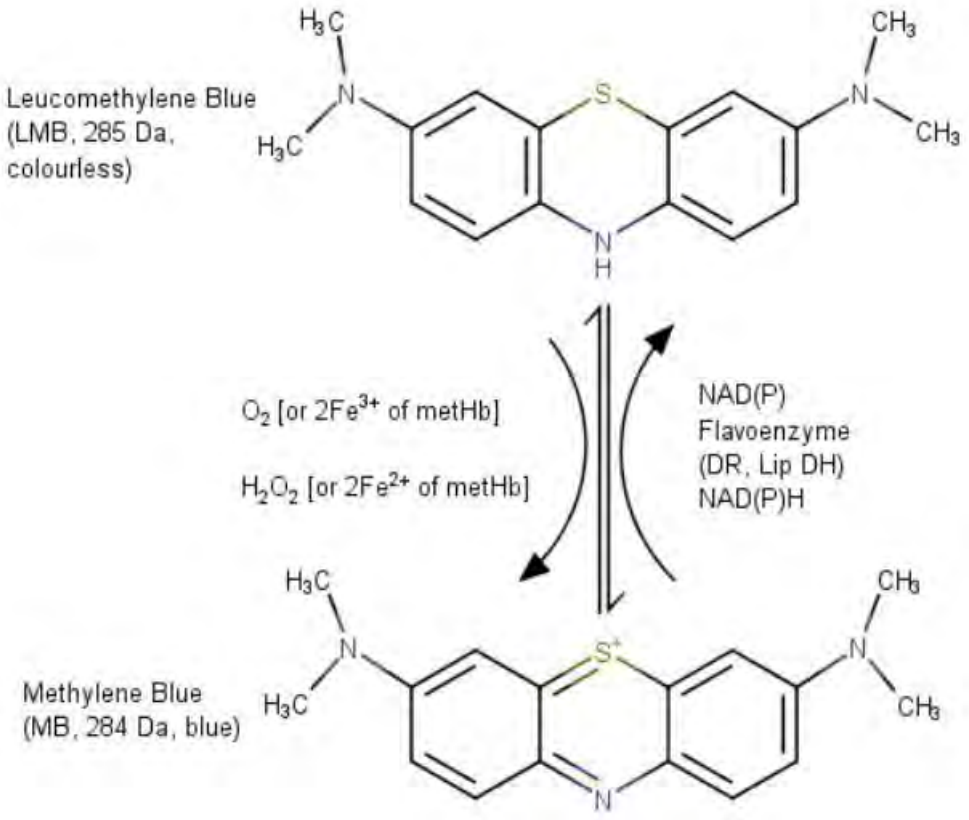


Figure 2.3: Reversible redox-oxidation system formed between methylene blue and leuco-methylene blue (Schirmer *et al.,* 2011).

2.1.3. Pleiotropism and mechanism of action

MB may interact with as many targets as the number of oxidoreductases that are available which supports the pleiotropism of MB (Salaris *et al*., 1991; Visarius *et al*., 1997). Thus, many targets have been identified with which MB and its demethylated metabolites interacts. The most prominent targets are nitrogen oxidase synthases (NOS), guanylate cyclase, methaemoglobin, monoamine oxidase A, acetylcholine esterase, and disulphide reductases such as glutathione reductase or dihydrolipoamide dehydrogenase (Schirmer *et al.,* 2011). The mechanism of action of MB in neurodegenerative diseases exhibits both facilitation of memory as well as mitochondrial neuroprotection (Rojas *et al.,* 2012). The mechanism is based on the dual effect MB has as an antioxidant and a metabolic enhancer. MB serves as a substrate for flavin-dependant disulphide reductases and at the same time acts as a non-competitive inhibitor thereof (Schirmer *et al*., 2011). During the interaction between MB with the flavoenzyme, MB is reduced to LeucoMB which is, due to its instability, spontaneously converted back to MB by molecular oxygen. Thus, MB is

made available for the next cycle, acting as a recycling catalyst. Superoxide and hydrogen peroxide are the main products yielded by this cycle (Schirmer *et al*., 2011).

2.1.4. Methylene blue compared to azure B – is methylene a prodrug for azure B?

During the metabolism of MB, N-demethylated compounds known as, azure A, azure B and azure C, are produced (as seen in figures 2.4 and 2.5). These metabolites also exhibit pharmacological effects (Akoury *et al.,* 2013; Warth *et al*., 2009; Wainwright *et al.,* 2007). It is clear that MB acts as a prodrug for its demethylated metabolites (Schirmer *et al.,* 2011). Studies on the physiochemical properties of each metabolite showed that only a slight difference exists between them in regards to partition coefficient (LogP) values and size. It was discovered that in comparison to oxidised MB, oxidised azure B can penetrate lipid membranes due to its ability to maintain a neutral quinoneimine form (Schirmer *et al.,* 2011). Earlier studies done on MB have not included the full mechanism by which it is metabolised. Thus, due to the lack of variance studies done on the effects of MB and azure B respectively, results seen in previous studies that were associated to MB is more likely to be connected to the activity of azure B (Bruchey & Gonzales-Lima, 2008; Schirmer *et al.,* 2011). Recent studies have proven the superiority of azure B to MB in regards to their effects. Because no documentation on the effects of azure B in humans could be found, Schirmer, who weighed 60 kg at the time, administered to himself 120 mg azure B dissolved in 30 ml water, orally. He experienced the typical effects documented for MB such as the bitter taste and immediate blue discolouration of the teeth and mucus membranes (Schirmer *et al.,* 2011). After a few hours have passed, he noticed discolouration of his urine with maximum intensity 12 hours after administration. Considering these findings, it is evident that MB preparations which were used for the past 10 decades, were contaminated with azure B – which was not noted before (Schirmer *et al.,* 2011).

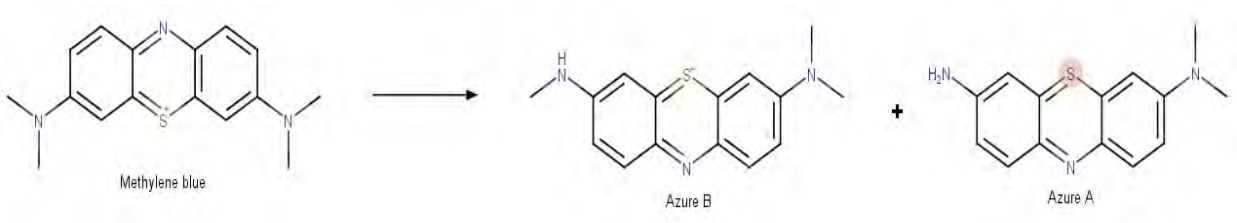


Figure 2.4: Metabolism of methylene blue to its metabolites – a structural comparison (Warth *et al.,* 2009).

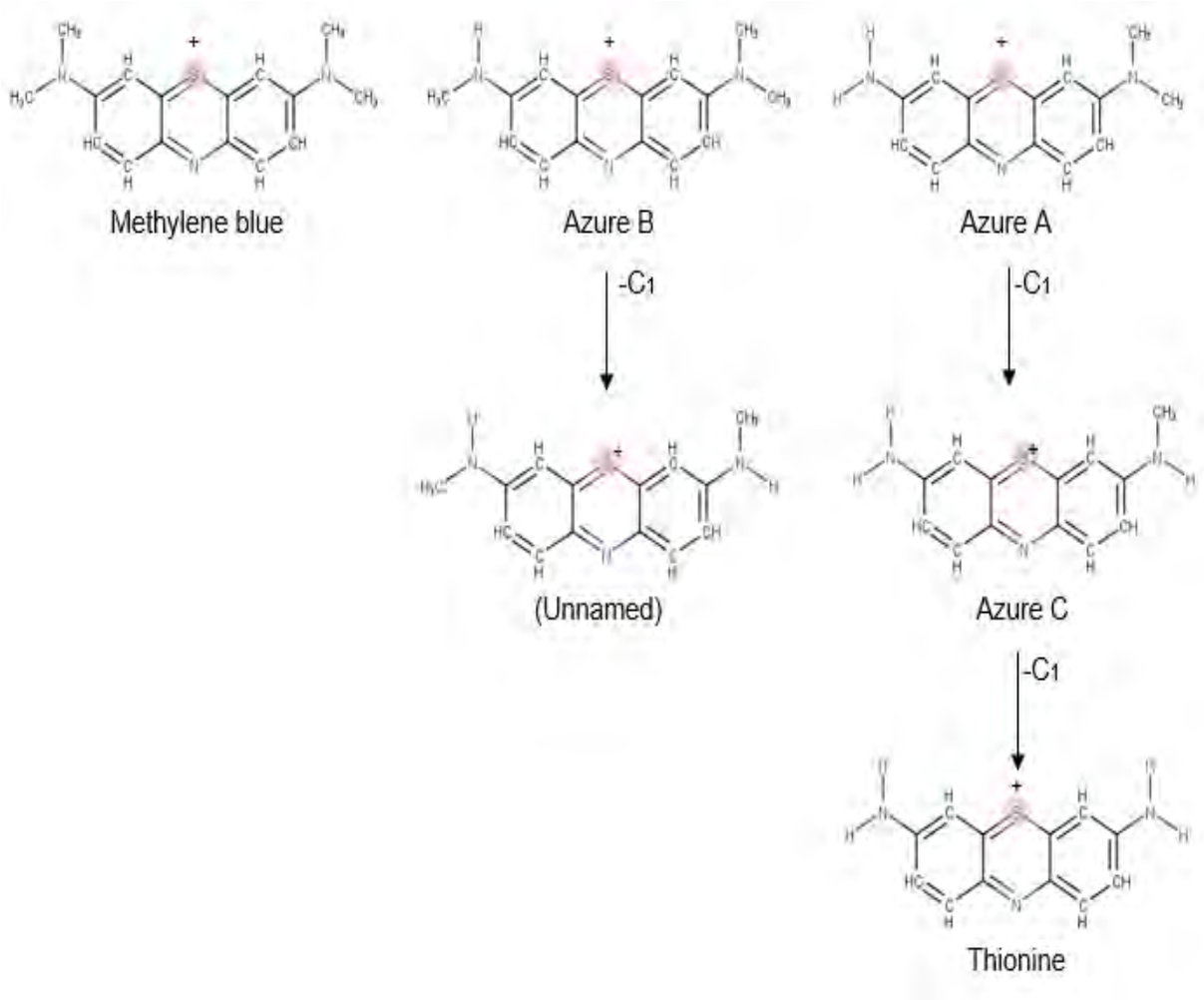


Figure 2.5: Metabolism of methylene blue (Wainwright & McLean, 2017).

2.1.5. Targets in the human body from an anti-Alzheimer's disease perspective

Figure 2.6 illustrates the potential effects and mechanisms that MB may have on the progression of AD. Also, typical pathological changes associated with AD, as well as changes in neurotransmitter levels, are indicated.

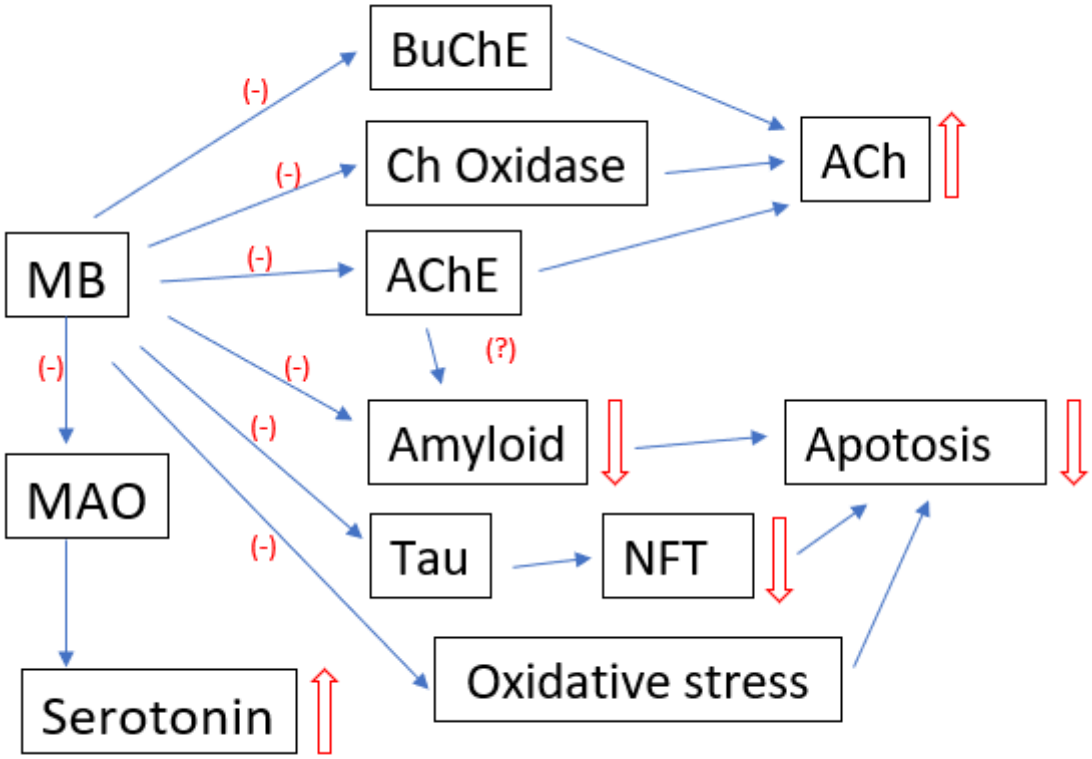


Figure 2.6: Summary of the different mechanisms of action that methylene blue exhibits (Cohen, 2013; Oz *et al.,* 2011).

AChE: acetylcholine esterase; BuChE: butyrylcholine esterase; Choline Ox: Choline oxidase; MAO: monoamine oxidase; NFT: neurofibrillary tangles.

2.1.5.1. Methylene blue and the cholinergic system

The cholinergic system plays a vital role in memory and learning (Giacobini, 2003; Holzgrabe *et al.,* 2007; Oz *et al.,* 2009). Results in recent studies have indicated that cholinergic activation has been linked to protein metabolism in amyloid plaques, a typical phenomenon seen in the neuropathology of Alzheimer's disease (AD) (Nordberg, 2006). MB has shown to influence the communication between neurons to such an extent that it changes the neurotransmission of cholinergic neurotransmitters in the synapses (Pfaffendorf *et al.,* 1997; Wischik *et al.,* 2008). When MB is given in combination with a cholinesterase inhibitor, the effects of MB seemed to be potentiated. The two neurotransmitters mostly known to mediate the effects of the cholinergic system, is acetylcholine (Ach) and butyrylcholine (BuCh), of which Ach is the most prominent (Pfaffendorf *et al*., 1997). In the healthy brain homeostasis is achieved by the positive and negative coupling of hormones and neurotransmitters. The action of Ach is terminated by an enzyme known as acetylcholine esterase (AChE) (Deiana *et al*., 2009). In an AD brain, AChE

levels are abnormally high, causing the amount of Ach to be decreased to a critical low level. Thus, if the Ach levels can be restored to normal by inhibiting AChE, symptoms associated with reduced cholinergic neurotransmission will improve (Deiana *et al*., 2009; Holzgrabe *et al*., 2007; Pfaffendorf *et al*., 1997). Also, AChE seems to have an interaction with the plaques present in the AD brain, leading to the formation of extremely toxic AChE-amyloid-Aβ complexes (Nordberg, 2006). It has been demonstrated, that MB inhibits AChE (figure 2.4) (Cawein *et al*., 1964; Louters *et al*., 2006; Petzer *et al*., 2014; Pfaffendorf *et al*., 1997; Wischik *et al.,* 2008) however, if the conversion from MB to LeucoMB is increased, AChE inhibition will decrease because of LeucoMB's inability to inhibit AChE. MB has also shown inhibitory activity towards butyrylcholine esterase (BuChE) (figure 2.7), thus making it a better candidate for the treatment of cholinergic system associated neurodegeneration due to its dual action on both enzymes (Abi-Gerges *et al*., 1997).

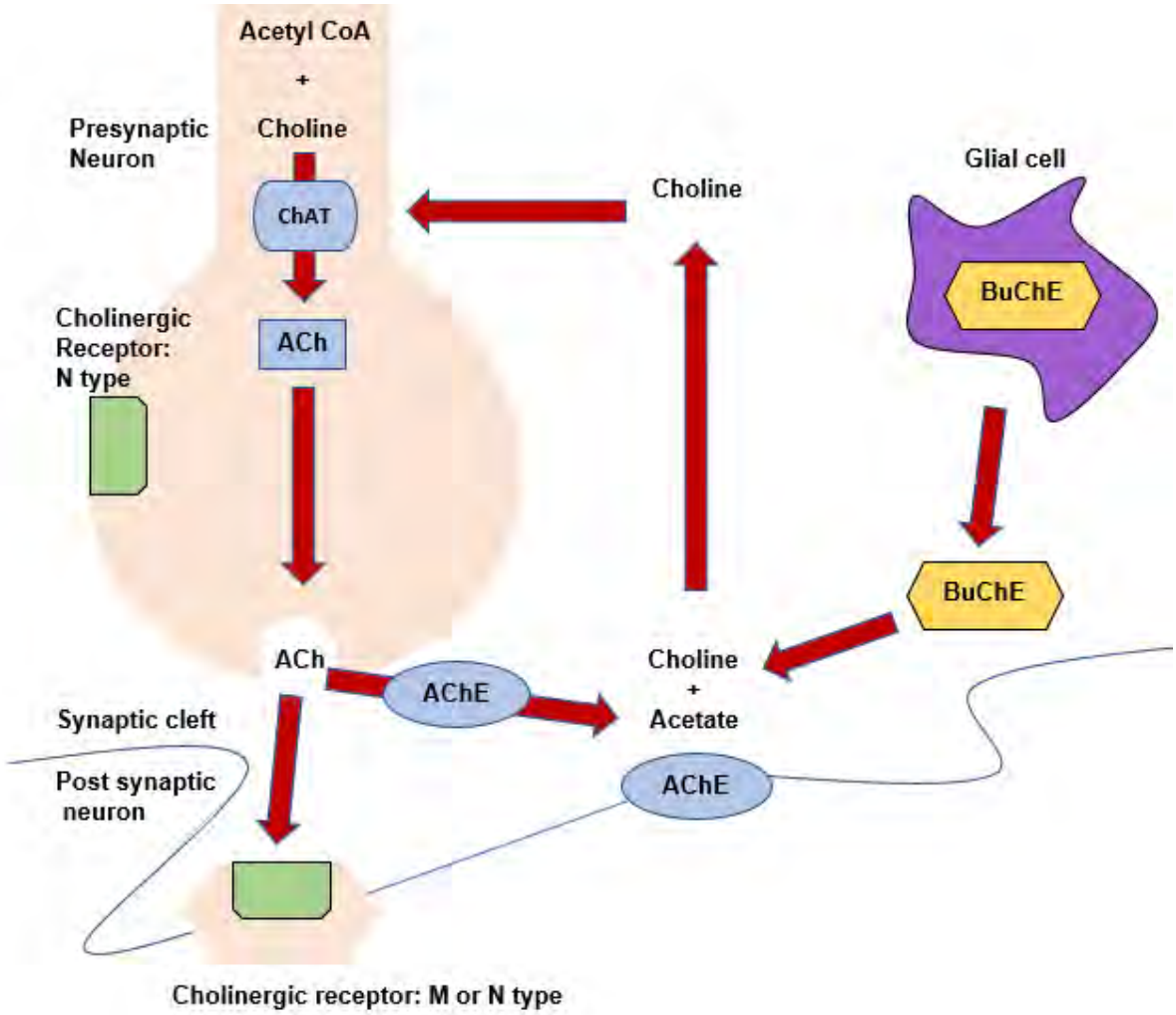


Figure 2.7: An illustration of the cholinergic system (Scarpini *et al.,* 2003).

ACh: acetylcholine; AChE: acetylcholinesterase; BuChE: butyrylcholine esterase; ChAT: choline acetyltransferase; CoA: coenzyme A.

2.1.5.2. Methylene blue and the serotonergic system

According to Naylor *et al.* (1986; 1987), symptoms associated with neurodegenerative disorders is not only the result of an insufficient cholinergic system, but also due to the imbalance of other neurotransmitters (Lorke *et al.,* 2006). The progression of AD is closely related to serotonergic (5-HT) deficits as well. Together with a remarkable decrease in 5-HT levels, the typical AD brain shows a decreased expression of 5-HT receptors (Lorke *et al.,* 2006). In some neurons, neurotransmitters such as Ach are prevented from being released due to the presence of 5-HT6 receptors (Alz.org., 2017). As a reversible inhibitor of monoamine oxidase (MAO) type A (figure 2.4), which is an enzyme responsible for the metabolism of 5-HT, MB expressed the ability to increase and restore cellular 5-HT levels. However, MB should not be administered to patients that already receive drugs that elevates 5-HT levels such as selective serotonin reuptake inhibitors. When MB is administered in combination with these drugs the very toxic serotonin syndrome could possibly be induced (Khavandi *et al*., 2008; Lorke *et al.,* 2006; Ramsay *et al.,* 2007).

2.1.5.3. Methylene blue and the NO-cGMP cascade

Nitric oxide (NO), previously known as Endothelial-Derived Relaxing Factor, are produced by enzymes known as nitric oxide synthases (NOS) and plays an important role in the cardiovascular-, immune-, central- and peripheral- nervous systems (Hibbs *et al*., 1987; Moody *et* al., 2001; Oosthuizen *et al*., 2005; Palmer *et al*., 1987). One of the main targets of NO is guanylate cyclase, which is activated by the binding of NO to the iron atom in the heme group leading to the formation of cyclic guanosine monophosphate (cGMP) (Dawson & Snyder, 1994). NO thus exerts its effects by producing cGMP (Moncada *et al*., 1991). According to Garthwaite (1911), MB can change the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway proposing that MB could inhibit NOS and guanylate cyclase non-selectively (Atamna & Kumar, 2010; Luo *et al*., 1995; Mayer *et al.,* 1993a,b; Moore & Handy, 1997; Schirmer *et al.,* 2011; Volke *et al*., 1999). Mayer *et al.* (1993a,b) performed a study where they discovered that MB directly inhibits NOS. The inhibition of NOS yields a lower level of cGMP production, resulting in the modification of the cGMP signalling. With the alteration of this pathway, depression and related illnesses have shown to improve (Brink *et al.*, 2008; Harvey *et al*., 1994; 2010; Harvey, 1996; Liebenberg *et al.*, 2010; Wegener *et al.*, 2010). The typical side effects associated with NOS inhibitors are completely avoided when MB is used instead (Hobbs *et al*., 1999; Ignarro *et al*., 1999; Narsapur & Naylor, 1983).

2.1.5.4. Methylene blue and monoamine oxidase

Monoamine oxidase (MAO) enzymes exist as two isoforms namely, MAO type A and MAO type B (Edmondson *et al*., 2007). MAO are responsible for the metabolism of catecholamines (Baldessarini, 2001; Ghaemi *et al*., 2001), where MAO-A displays specificity towards serotonin and noradrenalin and MAO-B displays specificity towards dopamine (Glover *et al*.,1977; Murphy *et al*., 1987). Recently, it has been shown that MB exhibits MAO-inhibition activity (Oxenkrug *et al*., 2007; Ramsay *et al*., 2007), which may explain its antidepressant effects (Aeschlimann *et al*., 1996; Harvey *et al*., 2010; Ramsay *et al*., 2007). Both MAO-A and MAO-B are inhibited by MB (figure 2.4), however, MB inhibits MAO-A with higher potency in comparison to MAO-B (Harvey *et al*., 2010; Ramsay *et al*., 2007).

2.1.5.5. Methylene blue and the mitochondria

Most of the effects that MB exhibit are due to its interaction with the mitochondria (Atamna *et al*., 2008, 2010; Louters *et al*., 2006). Closely related to the effect that MB has on the mitochondria, is its ability to prevent the formation of reactive oxygen species (ROS) (Kelner *et al.,* 1988a,b; Necula *et al.,* 2007a; Salaris *et al.,* 1991). ROS is a major role player in the pathology of depression and related illnesses (Bernstein *et al*., 1998; Harvey, 1996; Harvey *et al*., 2010). The mitochondria located in the muscle of depressed patients, produces much lower levels of adenosine triphosphate (ATP) in comparison to those in a healthy individual (Gardner *et al*., 2003). MB not only acts as a metabolic enhancer but also improves mitochondrial function (Hassan & Fridovich 1979; Peter *et al.,* 2000; Watts *et al*., 2013). This mechanism of MB is the basis on which cognitive disorders such as AD is treated (Atamna & Kumar, 2010; Peter *et al.,* 2000; Rojas *et al*., 2012; Watts *et al*., 2013). The pathology associated with AD is closely linked to inefficient mitochondrial respiration (Atamna & Kumar, 2010; Bennett *et al*., 1992; Gonzalez-Lima & Bruchey, 2004; Gonzalez-Lima *et al*., 1997, 1998; Liang *et al*., 2008). Mitochondrial respiration is enhanced when an increase in the action of cytochrome oxidase occurs. The oxidation-reduction relationship that exist between MB and LeucoMB enables MB to oxidise reduced coenzyme Q and at the same time LeucoMB to reduce cytochrome oxidase C (complex IV) (Atamna *et al.,* 2008, 2010; Callaway *et al.,* 2004; McCord & Fridovich, 1969; Scott & Hunter, 1966). As a result, oxygen consumption is more efficient and ATP production is optimised (Wong-Riley,1989; Zhang *et al*., 2006).

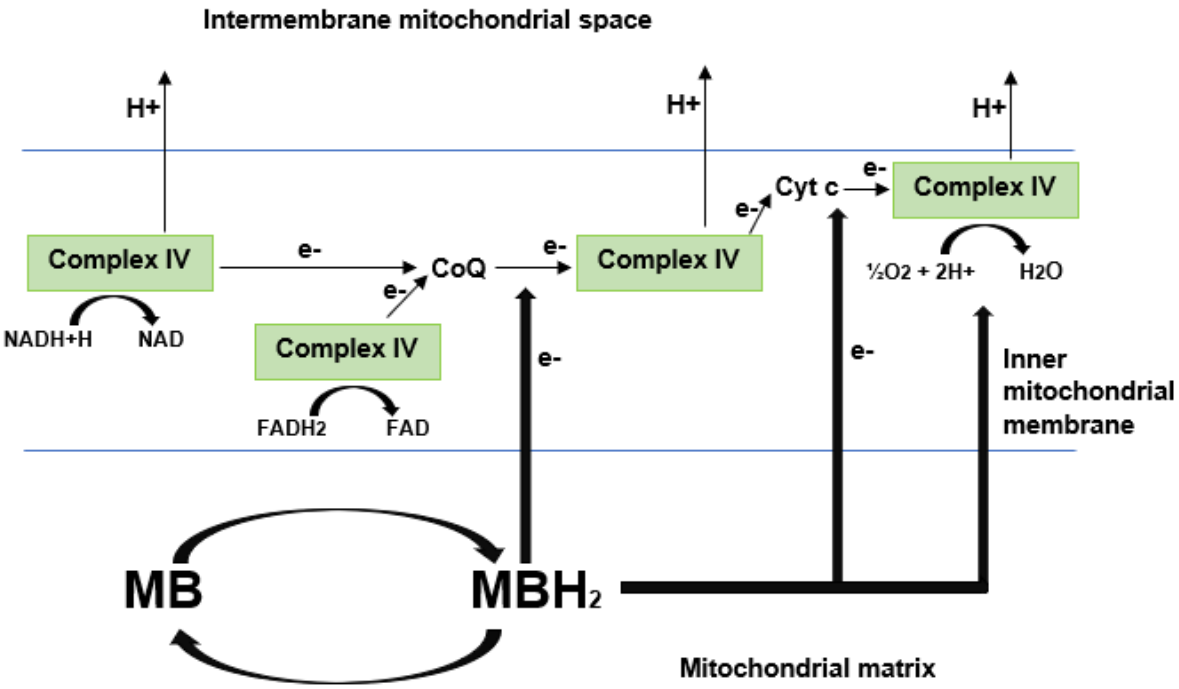


Figure 2.8: The mechanism of action that methylene blue exhibits in the mitochondria, where it acts as a neuroprotective agent and memory enhancer (Rojas *et al.,* 2012).

Figure 2.8 illustrates the pathway of oxidative phosphorylation in the inner mitochondrial membrane. MB, acting as an alternative electron acceptor, enables ATP production without ROS formation by bypassing complex I to III (Rojas, *et al.,* 2012; Watts *et al*., 2013; Yang *et al.,* 2017).

2.1.5.6. Methylene blue and oxidative stress

Previously mentioned is the ability of MB to act as an antioxidant (Ohlow & Moosmann, 2011; Peter *et al.,* 2000; Rojas & Gonzalez-Lima, 2010; Watts *et al*., 2013), not only in the mitochondria but also in all other cells in general (Rojas *et al*., 2012; Salaris *et al*., 1991). The mechanism by which this activity is established lies in its ability to serve as an artificial electron acceptor to complex I to IV (Kelner *et al.,* 1988; Lindahl & Oberg, 1961; McCord & Fridovich, 1969; Salaris *et al.,* 1991; Scott & Hunter, 1966; Visarius *et al*., 1997; Watts *et al*., 2013). During inefficient mitochondrial respiration, superoxide is formed as a product of electron leakage from the electron transport chain (Kelner *et al.,* 1988; Salaris *et al.,* 1991). These electrons interact with oxygen to produce superoxide which leads to an increase in oxidative stress. MB reduces the formation of superoxide by acting as an alternative electron acceptor to oxygen, rummaging the leaking electrons (Hassan & Fridovich, 1979; Kelner *et al.,* 1988; McCord & Fridovich, 1969; Necula *et al.,* 2007a; Salaris *et al.,* 1991; Vutskits *et al*., 2008). Also, MB has shown to enhance the rate of

mitochondrial ATP production as well, without superoxide formation (Lindahl & Oberg, 1961; Tretter *et al*., 2014; Wen *et al.,* 2011). However, important to keep in mind is that the relieve of oxidative stress is dose dependant – MB given in to high doses may cause oxidative stress and act as a pro-oxidant (Oz *et al.,* 2009; Rojas *et al.,* 2012; Watts *et al*., 2013).

2.1.6. Current indications of methylene blue

MB is currently indicated for various types of conditions (Oz *et al.,* 2011; Watts *et al*., 2013). The US Food and Drug Administration (FDA) has approved the use of MB for enzymopenic hereditary methemoglobinemia and acute acquired methemoglobinemia, prevention of urinary tract infections in older patients, thyroid surgery, intraoperative visualisation of nerves, nerve tissues, and endocrine glands and of pathologic fistulae (Cawein *et al*., 1964; Küpfer *et al*., 1994; Oz *et al.,* 2011; Paban *et al.,* 2014; Schirmer *et al.,* 2011; Watts *et al*., 2013). There are currently 22 clinical trials registered which involves MB treatment (http//:clinicaltrials.gov). Topical MB is used as the treatment of choice for priapism as well as for intractable pruritus ani (Schirmer *et al.,* 2011). Much interest in MB lies in its potential as an antimalarial (Coulibaly *et al*., 2009; Lo *et al.,* 2014; Müller, 1996, 1998; Peter *et al*., 2000; Schirmer *et al*., 2003; Watts *et al*., 2013) and anti-AD drug. According to data gathered during previous studies, the conclusion was made that the redox cycling properties of MB and the resulting effects on the mitochondria, form the basis of the mechanism of action of MB. A hundred years after its discovery, studies on MB has shown much promise due to its nootropic and neuroprotective properties (Rojas *et al.,* 2012; Schirmer *et al*., 2003; Watts *et al*., 2013). In Table 2.1 a summary of dosing regimes for the most common indications of MB is given.

Table 2.1: A summary of the most common indications of methylene blue and the corresponding doses (Oz *et al.,* 2011).

Clinical indications of MB	Dose	Reference
Methemoglobinemias	1-2 mg/kg I.V.	Cawein *et al*., 1964; Oz *et al.,* 2011; Peter *et al.,* 2000; Schirmer *et al.,* 2011; Watts *et al*., 2013.
Ifosfamide-induced encephalopathy	50 mg I.V. every four hours until symptoms resolve	Alici-Evcimen & Breitbart, 2007; Küpfer, *et al.,* 1994; Necula *et al.,* 2007a;

		Pelgrims *et al.,* 2000; Schirmer *et al.,* 2011.
Treatment of vasoplegic syndrome	2 mg/kg 20-minute infusion time	Schirmer *et al.,* 2011; Shanmugam, 2005; Warth *et al.,* 2009.
Parathyroid imaging	3-7.5 mg/kg I.V.	Gordon *et al.,* 1975; Rowley *et al.,* 2009.
Sentinel lymph node biopsy	Local application of 1-5 ml 1% MB solution	Varghese *et al.,* 2007.
Treatment of malaria	10 mg/kg twice a day orally	Coulibaly *et al.,* 2009.
Urinary tract infections prevention in the elderly	65 mg/day three times a day orally	Schirmer *et al.,* 2011.

2.1.6.1. Methemoglobinemia

In the red blood cells of individuals with methemoglobinemia, methemoglobin is produced when a normal ferrous ion binds to the heme complex in haemoglobin and oxidises to a ferric ion. The inability of the ferric ion to interact with oxygen, causes hypoxia due to a lack of enough oxygen that is carried to organs (Cawein *et al*., 1964; Do Nascimento *et al*., 2008; McCord & Fridovich, 1969; Singh *et al*., 2012; Wright *et al.,* 1999). MB is known to be the first line of treatment for a variety of different methemoglobinemia (Atamna & Kumar, 2010; Cawein *et al*., 1964; Lo *et al.,* 2014; Oz *et al.,* 2011; Peter *et al.,* 2000; Watts *et al*., 2013). The enzymes that are present in red blood cells can reduce MB to LeucoMB. In its turn, LeucoMB reduces the inactive methemoglobin to hemoglobin (Cawein *et al*., 1964; Do Nascimento *et al*., 2008; McCord & Fridovich, 1969; Schirmer *et al.,* 2011; Singh *et al*., 2012). This forms the basis for the mechanism by which inborn enzymopenic methemoglobinemia is treated. However, to produce sufficient amounts of LeucoMB, red blood cells should host a great number of reduced NADPH for this treatment to be effective (Do Nascimento *et al*., 2008; Singh *et al*., 2012).

2.1.6.2. Encephalopathy

A variety of cancer and related illnesses are treated with the well-known alkylating agent named ifosfamid (Alici-Evicimen & Breitbrat, 2007). The severity of ifosfamid associated side effects are also well-known to medical authorities, however, after the consideration of risk versus benefit, many patients are still treated with ifosfamid. Among these, the most alarming side effect is neurological toxicity presented as encephalopathy (Aeschlimann *et al*., 1996; Küpfer *et al*., 1994,

1996; Watts *et al*., 2013). The co-administration of MB has shown to not only treat, but also prevent ifosfamide associated encephalopathy (Aeschlimann *et al*., 1996; Alici-Evcimen & Breitbart, 2007; Ajithkumar *et al*., 2007; Atamna & Kumar, 2010; Lo *et al.,* 2014; Oz *et al*., 2011; Querfurth & LaFerla, 2010; Paban *et al.,* 2014; Rojas *et al.,* 2012; Schirmer *et al.,* 2011). As mentioned before, MB serves as an alternative electron acceptor, scavenging leaking electrons from the mitochondrial respiratory chain. As a result, the abnormal ratio of nicotinamide adenine dinucleotide (NAD+) to NADH that occurs with ifosfamid therapy, is restored (Ajithkumar *et al*., 2007; Küpfer *et al*., 1996; Oz *et al*., 2011). In addition, abnormal hepatic glucose production and intracellular redox balance is also restored (Ajithkumar *et al*., 2007; Küpfer *et al*., 1996; Oz *et al*., 2011). Therefore, MB is indicated as prophylaxis for patients that receives ifosfamid therapy one day prior to chemotherapy (Aeschlimann *et al*., 1996; Di Cataldo *et al*., 2009; Küpfer *et al*., 1996; Necula *et al.,* 2007a; Pelgrims *et al*., 2000).

2.1.6.3. Psychotic disorders

An imbalance of neurotransmitters in the central nervous system, especially dopamine, causes a psychotic disorder known as schizophrenia (Carlsson *et al*., 2001). A dopamine imbalance is usually the result of a cascade of events which includes altered mitochondrial function, altered glutamate activity, oxidative stress and immune-inflammatory reactions (Moller, *et al.,* 2011, 2013). In a study done by Klamer *et al.* (2004), it was discovered that MB is effective in treating psychotic behaviour in an animal model of schizophrenia (Atamna & Kumar, 2010). These animals presented with psychotic effects such as hyperactivity, increased stereotypic behaviours and episodic explosive jumping or popping that were remarkably reduced by MB (Deutsch *et al*., 1993). Knowing that NO may be involved in regulating glutamate, serotonin and dopamine mediated neurotransmission, the theory of using NOS inhibitors, such as MB, and NO-cGMP pathway modifiers in the treatment of psychotic disorders is established (Kano *et al*., 1998; Wegener *et al*., 2000; Smith & Whitton, 2000; 2001). Due to the involvement of NO and the NO-cGMP cascade in various neuropathological disorders including schizophrenia (Das *et al*., 1995; Karatinos *et al*., 1995; Karson *et al*., 1996; Bernstein *et al*., 2011), the reduction in psychotic behaviour of these animals suggests that if NOS or NO function is inhibited, psychotic symptoms may be reduced.

2.1.6.4. Mood disorders

Since 1899, MB have been used as treatment against a variety of neuropsychiatric illnesses (Coulibaly *et al*., 2009; Lo *et al.,* 2014; Watts *et al*., 2013). Depression is a serious mental disorder due to its recurrent nature (Narsapur & Naylor, 1983; Oz *et al*., 2012; Reif *et al*., 2006). Evidence

that MB may exhibit antidepressant and anxiolytic effects were reported during a pre-clinical experiment done by Eroglu & Caglayan (1997). Another test done by Narsapur and Naylor (1983) supported these findings when the symptoms of patients suffering from manic depression, were improved. A follow up study done by Naylor *et al*. (1987), revealed that a chronic dose of 15 mg MB per day can alleviate the symptoms of severely depressed patients. A test done by Harvey *et al.* (2010) on animal models of depression, also supported the postulation that MB exhibits anti-depression effects. These effects may be mediated by many mechanisms, where the increase of certain monoamines in the synaptic cleft due to the inhibition of MAO-A, is one of them (Aeschlimann *et al*., 1996; Harvey *et al*., 2010). This mechanism is supported by studies done by Volke *et al*. (1997) and Wegener *et al*. (2000). The monoamine hypothesis of depression, where unbalanced and decreased levels of monoamines in the central nervous system causes depression, is supported by the mechanisms by which MB relieves depression symptoms (Hyman & Nestler, 1993; Randrup & Braestrup, 1997; Schildkraut, 1965). Another hypothesis of depression is based on the formation of NOS in the NO-cGMP pathway (Dhir & Kulkarni, 2011; Eroglu & Caglayan, 1997; Harvey *et al*., 1990; 1994; Suzuki *et al.,* 2001). MB exhibits the ability to modulate this pathway by the inhibition of NOS, thus relieving depression symptoms (Luo *et al*., 1995; Moore & Handy, 1997; Volke *et al*., 1999).

2.1.6.5. Malaria

The affordability, accessibility and availability of MB have recently increased the interest of authorities in MB as a possible treatment of malaria (Akoachere *et al*., 2005; Coulibaly *et al*., 2009; Lo *et al.,* 2014; Müller, 1996, 1998; Peter *et al*., 2000; Walter-Sack *et al.,* 2009; Watts *et al*., 2013). The success in controlling any disease lies in preventing the pathogen from spreading. A study on the life cycle of the *Plasmoduim falciparum* parasite allowed scientists to identify the form responsible for transmission, known as gametocytes. Many drugs were designed for the treatment of malaria, but only a few of them are active against gametocytes. However, MB has shown to successfully kill *Plasmoduim falciparum* gametocytes as well as *in vitro* and *in vivo* malaria (Coulibaly *et al*., 2009; Vennerstrom *et al*., 1995). Parasitic disulphide reductase flavoenzymes known as glutathione reductase and thioredoxin reductase, is the main target on which MB has its anti-malarial effect (Buchholz *et al*., 2008; Färber *et al.,* 1998; Haynes *et al*., 2010). The effect is accomplished by the conversion of MB to LeucoMB due to the interaction of MB with the reduced flavoenzyme cofactor known as flavin adenine dinucleotide ($FADH_2$). Due to ROS production, LeucoMB is converted back to MB by oxidation, depleting reduced NADPH which is required for the reduction of FAD to $FADH_2$ (Buchholz *et al*., 2008; Färber *et al.,* 1998). As a result, the ability of the parasite to counter oxidative stress is significantly weakened which leads to parasite death. A big advantage associated with MB therapy, is its effectiveness and

safety in patients suffering from glucose-6-phosphate dehydrogenase deficiency. Recently, the combination of artemisinin and MB have shown much potential. It was discovered that these two drugs potentiate each other's mechanism of action, enabling them to demolish malaria parasites (Akoachere *et al*., 2005; Zoungrana *et al*., 2008).

2.2. Alzheimer's disease and related neurodegenerative disorders.

2.2.1. General background

Dementia is very common under elderly people (Barten & Albright, 2008; Gura, 2008; Swaab, 2014). AD, which is the leading cause of dementia (60-80% cases), is an illness characterised by progressive cognitive impairment and extreme neuropsychiatric disruptions (Barten & Albright, 2008; Gura, 2008; Necula *et al.,* 2007a; Oz *et al.,* 2009, 2011; Paban *et al.,* 2014; Scarpini *et al.,* 2003; Stoppelkamp *et al.,* 2011). AD is among the most traumatic illnesses, not only for the person suffering from it, but also for the family and caregivers. In comparison to normal ageing of the brain, an AD patient's brain function deteriorates abnormally fast and to an extent where the person becomes infant-like, completely depended on others and not able to control certain bodily functions (Atamna & Kumar, 2010; Swaab, 2014). A patient's personality changes with the progression of the disease until its completely lost. The family is then left with a physical body of their loved one without a personality, that needs taking care of. Normal brain aging and AD have a lot in common, for instance, the changes that occurs in the brain of an AD patient is also observed in the brain of a healthy individual. The difference however, lies in the extend of brain deterioration and the age of onset (Swaab, 2014).

2.2.2. Possible causes of Alzheimer's disease

The exact cause of AD remains unknown; however, theories have been developed based on typical pathological changes that was observed during autopsies on AD brains. In the last few decades the possibility of a genetic component in AD have also been studied (Velez-Pardo *et al.,* 1998). Only an estimated 1% of AD cases are inheritable due to mutations found on the gene for Beta Amyloid Precursor Protein (β-APP) and presinilin 1 and 2. Another 17% of AD cases are caused by an inheritable factor known as apolipoproteinE-ε4 (Barten & Albright, 2008; Swaab, 2014). Having the apolipoprotein E-ε4 gene, does not necessarily mean that the person will eventually have AD, but this is the gene that has the biggest impact regarding the genetic risk of possibly developing AD (Alz.org., 2017). However, an agreement is made stating rather than one cause, complex interactions between a variety of risk factors leads to the development of AD (Alz.org., 2017, Swaab, 2014). The most important risk factors include old age, genetic composition and family history, however, other factors such as a lack of exercise and healthy

eating, high blood pressure and environmental factors also plays a role (Alz.org., 2017). Recently, the main interest lies in the deposition of extracellular amyloid beta protein as a possible cause for AD (Akoury *et al.,* 2013; Atamna & Kumar, 2010; Barten & Albright, 2008; Necula *et al.,* 2007b; Swaab, 2014). The stacking of this toxic protein is caused by various gene-environment interaction related risk factors. As a result of amyloid toxicity, tau proteins responsible for stabilisation of microtubules, loses their form, starts lumping together and collapses, which leads to the formation of tangles (Akoury *et al.,* 2013; Alz.org., 2017; Barten & Albright, 2008; Swaab, 2014). These tangles interfere with the functionality of neurons to such an extent that they eventually die (Mudher & Lovestone, 2002; Necula *et al.,* 2007b; Paban *et al.,* 2014). These pathological findings are typically seen when autopsies are done on AD brains, which supports the amyloid hypothesis. Ultimately dementia sets in due to mass cell death. The disease is spread through the brain, due to the transfer of the toxic amyloid protein from dying cells to nearby tissue. According to the amyloid-cascade-hypothesis, AD progression always follows a fixed pattern starting in the entorhinal cortex where after it spreads to the limbic system and eventually to the brain cortex (Necula *et al.,* 2007b; Swaab, 2014).

Another hypothesis states that AD is merely an accelerated form of brain ageing. Swaab (2014) agrees with this argument. In his book he compares brain cells with the engine of a vehicle. Just as an engine wears itself down over time, brain cells damage themselves similarly. However, brain cells can restore the damage to some extent, but never completely. The leftover damaged cell pieces stack up over time, forming the foundation of the ageing process. Brain ageing will be quicker and more severe in people that underwent trauma to the brain or in cases where the brain is not able to restore damaged cells fast enough, which leads to the formation of plaques and tangles. In these cases, AD will start to develop (Swaab, 2014).

2.2.3. Current treatments of Alzheimer's disease

The basis for drug development was the "cholinergic hypothesis" (see 2.1.5.1). The drug designs focus on drugs with the ability to restore and optimise neurotransmitter levels that are abnormal in AD (figure 2.9) (Scarpini *et al.,* 2003; Wischik *et al.,* 2008). For example, drugs that are capable of increasing Ach levels and/or enhance the stimulation of nicotinic and muscarinic receptors. According to Scarpini *et al.* (2003) the discrepancies seen in early stages of AD are proven to be the cause of insignificant signalling that occurs in the cholinergic system.

Drugs already available for the treatment of AD:

- AChE inhibitors: These drugs improve cognitive as well as behavioural symptoms associated with AD (Scarpini *et al.,* 2003).
- Traditional N-Methyl-D-aspartate (NMDA) glutamate receptor blockers (Atamna & Kumar, 2010).
- Memantine: This drug has only recently been approved. It belongs to the class of glutamate modulating drugs. Its mechanism of action is based on the blockage of NMDA channels (Scarpini *et al.,* 2003).

Due to a lack of effective AD treatment, AChE inhibitors are given to relieve some AD-related symptoms, but it does not work effectively (Scarpini *et al.,* 2003). However, due to its tolerability in comparison to drugs such as muscarinic agonist, presynaptic releasing agents and precursors of AChE, they are the recommended first choice treatment for patients suffering from mild to moderate AD (Scarpini *et al.,* 2003). However, the drugs currently available for the treatment of AD, does not treat the underlying cause (Alz.org., 2017).

Many other approaches have recently been researched, such as amyloid modulating drugs that includes metal chelates, cholesterol lowering- and anti-inflammatory drugs. Also, inhibitors of amyloid precursor protein (APP), β- and γ-secretase (Scarpini *et al.,* 2003).

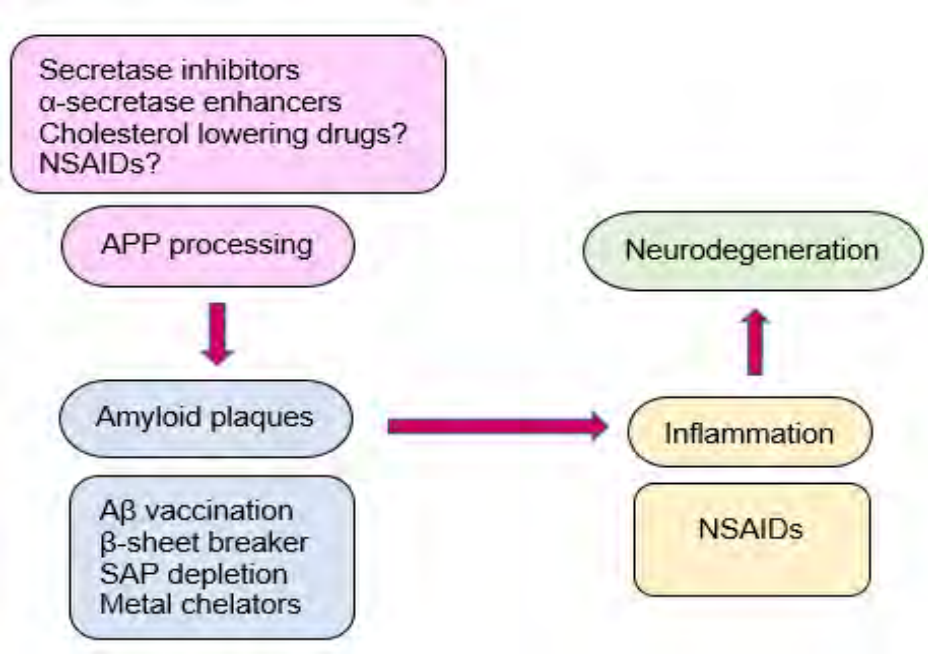


Figure 2.9: Generally accepted hypothesised pathology and associated treatment in Alzheimer's disease (Scarpini *et al.,* 2003).

NSAIDs: nonsteroidal anti-inflammatory drugs; APP: amyloid precursor protein; SAP: serum amyloid P component

Other possible targets for the development of future drugs include:

- Beta amyloid
- Tau protein
- Beta-secretase (BACE)
- The 5-HT6 receptor and
- Inflammation.

2.2.4. Methylene blue and Alzheimer's disease

Due to the lack of finding drugs that are effective against AD and relatively safe in terms of side effects, the possibility of using MB have recently been a major point of interest (Atamna & Kumar, 2010; Gura, 2008; Sullivan, 2008; Wischik *et al.,* 2008). Studies done on healthy animals and humans have shown promising results regarding the ability of MB to improve memory and slow down AD progression (Atamna & Kumar, 2010; Luna-Muñoz *et al*., 2008; Oz *et al.,* 2009; Rojas *et al.,* 2012; Sullivan, 2008; Watts *et al*., 2013). MB have also shown to inhibit of Aβ peptides and tau protein aggregation (figure 2.10 and 2.11) (Akoury *et al.,* 2013; Cawein *et al*., 1964; Louters *et al*., 2006; Luna-Muñoz *et al*., 2008; Necula *et al.,* 2007a; Paban *et al.,* 2014; Schirmer *et al.,* 2011; Sullivan, 2008; Wischik *et al.,* 2008). Also contributing to the benefits of using MB in AD, is its ability to inhibit AChE (Petzer *et al*., 2014; Pfaffendorf *et al*., 1997; Wischik *et al.,* 2008). Another mechanism of MB to counter AD symptoms lies in its ability to inhibit Aβ42 oligomerization and Aβ42 fibrilisation (Akoury *et al.,* 2013; Necula *et al*., 2007; Oz *et al.,* 2009; Taniguchi *et al*., 2005; Wischik *et al*., 1996). In a review done by Atamna & Kumar (2010), the ability of MB to increase cytochrome c oxidase (complex IV) and heme synthesis as well as improve mitochondrial respiration, have also been proven.

A study done by Paban *et al.* (2014) which involved APP/PS1 mice that presented with memory and learning deficits associate with beta amyloid accumulation and dysregulatory metabolic function due to old age. MB was administered to these mice for a period of three months. Paban *et al.* (2014) observed that the mice that received MB treatment, showed improved spatial and non-spatial memory, had an increase in social behaviour and were not as apathic. Paban *et al.* (2014) also concluded that MB given in doses ranging from no more than 1-4 mg/kg (Clifton. & Leikin, 2003) leads to memory improvement in many ways which includes: spatial learning, inhibitory avoidance, object recognition, habituation to familiar environment and discriminational memory. Also indicated in the study was the hormetic dose-response behavioural effects that MB exhibits in normal rats. These findings supported results gathered in a previous study done by Bruchey and Gonzales-Lima (2008). In another MB-related study, Medina *et al.* (2010) discovered that soluble beta amyloid levels in triple transgenic Alzheimer's disease (3xTg-AD) mice were

remarkably decreased with the treatment of MB. Other mechanisms of action by which MB counters the symptoms of AD were proposed due to its pleiotropism (Schirmer *et al.,* 2011). It is postulated that the conversion of MB between the oxidised and reduced forms (Akoury *et al.,* 2013), enables it to act as a neuroprotective agent against neurodegeneration, operating via mitochondria and cytochrome c oxidase (Schirmer *et al.,* 2011).

According to Akoury *et al.* (2013), MB has been used in phase 2 clinical trials on humans and have recently been progressed to phase 3 clinical trials. Schirmer *et al.* (2011) postulates that if the effects of MB can be confirmed, MB can possibly be taken prophylactically for many years against AD and other neurological disorders (see Table 2.2).

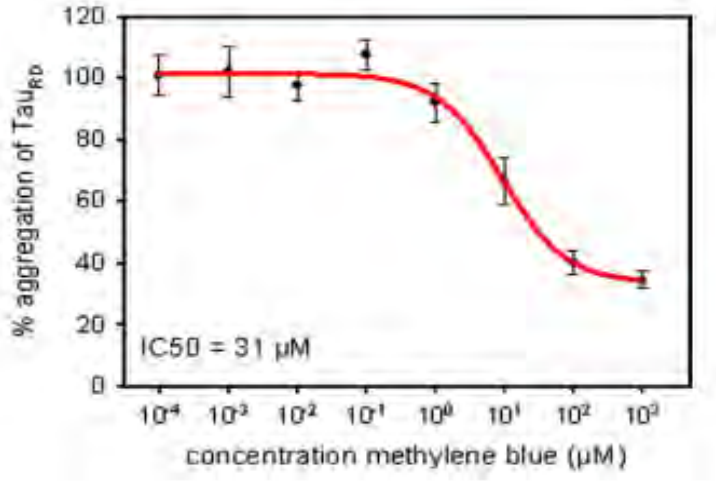


Figure 2.10: Methylene blue inhibits the aggregation of tau protein (Schirmer *et al.,* 2011).

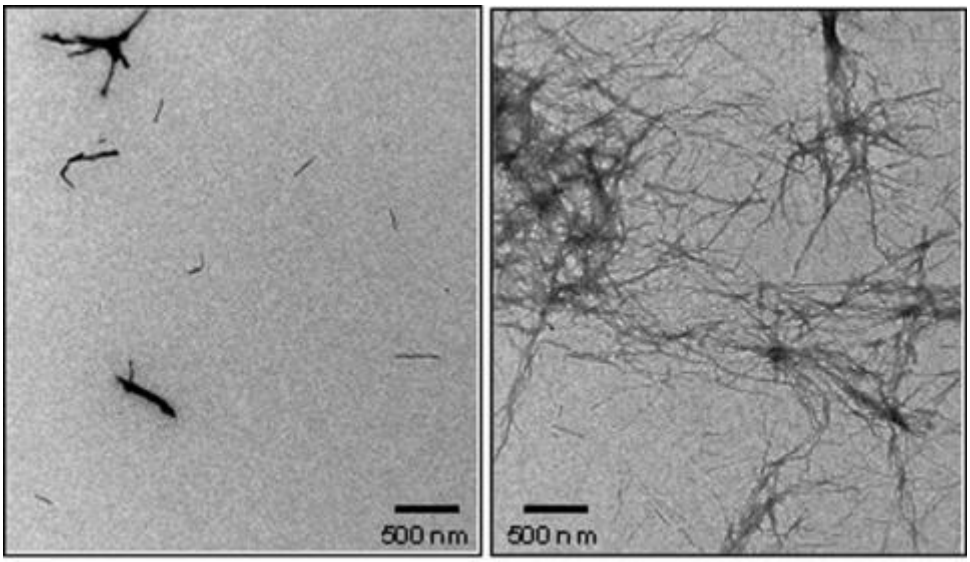


Figure 2.11: Tau filaments as seen on electron micrographs taken before and after the administration of methylene blue (Schirmer *et al.,* 2011).

Table 2.2: Summary of possible nervous system related indications of methylene blue and their corresponding doses (Oz *et al.,* 2011, Schirmer *et al.,* 2011).

Potential indication of MB in clinical neuroscience	Dose	Reference
Alzheimer's disease	10 – 12 nM *in vitro* *(60 mg three times a day according to* Wischik *et al.,* 2008*)*	Atamna *et al.,* 2008; Hattori *et al.,* 2008; Necula *et al.,* 2007a,b; Taniguchi *et al.,* 2005; Wischik *et al.,* 1996.
Depression and anxiety	50 mg per day in patients (2-60 mg/kg I.P. in rats)	De-Oliveira & Guimaraes, 1999; Eroglu & Caglayan, 1997; Naylor *et al.,* 1986; 1987; Patil *et al.,* 2005; Volke *et al.,* 2003.
Psychosis	100 mg twice per day orally or 520 mg once per day orally in patients (32-100 mg/kg I.P. in rats)	Deutsch *et al.,* 1997; Narsapur & Naylor, 1983; Naylor *et al.,* 1986; 1988; Thomas & Callender, 1985.
Cognitive deficits	(0.15-4 mg/kg I.P. in rats)	Callaway *et al.,* 2002, 2004; Deiana *et al.,* 2009; Gonzalez-Lima & Bruchey, 2004; Wrubel *et al.,* 2007.
Neuroprotection	(70 µg/kg locally injected in rats)	Zhang *et al.,* 2006; Rojas *et al.,* 2009.
Pain	1 ml 1% MB solution locally in patients (20 mg/kg I.P. in rats)	Peng *et al.,* 2007.
Intractable itching	10-15 ml 0.5-1% MB solution locally in patients	Eusebio *et al.,* 1990; Mentes *et al.,* 2004; Sutherland *et al.,* 2009.

2.3. Summary

In this chapter the unique characteristics in terms of structure that MB exhibits, enabling it to take part, modulate, influence and inhibit certain processes associated with a variety of diseases, was discussed. The interesting redox chemistry of MB allows it to penetrate through different lipid membranes, extending its area of action and increasing the number of targets and mechanisms by which its action is established in the body. Also, different theories by which Alzheimer's disease is caused is briefly discussed along with the typical pathology associated with the disease and possible targets for drug delivery. The potential of MB as a possible drug against AD, have recently been highlighted (Harvey *et al*., 2010; Petzer *et al*., 2012; Querfurth & LaFerla, 2010; Ramsay *et al*., 2007). However, it has been established that pure MB is not commercially available, which influences all previous experiments done and conclusions made on the efficiency of MB as a treatment against AD (Schirmer, *et al.,* 2011). Instead, it remains unknown whether the neuroprotective and memory enhancing effects are associated with the action of MB or of azure B and what the influence of these metabolites are on each other's actions. Although it has been demonstrated that azure B has superior neuroprotective and memory enhancing abilities in comparison to MB, it is not clear which compound mediates the effects with the administration of commercially available MB, since a batch of MB is contaminated with a great amount of azure B. Due to the growing interest in MB and its metabolites as a possible AD treatment, the separation of MB from its metabolites will open many doors in regards to identifying which metabolite is responsible for the actual effect and how the purity of the compound has affected previous studies. Thus, the current study will attempt to design a simple and cost effective analytical method to separate MB from its metabolites to ensure more accurate results in future studies on MB and its metabolites.

CHAPTER 3 - THIN LAYER CHROMATOGRAPHY

3.1. General background.

Chromatography is a group of analytical techniques used for the purpose of separating and identifying individual compounds in a mixture (Clark, 2007b; Hansen *et al.,* 2012). The simplest of these techniques is thin layer chromatography (TLC) due to the fact that additional apparatus is not needed to move the mobile phase (MP) through the stationary phase (SP). A short definition for TLC is the analyses of mixtures by the separation of each compound present in the mixture (Clark, 2007b). Many samples can be placed on one TLC plate at the same time, making the technique fast in comparison to other chromatography techniques (Hansen *et al.,* 2012). For many years, TLC have been used to identify possible impurities that are present in synthesised drugs. Different stationary phases are commercially available and accessible, making this technique very popular and cost effective (Hansen *et al.,* 2012).

According to Hansen *et al.* (2012), the first choice of chromatography is normal phase chromatography (NPC) where the MP consists of organic solvents and the SP is made of a polar silica material (Clark, 2007b). However, lately the implementation of reverse phase chromatography (RPC) has increased in popularity. During RPC an aqueous MP in combination with a less polar SP is used (Hansen *et al.,* 2012). Different detection methods are available for the visualisation of the compounds after separation by TLC. Compounds with specific properties can be visualised by specific methods for instance, a compound with florescence ability can be visualised by UV-light (Clark, 2007b; Hansen *et al.,* 2012). Another advantage of this technique is the ability of it to display all the components that are present in a mixture, including those that have a strong interaction with the SP, displaying no or only a short migration distance. In comparison to other techniques such as HPLC, the detection limits associated with TLC are much higher making it impossible for volatile substances to be analysed (Hansen *et al.,* 2012).

Silica gel is the most common material used as a SP in TLC (figure 3.1). For more polar compounds, a cellulose SP is used. Because of the similarity between the silica used in TLC and high performance liquid chromatography (HPLC), the TLC plates that contains silica as a SP can be used in NPC and RPC (Hansen *et al.,* 2012). Sometimes the SP may contain certain chemical groups to facilitate the separation of compounds that have certain chemical properties. The modification of the silica gel group's chemical properties will allow us to create a more hydrophobic SP. In RPC the silica is modified by the addition of octyl or octadecyl groups and is used in combination with a polar MP such as acetonitrile or methanol (Hansen *et al.,* 2012).

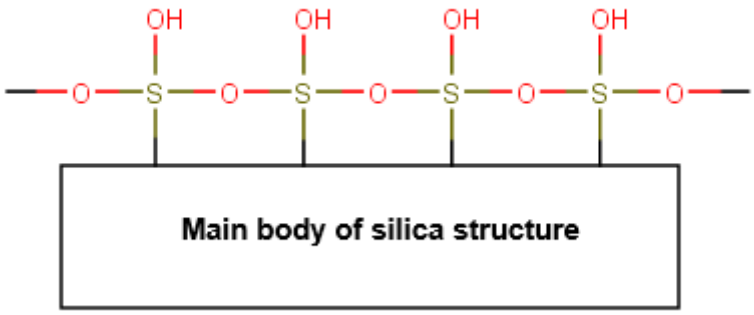


Figure 3.1: Illustration of the surface of silica gel (Clark, 2007b).

The silica atoms on the surface of the SP is bound to hydroxyl molecules instead of oxygen atoms, making the surface very polar (Clark, 2007b). The hydroxyl molecules allow the SP to form hydrogen bonds with certain compounds. Also, the presence of the hydroxyl molecule enables the SP to interact with certain analytes by van der Waals forces and dipole-dipole forces (Clark, 2007b).

Volatile liquids that are able to evaporate after the separation is used as mobile phase, thus the influence of spectroscopic characteristics such as UV transparency that plays a role in HPLC, is excluded (Hansen *et al.,* 2012). There are also no limitations in regards to pH due to the fact that TLC plates are only used once. Both the MP and the chemical properties of the analytes have an influence on retention. The MP's influence on the retention correlates with its solvent strength. For optimum retention that range from 0.2-0.8, the solvent strength of the MP should be adjusted accordingly (Hansen *et al.,* 2012). The retardation factor (Rf) is calculated by dividing the distance travelled by the spot from of the starting point to its centre, with the distance of the front line of the MP. The Rf value is used for identification purposes. When the same experiment is repeated under the exact same circumstances, the Rf value of each compound would be the same (Clark, 2007b). However, any change such as temperature, solvent composition etc, would affect the Rf values. Low Rf values are seen when the solvent is too weak, thus strong retention is observed. Where the opposite is true for strong solvents. Usually a mixture of solvents with different solvent strengths are combined for optimum selectivity and retention. When an analyte consists of very polar groups, its retardation would be very significant due to its affinity to the more polar SP. For example, analytes containing amino groups are strongly retarted due to its strong interaction with the acid silanol groups of the SP, sometimes resulting in tailing. To counteract or suppress this observation the MP should be adjusted by either adding a basic solvent such as ammonia or a volatile amine such as diethylamine, or by adding a volatile acid such as formic acid or acetic acid (Hansen *et al.,* 2012). The added acid will hinder the ionisation of the silanol groups and as a

result the ionic interaction will be decreased. When RPC is implemented, hydrophobic analytes now have a greater affinity for the SP which is less polar than NPC, and hydrophilic analytes will elute easier and quicker. Water-based mobile phases are used in RPC, thus solvents which are not miscible with water cannot be used (Hansen *et al.,* 2012).

3.2. Procedure (figure 3.2)

- A pencil line is drawn 1-2 cm from the bottom edge of the TLC plate. Any labelling should also be done in pencil (Clark, 2007b).
- A tiny dot is made on the pencil line with sample solvent, using a micropipette (Hansen *et al.,* 2012).
- To compensate for possible band broadening, samples should not be placed less than 1cm apart from each other (Hansen *et al.,* 2012).
- After the solvent in which the sample was dissolved, has evaporated, the TLC plate is then placed inside a beaker that contains a shallow layer of MP (Clark, 2007b; Hansen *et al.,* 2012).
- Important to remember is that the MP should not cover the pencil line that contains the analyte. Also, the beaker should be covered to ensure that the system is saturated with the vapour of the MP and that the MP will not evaporate from the TLC plate during the separation procedure (Clark, 2007b).
- The mobile phase will slowly travel upwards on the plate (Clark, 2007b; Hansen *et al.,* 2012). The compounds that are present in the sample mixture, will move with the MP at different rates (see figure 3.3), mediating the chromatographic separation process (Clark, 2007b).
- The plate is removed from the tank when the MP has migrated at least two-thirds or three-quarters of the length of the plate and the position of the MP front is marked with pencil (Clark, 2007b; Hansen *et al.,* 2012).
- Depending on the properties of the compounds that were present in the samples, different methods can be implemented for visualisation purposes (Hansen *et al.,* 2012).
- The retention factor (Rf) is calculated to identify compounds (see figure 3.4) (Clark, 2007b; Hansen *et al.,* 2012).

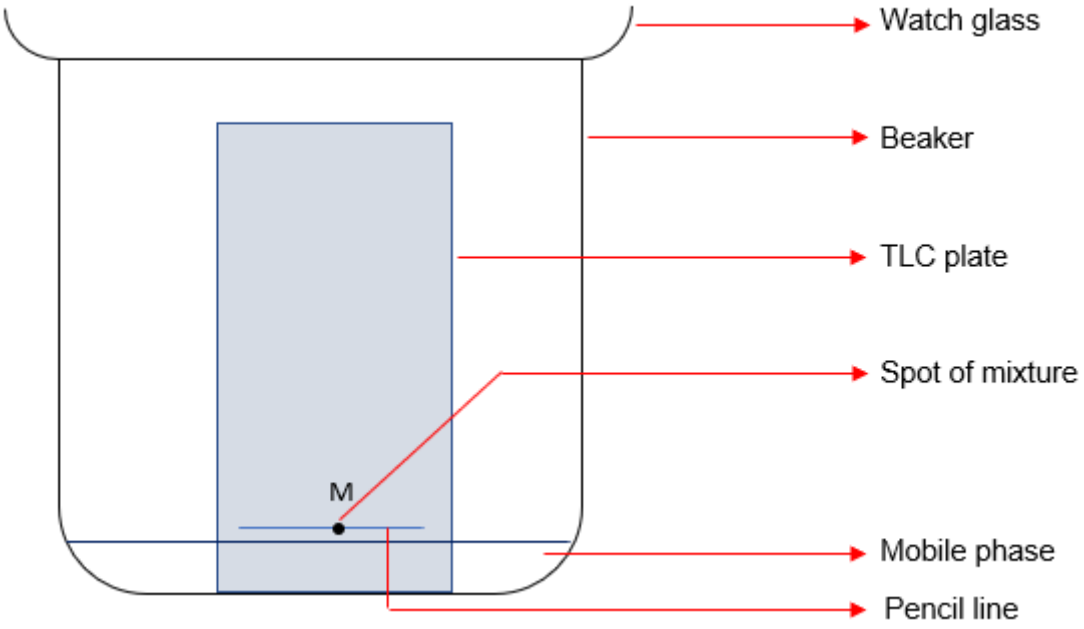


Figure 3.2: Simple illustration of how the TLC system is set up (Clark, 2007b).

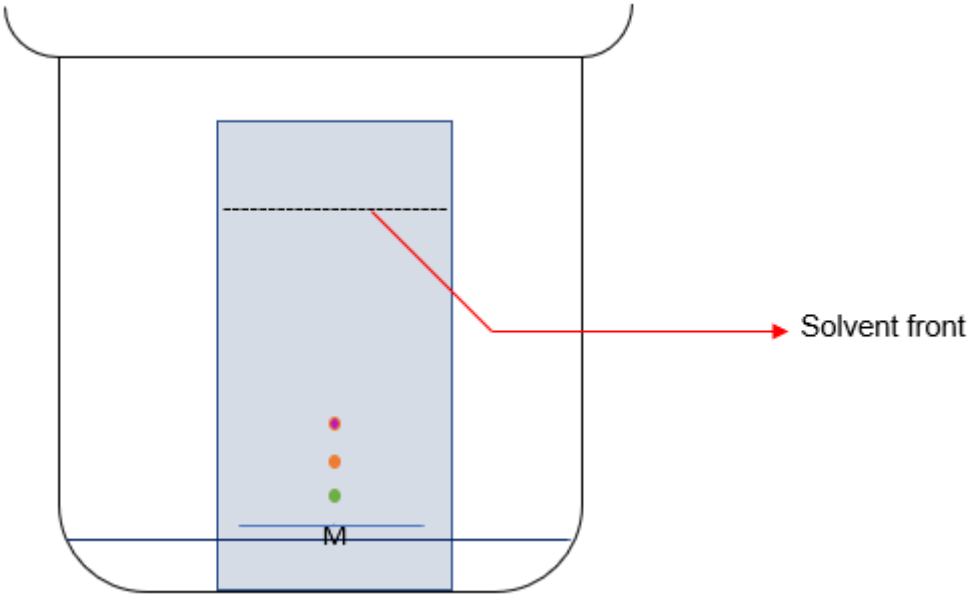


Figure 3.3: Illustration of what the scientist will see during development of the TLC chromatogram (Clark, 2007b).

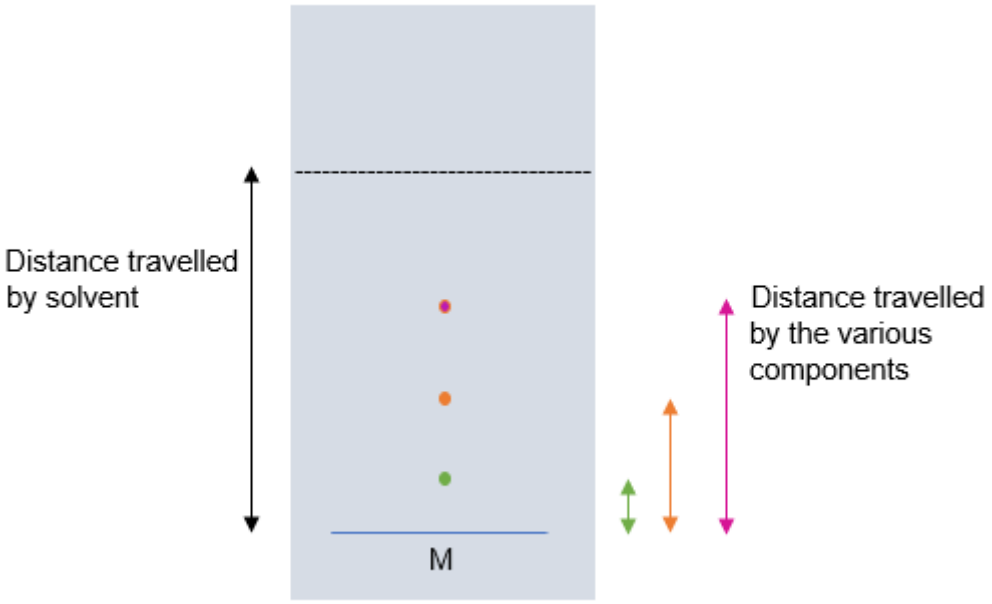


Figure 3.4: Measurement of each component's Rf value for the purpose of identification (Clark, 2007b).

3.2. Method development.

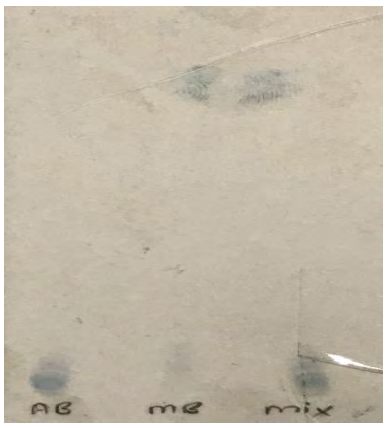
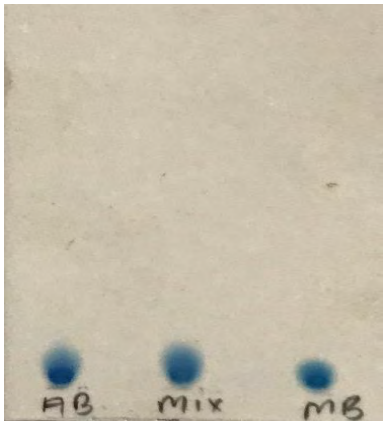
Using NPC TLC, a trial-and-error method was used to determine the MP and solvent that would possibly separate the compounds from each other. At first, the sample concentration was not taken into consideration, and therefore misleading conclusions that the compounds could not be separated by TLC, was made (refer to Addendum A).

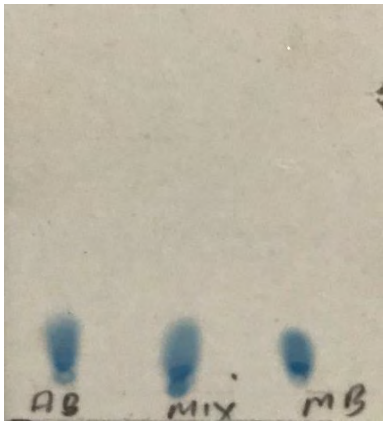
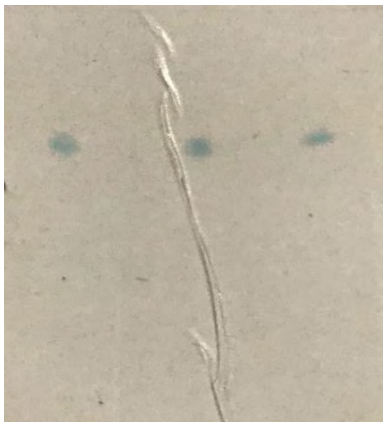
As a result, a known sample concentration of 20 µg/ml was then used of RPC TLC plates and separation of the compound was observed when a suitable MP was identified (Table 3.1).

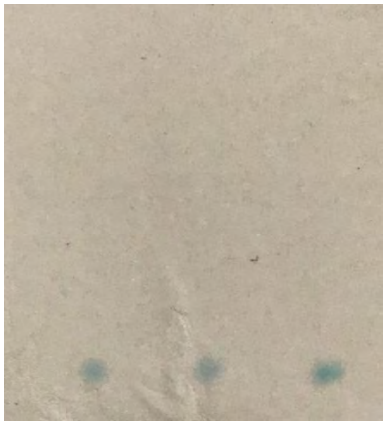
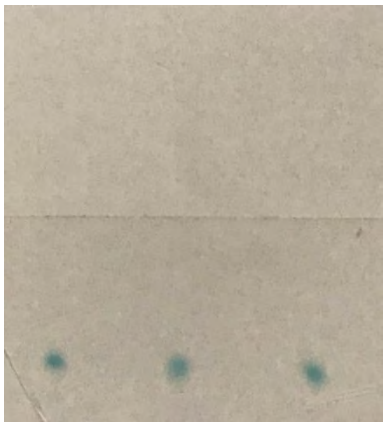
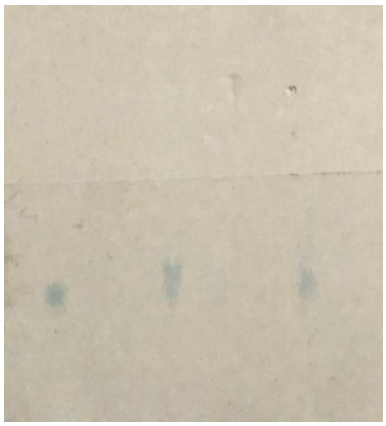
NPC TLC plates were again tested for effective separation and the results are shown in Table 3.2.

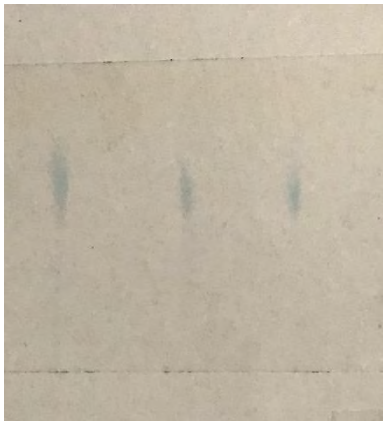
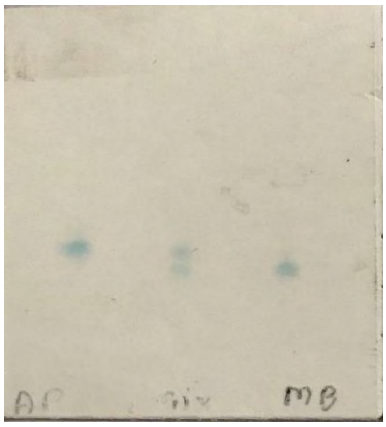
3.3. Results

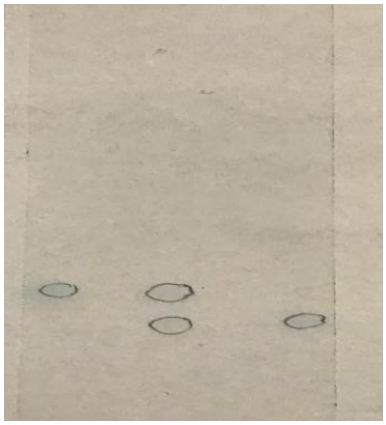
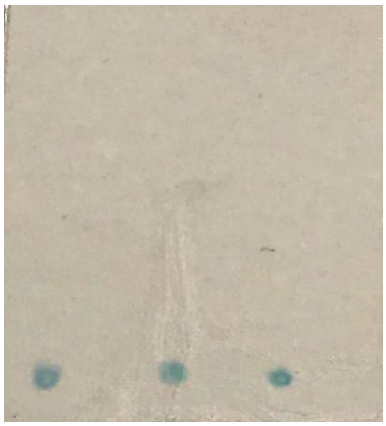
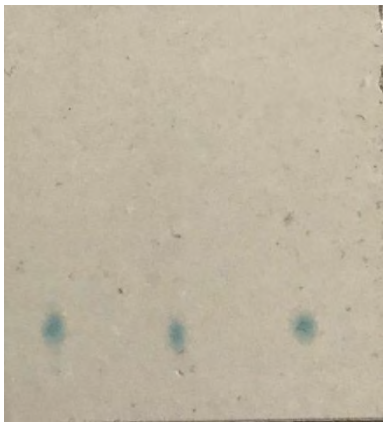
Table 3.1: Results for reverse phase TLC (Known sample concentration of 20µg/ml).

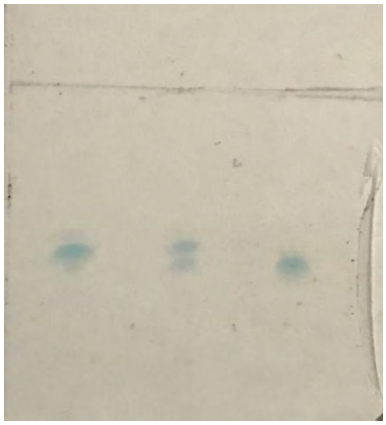
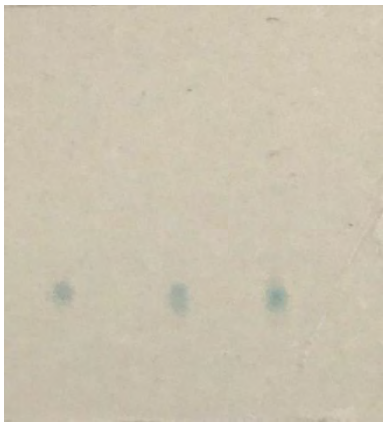
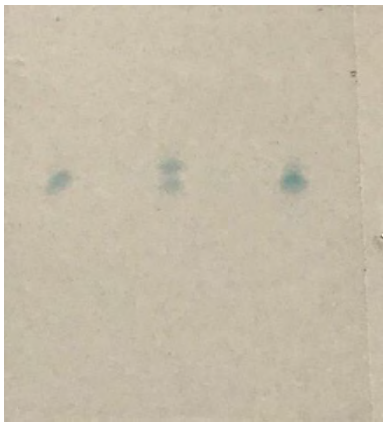
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:1 5 mM ammonium acetate in 10:90 water-methanol to acetonitrile
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:1 5 mM ammonium acetate in 10:90 water-methanol to 50:50 acetonitrile-methanol

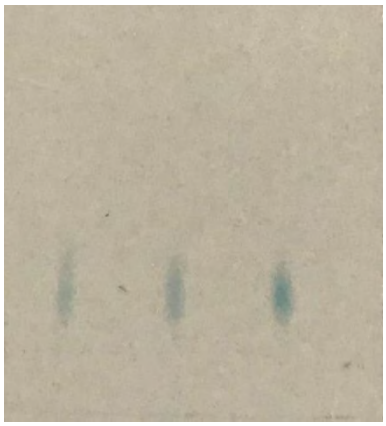
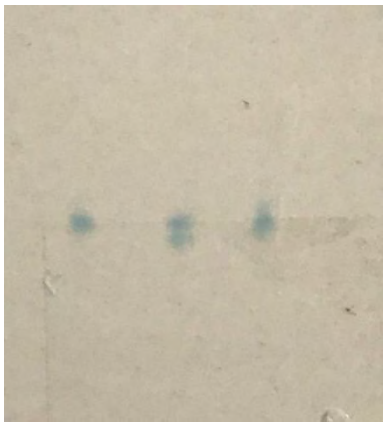
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:3 5 mM ammonium acetate in 10:90 water-methanol to acetonitrile
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 chloroform to methanol
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 chloroform to 50:50 acetonitrile-methanol

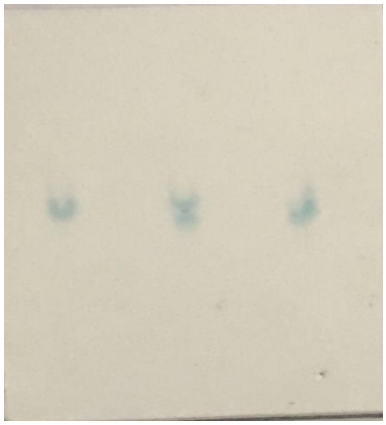
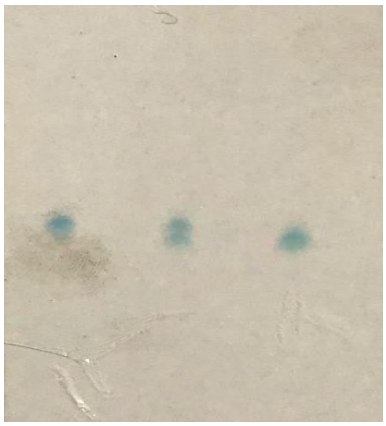
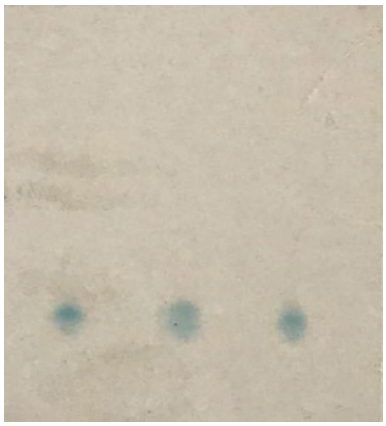
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 5 mM ammonium acetate in 10:90 water-methanol to 50:50 acetonitrile-methanol
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:1 5 mM ammonium acetate in 10:90 water-methanol to 50:50 acetonitrile-methanol to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 5 mM ammonium acetate in 10:90 water-methanol to chloroform

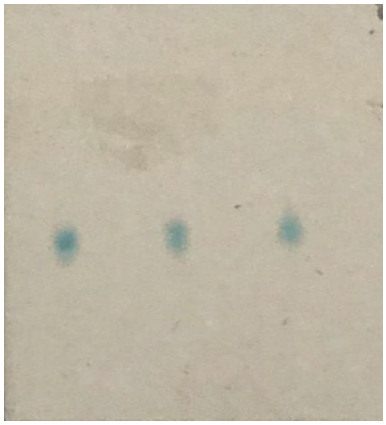
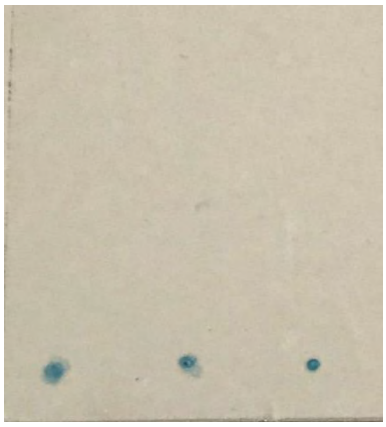
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 dichloromethane to 50:50 acetonitrile-methanol
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 dichloromethane to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane

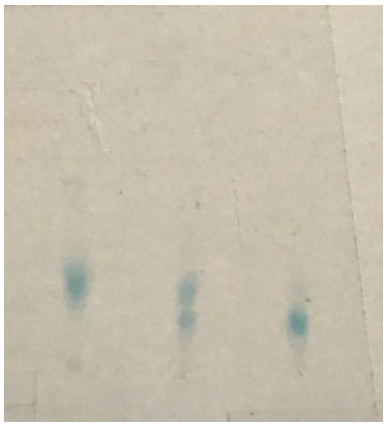
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 10 mM ammonium acetate in 10:90 water-methanol to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to 50:50 acetonitrile-methanol

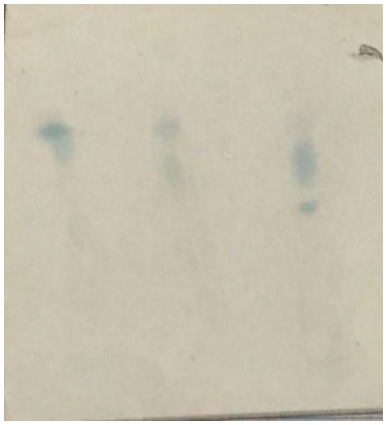
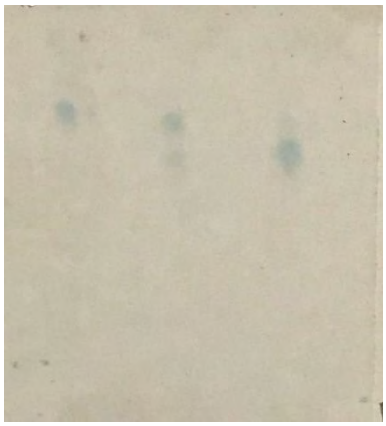
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:2 5 mM ammonium acetate in 10:90 water-methanol to chloroform to dichloromethane

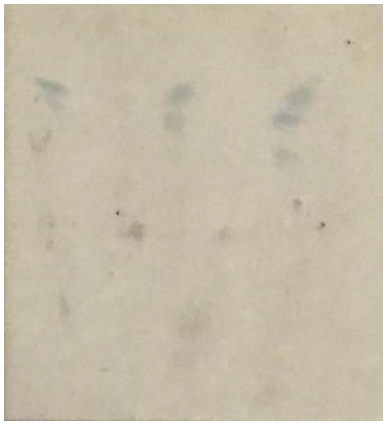
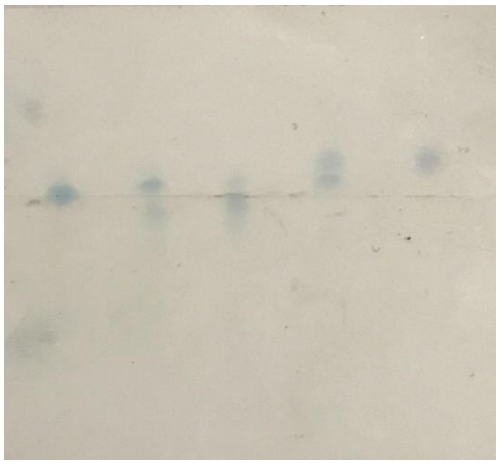
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:2:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:0.5:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to chloroform to ammonium acetate
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to chloroform

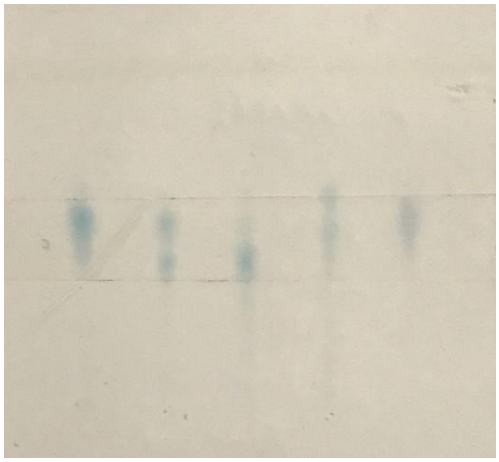
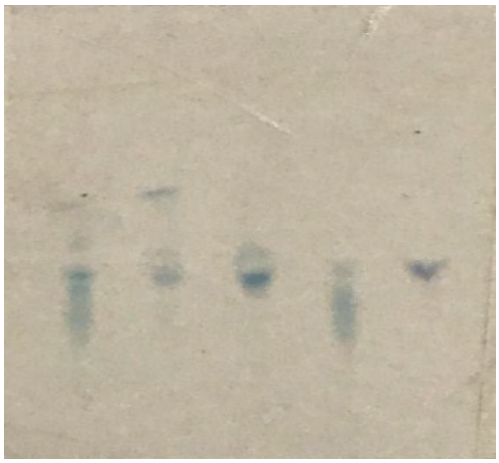
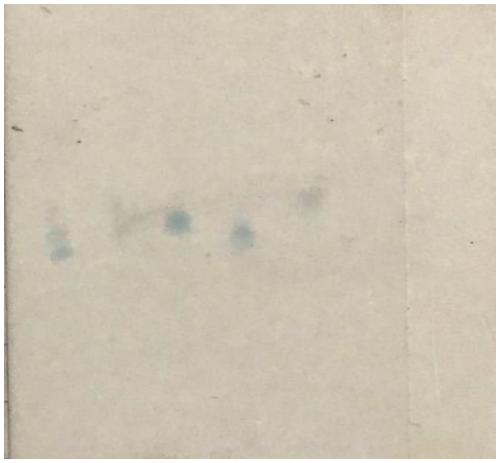
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:0.1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to methanol

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to acetonitrile
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 petroleum-ether to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1.5:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to toluene
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:2:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform
	• Sample: 1. AB 2. AB + MB 3. MB 4. AA 5. Thionin • Solvent: methanol • MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform

	• Sample: 1. AB 2. AB + MB 3. MB 4. AA 5. Thionin • Solvent: methanol • MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform
	• Sample: 1. AB + MB + AA 2. AA 3. AB 4. MA 5. Thionin • Solvent: methanol • MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform
	• Sample: 1. AB + MB + AA 2. AA 3. AB 4. MA 5. Thionin • Solvent: methanol • MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform

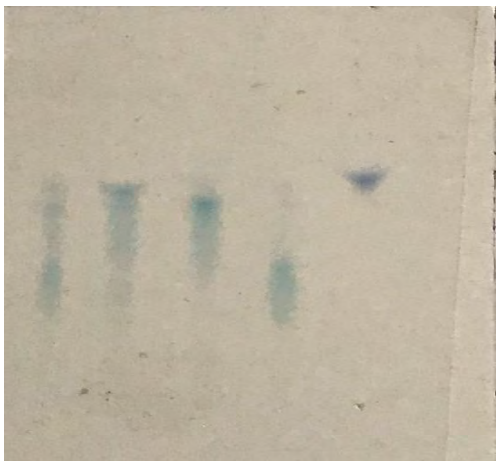
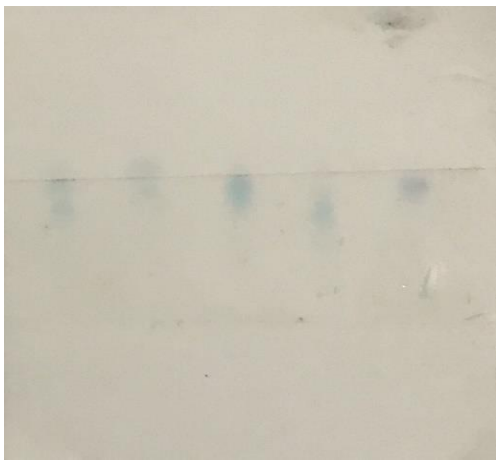
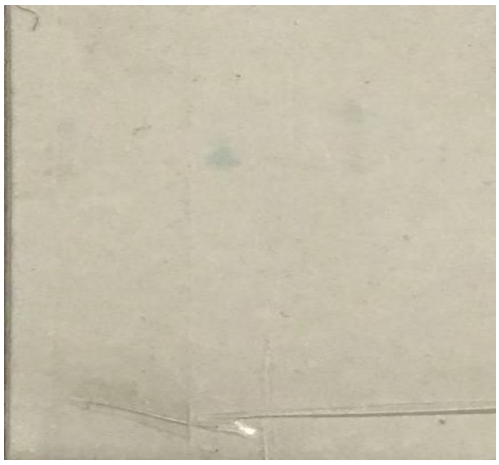
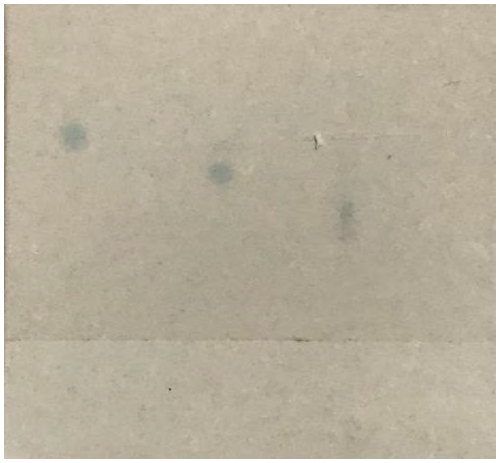
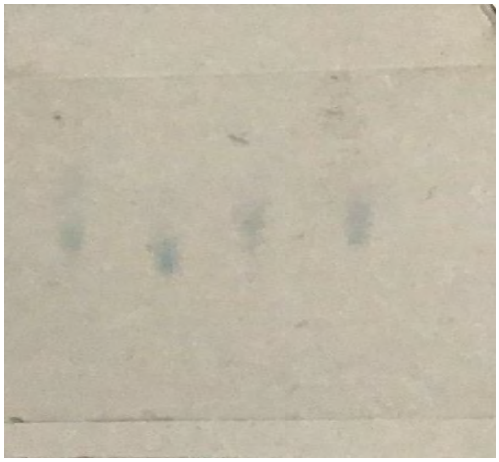
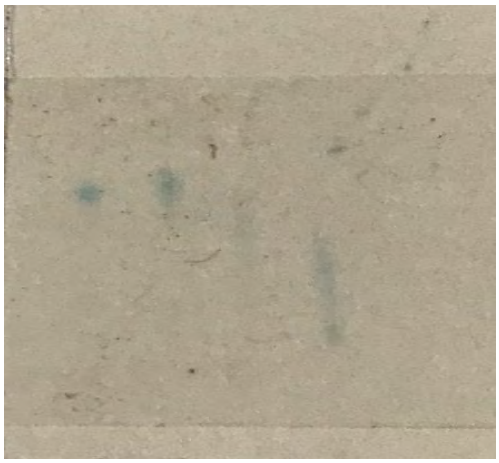
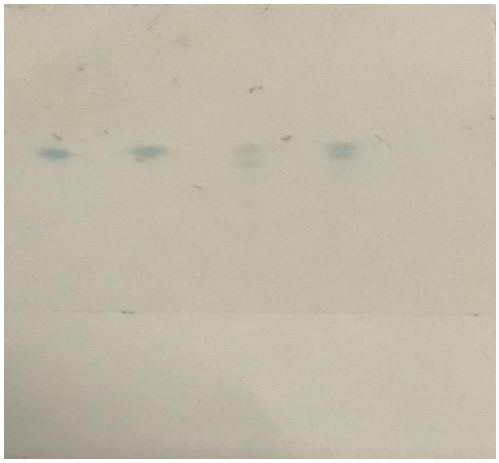
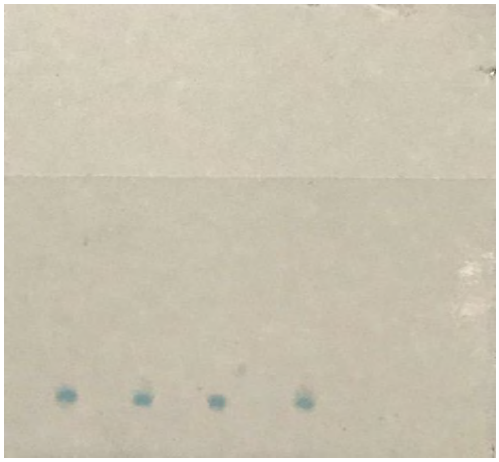
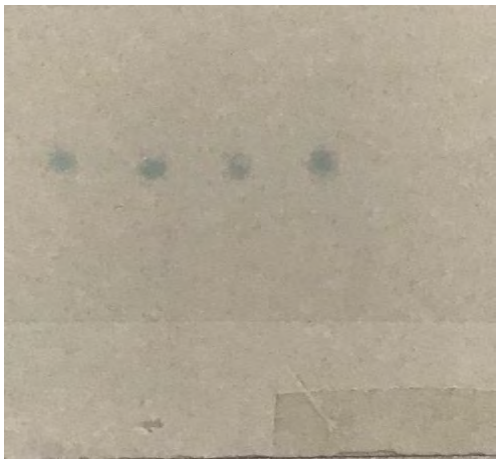
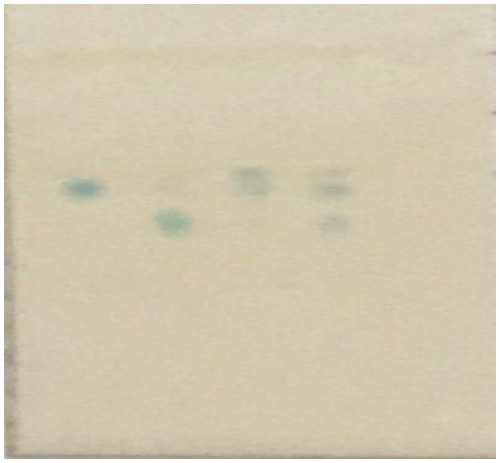
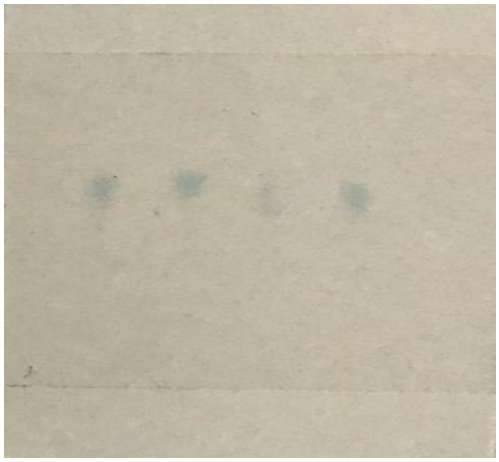
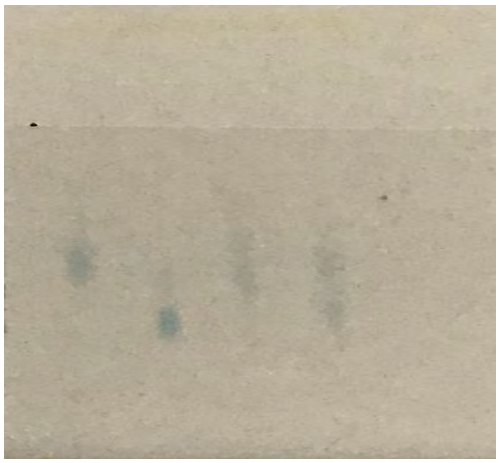
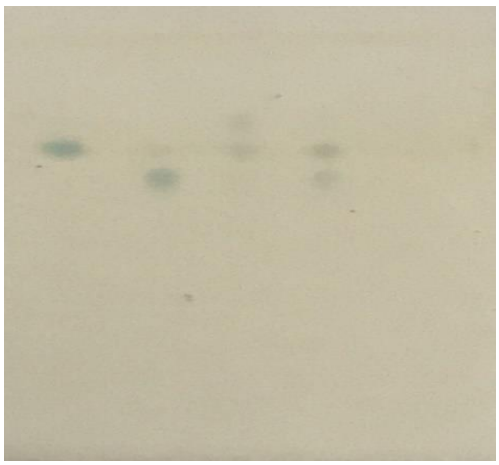
	• Sample: 1. AB + MB + AA 2. AA 3. AB 4. MA 5. Thionin • Solvent: methanol • MP: 1:2:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate
	• Sample: 1. AB + MB + AA 2. AA 3. AB 4. MA 5. Thionin • Solvent: methanol • MP: 1:2:1:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform

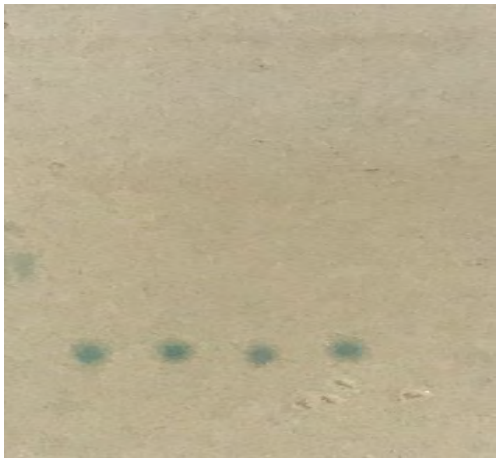
Table 3.2: Results for normal phase TLC (known concentration of 20µg/ml).

	• Sample: 1. AB 2. MB 3. AA • Solvent: methanol • MP: 2:1 chloroform to methanol
	• Sample: 1. AB 2. MB 3. AA • Solvent: methanol • MP: 2:1:0.5 chloroform to methanol to dichloromethane
	• Sample: 1. AB 2. MB 3. AA • Solvent: methanol • MP: 2:1:0.5 chloroform to dichloromethane to 5 mM ammonium acetate in 10:90 water-methanol

	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1:0.5 chloroform to methanol to dichloromethane
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1: chloroform to acetonitrile-methanol
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1: chloroform to methanol

	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 3:1 chloroform to methanol to acetonitrile
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1:0.3 chloroform to methanol to benzene
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1.3:0.2 chloroform to methanol to tetrahydrofuran

	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1:1 chloroform to 50:50 acetonitrile-methanol to dichloromethane
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:2:0.1 chloroform to methanol to benzene
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1.5:0.1:0.5 chloroform to methanol to tetrahydrofuran to dichloromethane

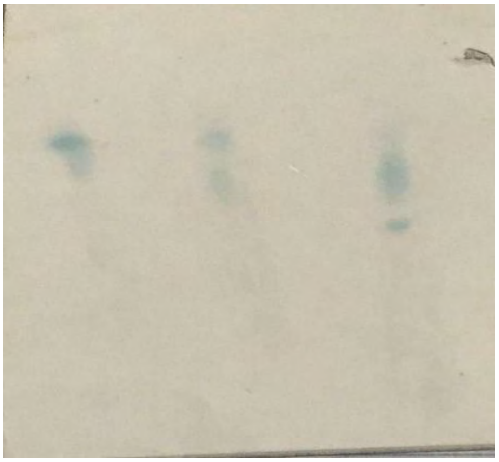
	• Sample: 1. AB 2. MB 3. AA 4. Mixture • Solvent: methanol • MP: 2:1.2:0.2 chloroform to acetonitrile to tetrahydrofuran

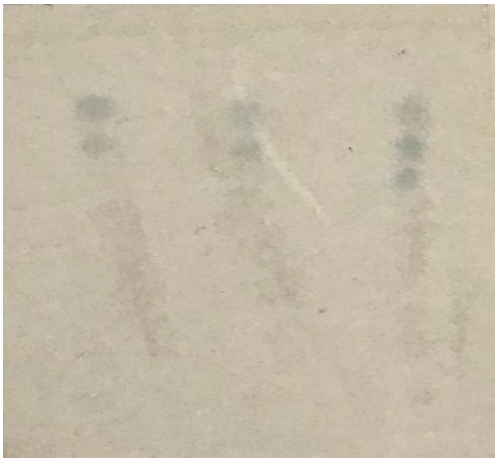
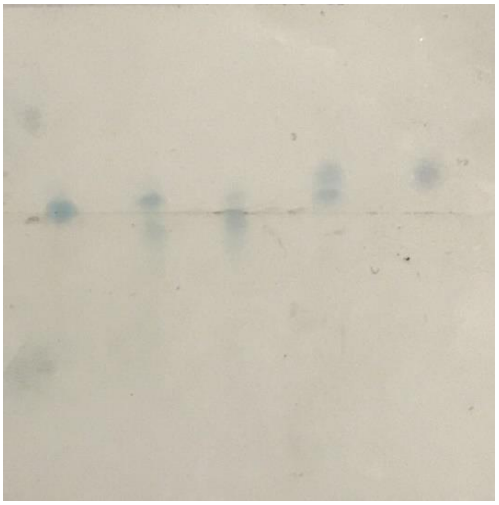
3.4. Conclusion

In Table 3.3, a short summary of successful TLC plates is given.

During this study, separation of the compounds was achieved when both NPC and RPC were implemented. However, slightly better separation is seen on NPC TLC plates. This study proves that MB can be separated from its metabolites with TLC.

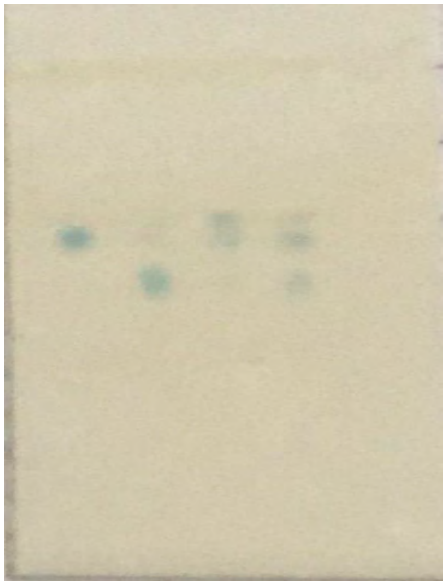
Table 3.3: Summary of successful TLC methods for the separation of MB from its metabolites.

TLC plate	Description
	• RPC TLC • Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:2:1 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate

<table>
<tr>
<td> 
  </td>
<td>

RPC TLC
Sample: 1. MB
2. AB
3. MB + AB
Solvent: methanol
MP: 1:2:1:0.5 5 mM ammonium acetate in 10:90 water-methanol to dichloromethane to ethyl-acetate to chloroform

Sample: 1. AB
2. AB + MB
3. MB
4. AA
5. Thionin

</td>
</tr>
<tr>
<td>  </td>
<td>

NPC TLC
Sample: 1. AB
2. MB
3. AA
4. Mixture
Solvent: methanol
MP: 2:1.3:0.2 chloroform to methanol to tetrahydrofuran

</td>
</tr>
</table>

CHAPTER 4 - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.1. General background

High performance liquid chromatography (HPLC) was discovered in the 1960s and was previously known as high pressure liquid chromatography due to the high pressures at which this method is carried out (Hansen *et al.,* 2012). Because of the big drawback of high pressures that could not be avoided, the decision was made to change the name of the method to HPLC, focussing on the extremely good separations obtained rather than on the high pressure.

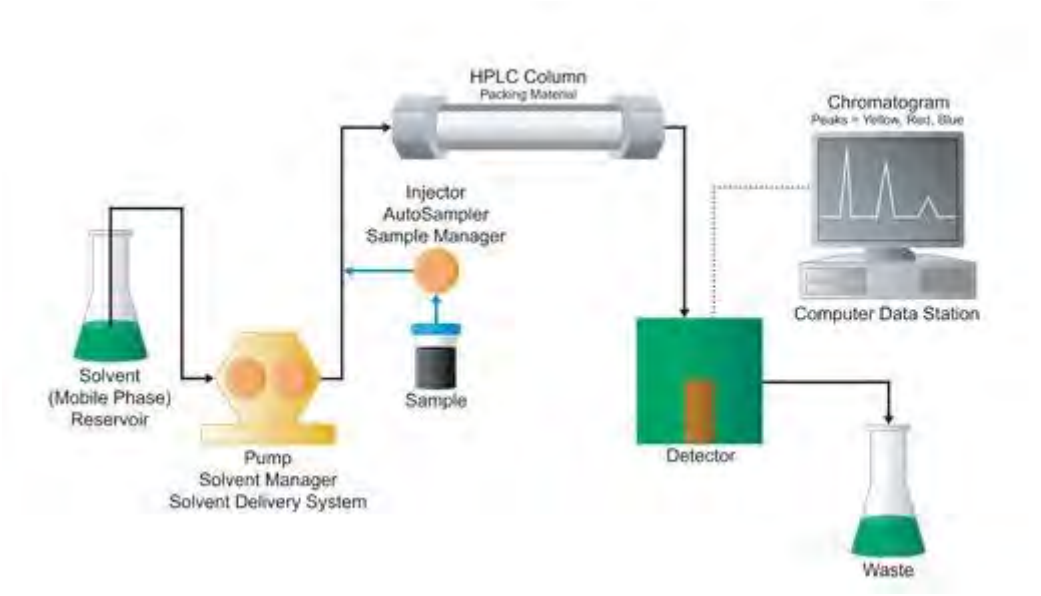


Figure 4.1: An illustration of a HPLC system (Arsenault & McDonald, 2007).

HPLC consists of three main parts namely: the solvent delivery system, the column in which the compounds are separated and the detector (figure 4.1). A great advantage of this analytical technique is that it can be regulated by a computer, thus automating the whole system (Hansen *et al.,* 2012). If the HPLC is equipped with an autosampler (Arsenault & McDonald, 2007), the system can work for long periods of time. However, trained personnel are a necessity to make sure that the system is setup correctly. The samples, which may vary between 5 to 100µg/ml depending on the type of HPLC system used and the analyte to be analysed, are injected directly into the flowing mobile phase (MP) by the injection system (Arsenault & McDonald, 2007). The column contains the stationary phase (SP) through which the MP is pumped (Arsenault & McDonald, 2007). As a result, the separated compounds are then delivered to the detector. The

MP is pumped at a consistent flow rate that can vary between 0.01 to 10ml/min (Arsenault & McDonald, 2007; Hansen *et al.,* 2012). Due to the small particle size of the SP (Clark, 2007a), a back pressure that may reach up to 300 bar, depending on the HPLC system that is used, is created when the MP is pumped through. Thus, for the HPLC to work, the pump should be able to pump the MP at a consistent flow rate against the created pressure. An increase in back pressure occurs due to the gradual blockage of the column by particles of samples that is injected into the column (Hansen *et al.,* 2012). Comprised analytical results will be obtained because of the change in flow rate with an increase in back pressure if not for the regulation mechanism in the pump that keeps the flow rate consistent. The compounds that is analysed should not be dissolved in a stronger eluting solvent than the MP. The electronic response on the separated samples generated by the detector, is processed by a computer and displayed as chromatograms (Arsenault & McDonald, 2007; Hansen *et al.,* 2012).

The importance of HPLC in drug development and research is listed as follows:

- accurate, robust and precise quantitative determination methods are provided when UV detection is implemented in the HPLC technique (Hansen *et al.,* 2012);
- decomposition quantification and drug stability in drug preparations is monitored very well by HPLC (Hansen *et al.,* 2012); and
- drug and their metabolites can be identified in biological material by HPLC (Hansen *et al.,* 2012).

It is important to know how certain parameters can influence the quality of separation, thus it will be briefly discussed below:

- Flow rate: The efficiency (*H*) of the MP can be expressed by the van Deemter equation which conveys the correlation between efficiency expressed by the height equivalent to a theoretical plate (*H*) and the band broadening phenomena (*A, B* and *C*) as a function of the flow rate (μ) (Arsenault & McDonald, 2007; Hansen *et al.,* 2012).

$$H = A + \frac{B}{\mu} + C\mu$$

Equation 4.1 - Van Deemter equation (Arsenault & McDonald, 2007; Hansen *et al.,* 2012)

  - The A term is independent of the flow rate and takes into account the difference in travel distance of identical particles (Hansen *et al.,* 2012).
  - The B term considers Brownian movements of the particles in the MP and is only applicable when lower flow rates are used (Hansen *et al.,* 2012).
  - The C term allows for the mass transfer between the MP and the SP to also be taken into consideration (Hansen *et al.,* 2012).

- *The column*: As the heart of separation by HPLC (Kazakevich & LoBrutto, 2007), the condition of the column is very important (Arsenault & McDonald. 2007). In the column the MP comes in contact with the SP that leads to the production of an interphase with a large surface. The particle size of the packing material determines the amount of theoretical plates that a column consists of (Hansen *et al.,* 2012; Kazakevich & LoBrutto, 2007). A high number of theoretical plates should be present in the column. Smaller particles yield more theoretical plates, but higher back pressures, thus shorter columns should be used. MP consumption through the column is also a factor to be considered and can be reduced by the reduction of the internal diameter and column length (Hansen *et al.,* 2012).

- *Pumps*: Piston pumps are the most common (figure 4.2) (Kazakevich & LoBrutto, 2007). The pump provides a continuous and constant flow of MP through the HPLC system (Arsenault & McDonald, 2007). Modern pumps are able to accommodate the mixing of different solvents in a chosen ratio.

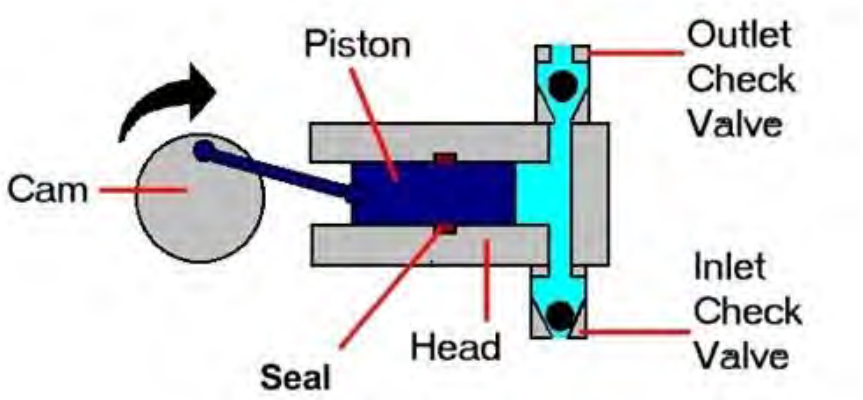


Figure 4.2: The main parts of a piston pump (Mechanical engineering, 2015).

Isocratic elution is accomplished by keeping the composition of the MP constant throughout the course of the whole process. When the composition of the MP is changed during the analytical process, gradient elution is implemented (Hansen *et al.,* 2012).

- *Detector*: The response given by the detector is converted into an electrical signal (Arsenault & McDonald, 2007; Kazakevich & LoBrutto, 2007). For quantitative analysis to be accomplished (measurement of peak area or height), the detector response should correlate with the concentration or the mass of the substance in the MP (Arsenault & McDonald, 2007; Hansen *et al.,* 2012; Kazakevich & LoBrutto, 2007).

  Two types of detectors are available:

  - General detectors respond to any changes that occurs in the MP (Hansen *et al.,* 2012).
  - Specific detectors measure changes of compounds that consists of specific properties (Hansen *et al.,* 2012).

  An ultraviolet (UV) detector is used for quality control of drugs, due to the higher limit of detection it has in comparison to others for certain analytes (figure 4.3). At a selected wavelength, the UV detector continuously registers and monitors the amount of UV light that is absorbed by the compounds. When the analyte appears in the detector flow cell, a change in absorbance is caused and a positive signal is obtained if the analyte absorbs more than the MP does.

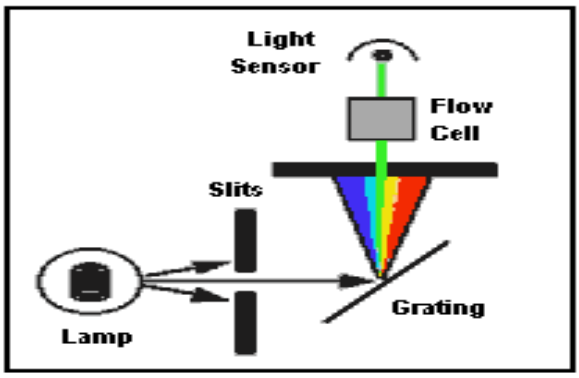


Figure 4.3: Illustration of a UV detector (L.C. Resources Inc., 2001).

- *Injectors*: Just before entering the top of the column, a certain amount of the sample is injected in to the flowing MP (Arsenault & McDonald, 2007; Hansen *et al.,* 2012; Kazakevich & LoBrutto, 2007). The HPLC should be equipped with a device that could withstand the high back pressure previously mentioned, and deliver the specified amount of sample with each injection (Hansen *et al.,* 2012).

- *MP*: Before a liquid could be considered as a potential MP, it should have the following properties:
    - no response should be given to the detector that is used; satisfactory purity;
    - Low viscosity (to ensure a low as possible back pressure);
    - low toxicity;
    - inflammable;
    - nonreactive; and
    - suitable for disposal (Hansen *et al.,* 2012).

- Solvents for sample preparation: The first choice of solvent is the MP that will be used in the HPLC procedure (Arsenault & McDonald, 2007). Preferably, the solvent should have a lower solvent strength than that of the MP and samples should never be dissolved in solvents that has a higher solvent strength in comparison to the MP (Hansen *et al.,* 2012). Solvents with lower solvent strengths should be used when larger sample volumes are injected.

4.1.1. Resolution – separation performance

Chromatographic resolution (Rs) is defined as the degree to which two compounds are separated from each other (Arsenault & McDonald, 2007; Hansen *et al.,* 2012). The resolution that a HPLC column can achieve is determined by two factors namely:

- chemical separation (selectivity) power that is generated by the physiochemical competition that exist between the MP and SP for the compounds in the solution (Arsenault & McDonald, 2007); and.
- mechanical separation (efficiency) power that is generated by:
    - length of the column;
    - size of the particles; and
    - measure of uniformity of the packed bed (Arsenault & McDonald, 2007).

Both selectivity and efficiency are taken into consideration with the one term, resolution (Arsenault & McDonald, 2007; Kazakevich & LoBrutto, 2007). Resolution can be calculated by looking at the relationship between the average width of two peaks at the baseline and the distance between the peak maxima's (Kazakevich & LoBrutto, 2007). The selectivity (peak positions), is a measure of the properties that the analytes themselves as well as the SP possesses. Efficiency (band broadening), is a measure of the column properties, as well as the condition of the instrument (Kazakevich & LoBrutto, 2007). For resolution to be improved either the selectivity or the efficiency

should be adapted. However, like mentioned earlier, selectivity has a bigger influence on resolution. In figure 4.4, the influence of sensitivity and efficiency is illustrated visually. I: Peaks are narrow and far from each other. II: Peaks are narrow, but very close to each other, however, separation is still acceptable. III: Peaks are wide and close to each other, but acceptable separation is still obtained. IV: Very bad chromatography – no separation is obtained (Kazakevich & LoBrutto, 2007).

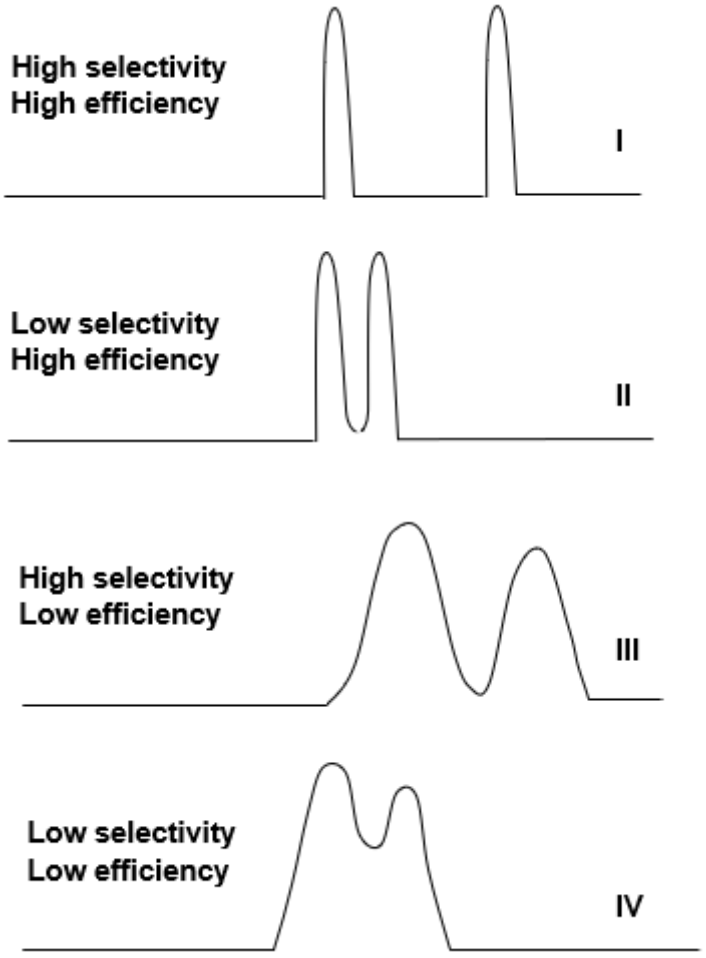


Figure 4.4: A visual illustration of how sensitivity and efficiency will ifluence chromatographic resolution (Kazakevich & LoBrutto, 2007).

4.1.2. Efficiency

The degree of band broadening, that occurs when the equally distributed analyte that is injected into and moves through the column, is known as efficiency (Hansen *et al.,* 2012; Kazakevich & LoBrutto, 2007). The mechanical separation power of a column is determined by the particle size and the length of the column provided that the column bed is uniformly packed and stable (figures 4.5 and 4.6) (Arsenault & McDonald, 2007). Efficiency is measured and compared by a plate

number (N) (Arsenault & McDonald, 2007; Hansen *et al.,* 2012; Kazakevich & LoBrutto, 2007). When the particle size decreases, a higher efficiency will be observed, but also a higher back pressure will be noted. Efficiency is increased with an increase in column length (Arsenault & McDonald, 2007; Kazakevich & LoBrutto, 2007). But in the case of longer columns some disadvantages such as higher back pressure, larger MP consumption and higher run times becomes a concern (Arsenault & McDonald, 2007). The opposite is true for shorter columns, however these columns have a lower efficiency and amount of plates. Efficiency is a property of a column, meaning that the same number of plates should be present for all compounds analysed on the same column (Arsenault & McDonald, 2007; Kazakevich & LoBrutto, 2007). High efficiency columns are able to analyse compounds with low selectivity by the generation of narrow chromatographic zones (Kazakevich & LoBrutto, 2007).

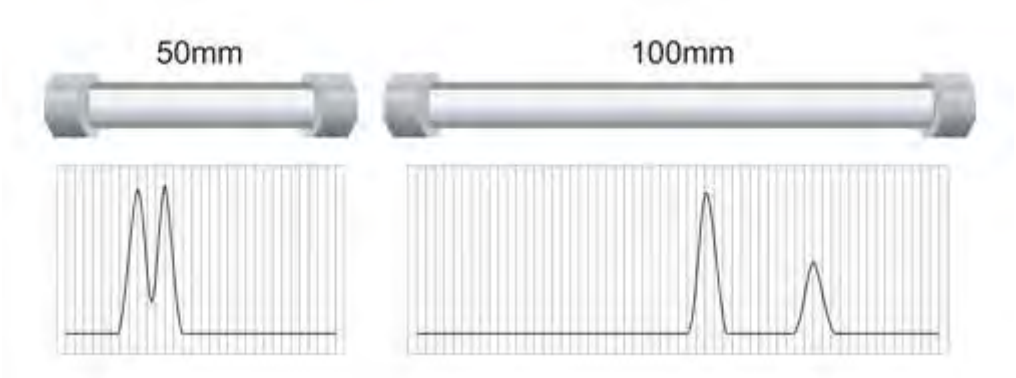


Figure 4.5: The influence of column length on efficiency (Arsenault & McDonald, 2007).

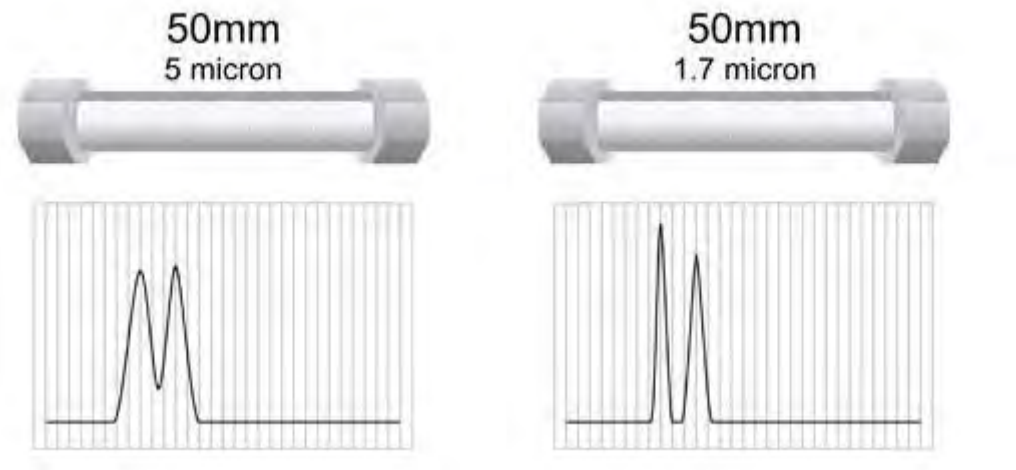


Figure 4.6: The influence of particle size of the packing material on efficiency (Arsenault & McDonald, 2007).

4.1.3. Selectivity

The degree by which a chromatographic system is able to discern between different compounds is known as selectivity (α) (Hansen, *et al.,* 2012; Kazakevich, 2007; Kazakevich & LoBrutto, 2007). It is known as the degree of chemical separation power (how the speed of each elute is changed) which is influenced directly by the combination of SP and MP used during the analytical separation process (Arsenault & McDonald, 2007). For resolution to be optimised, a change in selectivity will have the biggest impact. Thus, selectivity is the most important parameter for optimal separation (Arsenault & McDonald, 2007). As for the MP composition, the use of methanol instead of acetonitrile or visa versa, will influence the selectivity greatly when analysing isomers (Kazakevich & LoBrutto, 2007). The nature of the analytes and their interaction with the SP also has a direct influence on the selectivity (Kazakevich & LoBrutto, 2007). When the selectivity should be increased dramatically, the best option is to consider another SP. The ratio between retention factors or reduced retention times of two analytes are used for the determination of selectivity. Optimal selectivity is of utmost importance as it directly determines the degree and quality of the separation (Arsenault & McDonald, 2007).

4.1.4. Retention time

The identity and behaviour of the analyte is described by the retention time (Kazakevich & LoBrutto, 2007), which is a very easy measured parameter for a given system (figure 4.7). It is measured from the point of injection to the middle point of the peak on the base line (Clark. 2007a; Clark, 2016). The retention time of an analyte is directly influenced and dependent on the MP flow rate (Kazakevich & LoBrutto, 2007). Thus, a faster flow rate will result in smaller retention times (Clark, 2007a). The retention volume can be calculated by adding the MP flow rate to the analyte retention time (Kazakevich & LoBrutto, 2007). The retention volume is a more universal descriptor than the retention time for a given analyte.

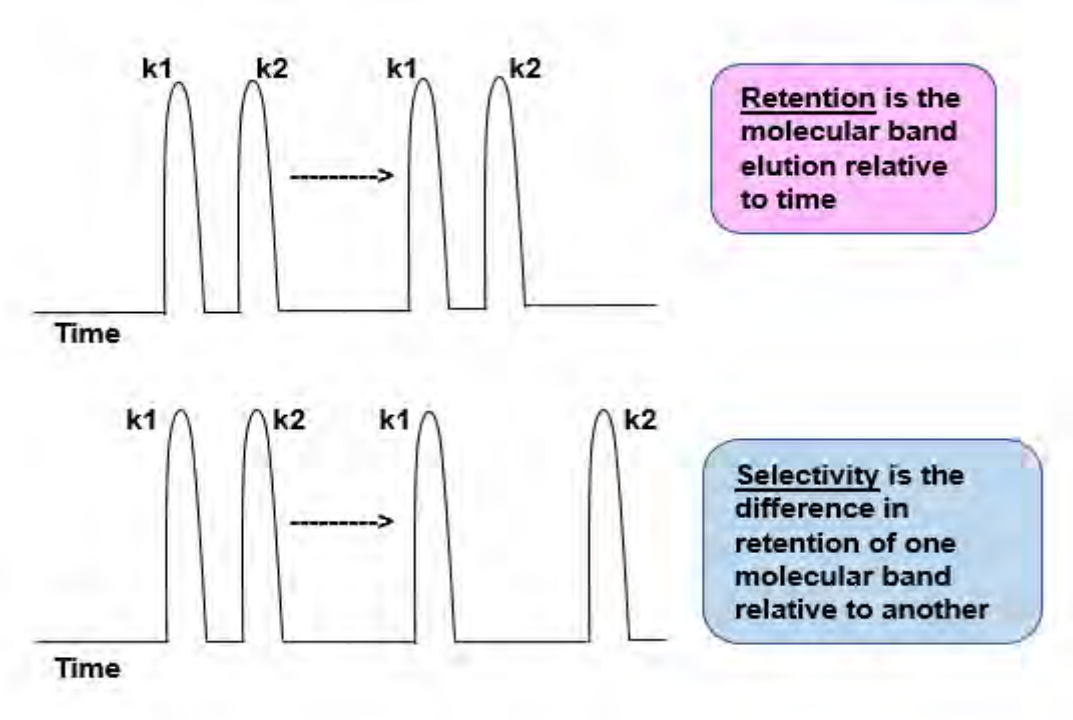


Figure 4.7: The difference between retention and selectivity (Dorman, 2011).

4.1.5. Retention factor

As mentioned before there are two parts that make up the retention of the analytes namely: the time that the analyte spends in the MP while it moves through the column and the time that the analyte spends interacting with the SP (Kazakevich & LoBrutto, 2007). The retention factor, which is also known as the capacity factor, is dimensionless and not influenced by the column dimensions or MP flow rate. Analytes that are retained in the column have positive retention factors and as for non-retained analytes a retention factor that is equal to zero is obtained (Kazakevich & LoBrutto, 2007). The “zero” retention factor is obtained when the void volume is taken in consideration meaning that these analytes have no interaction with the SP and are eluted within the void volume. A negative retention factor will be obtained by eluent molecules that interacts with the SP stronger than the interaction between analyte molecules with the SP (Kazakevich & LoBrutto, 2007).

4.1.6. Void volume

An analyte that does not interact with the SP will not appear in the detector directly after the sample has been injected (Kazakevich & LoBrutto, 2007). Because the SP is made up of porous material, a significant volume of liquid fills the pores between the SP particles, the analyte still

has to travel through this volume of liquid even though it has no interaction with the SP molecules (Kazakevich & LoBrutto, 2007). This liquid phase volume is known as the void volume (also known as the retention volume of non-retained components or hold-up volume) (Kazakevich & LoBrutto, 2007). The time a compound spends in this liquid phase (that causes the void volume) without interacting with the SP is called the hold-up time and is part of an analytes total retention time (Kazakevich & LoBrutto, 2007). Important to remember is that the retention time of an analyte is the total amount of time it spends in the column, thus for a retained compound the retention time is the product of the hold-up time and the time spend interacting with the SP (Kazakevich & LoBrutto, 2007). If the total retention time is the product of the hold-up time and the time spend retained on the surface of the SP, the reduced retention time would be the total retention time minus the hold-up time (figure 4.8).

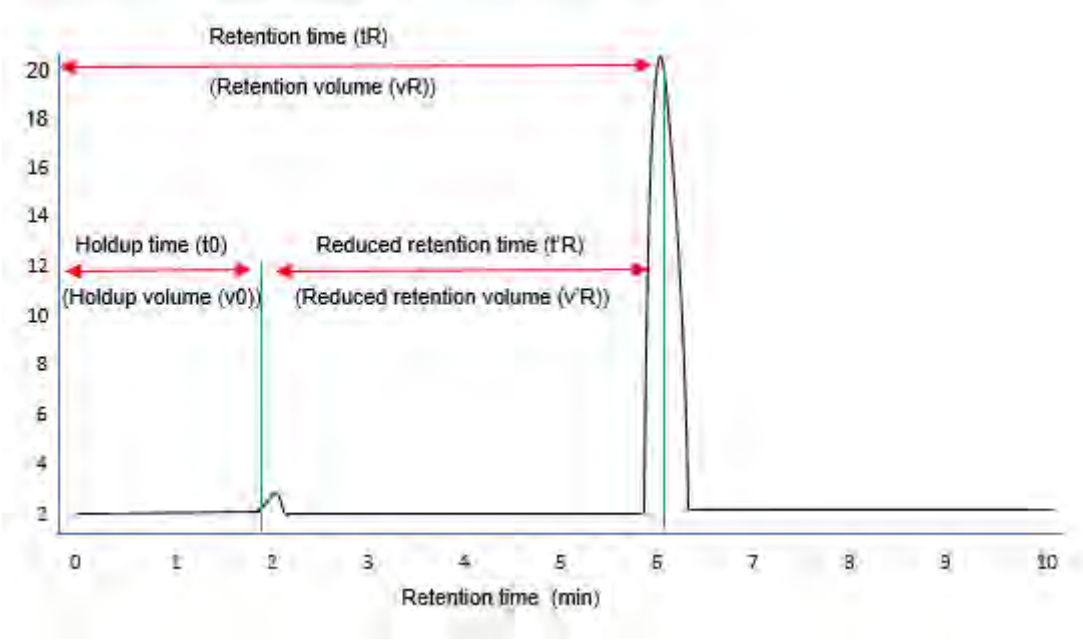


Figure 4.8: Descriptors of analyte retention (Kazakevich & LoBrutto, 2007).

4.1.7. Normal phase chromatography (NPC)

Strength differences of polar interactions between the analyte and the SP is explored during NPC. When the interaction between the SP and the analyte is strong, retention times will be longer (Hansen *et al.,* 2012; Liu & Vailaya, 2007). The separation process is based on the competition between the analyte and the MP for absorption sites on the SP. When the MP interacts stronger with the SP in comparison to the analyte, retention times will decrease (Hansen *et al.,* 2012). The MPs used in NPC are nonpolar solvents (Arsenault & McDonald, 2007; Hansen *et al.,* 2012; Liu & Vailaya, 2007). Mostly, a small amount of polar modifier is added which allows for analyte

retention in the column to be controlled. As for the SP, porous oxides such as silica or alumina of which the surface molecules are covered with OH-groups, are most commonly used (Hansen, *et al.,* 2012; Liu & Vailaya, 2007). The retention of the analytes on these types of SP's are greatly influenced by composition of the MP (Liu & Vailaya, 2007). Also becoming more popular in NPC, is chemically modified SPs such as the addition of trimethoxy glycidoxypropyl silane groups on the surface molecules of the silica, making the SP less polar (Clark, 2007a; Hansen *et al.,* 2012). The selection of the most suitable SP is depended on the solubility of the samples in specific MPs (Arsenault & McDonald, 2007; Liu & Vailaya, 2007). Highly hydrophobic compounds, which are insoluble in aqueous or polar solvents, are analysed by NPC (Clark, 2007a).

4.1.8. Reverse phase chromatography (RPC)

Due to the fact that nearly 90% of all low-molecular-weight samples are analysed by RPC (Arsenault & McDonald, 2007; Kazakevich & LoBrutto, 2007), it is the most popular type of chromatography by far (Hansen *et al.,* 2012). The main mechanism of separation is hydrophobic interactions and the main forces that mediates RPC is dispersive forces (Clark, 2007a; Hansen *et al.,* 2012). In contrasts to NPC, the surface of the SP is hydrophobic and the MP is polar (Arsenault & McDonald, 2007; Clark, 2007a; Hansen *et al.,* 2012). MPs are mainly water-based (Arsenault & McDonald, 2007; Clark, 2007a). The main reason behind its popularity is due to the ability to separate compounds with very similar chemical structures. From an energetic point of view, this advantage can be explained by looking at the strength of the dispersive forces. They are of the weakest intermolecular forces, allowing the general interaction energy in the whole HPLC system to be significantly lower in comparison to NPC. Thus, very closely related compounds with similar properties can be discerned. The SPs used in RP are porous rigid materials (silica) that have been chemically modified (figure 4.9) by adding hydrophobic groups on their surface molecules (Clark, 2007a; Hansen *et al.,* 2012). A higher absorbent surface area leads to greater retention and mostly better separation. This applies to all types of HPLC that have positive analyte surface interaction.

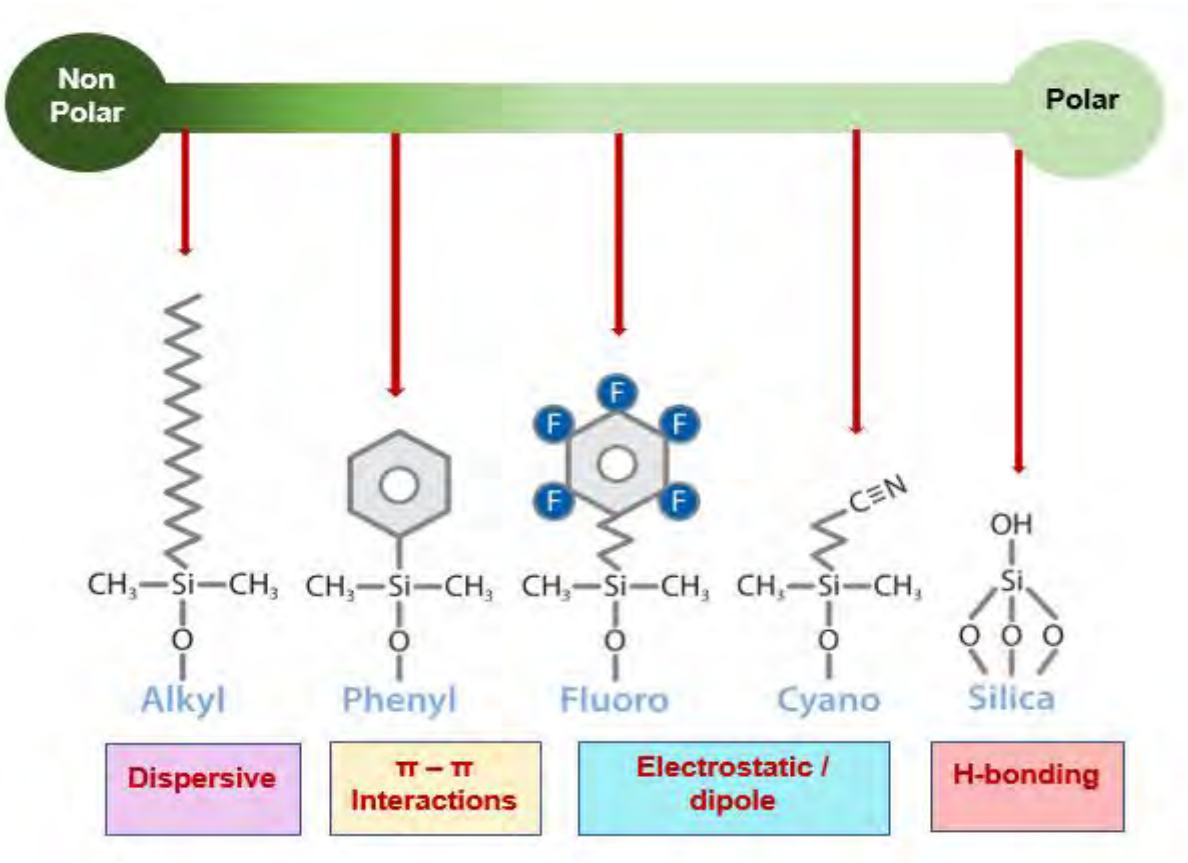


Figure 4.9: Different reverse phase chromatography stationary phase ligands that are most commonly used (Dorman, 2011).

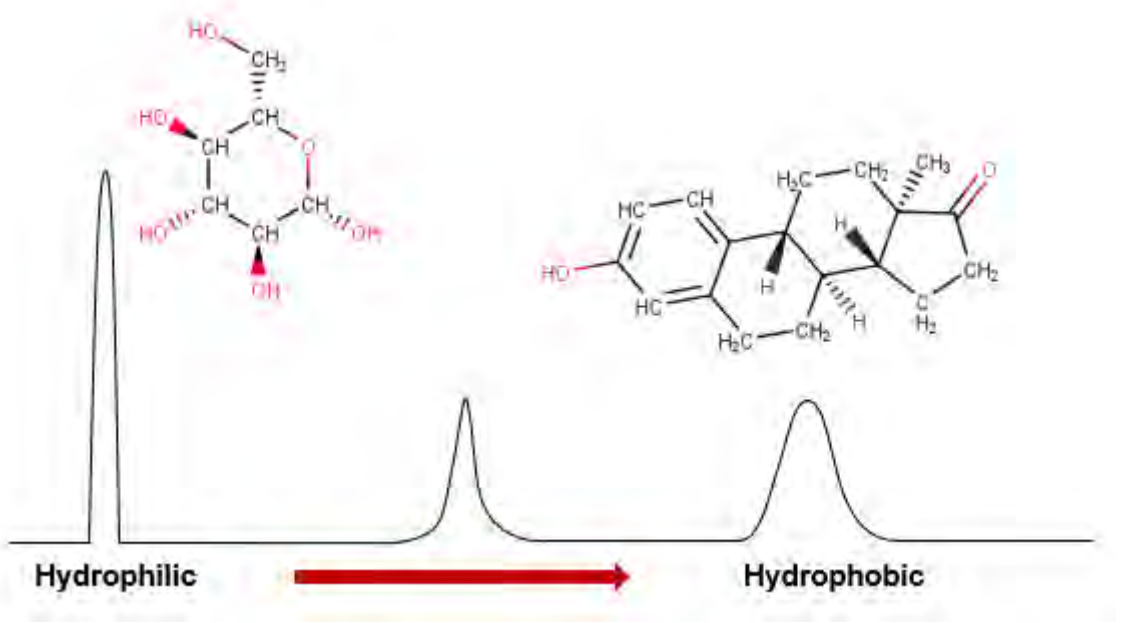


Figure 4.10: Peak differences of a hydrophilic and hydrophobic analyte analysed by reverse phase chromatography (Dorman, 2011).

Like mentioned before, RPC consists of more polar MPs (Clark, 2007a). The separation is based on the solubility of the analyte in the MP in comparison to the SP (Clark, 2007a). When a compound is more hydrophilic it will rather interact with the hydrophilic (more polar) MP, thus is will elute much quicker and have a shorter retention time (Clark, 2007a). If a compound is more hydrophobic, it will rather interact with the hydrophobic SP, making its retention times much longer (depending on the strength of retention – figure 4.10).

4.2. Current methods for the analyses of MB

The quantification of MB and/or its metabolites have been accomplished by various published methods such as HPLC, capillary electrophoresis (CE), chemiluminescence and spectrophotometry. However, these methods have great disadvantages and suffer from multistep extractions, high detection limits and long HPLC run times (BelazDavid *et al.,* 1997; Borwitzky *et al.,* 2005; Burhenne *et al.,* 2008; Gaudette & Lodge 2005; Kim *et al.,* 2014; Turnipseed *et al.,* 1997; Xu *et al.,* 2009). Recently, LC-MS/MS-based methods were implemented for the analyses of MB and its metabolites (BelazDavid *et al.,* 1997; Borwitzky *et al.,* 2005; Gaudette & Lodge 2005; Kim *et al.,* 2014; Turnipseed *et al.,* 1997). The method developed by Xu *et al.* (2009) did not measure the level of MB in biological fluids (Kim *et al.,* 2014). And according to Burhenne *et al.* (2008) the other methods had great disadvantages which include the requirement of intensive labour for sample preparation as well as large volumes of biological samples and organic solvents (Kim *et al.,* 2014). According to Kim *et al.* (2014) and Yang *et al.* (2012) reported that the use of capillary electrophoresis and mass spectrometry methods also did not overcome the disadvantages of existing. The methods that were just mentioned also have the disadvantage of low sensitivity and because of their difficulty level, they cannot be implemented by untrained personnel (Burhenne *et al.,* 2008; Kim *et al.,* 2014). Kim *et al.* (2014) developed a LC-MS/MS method by which MB and AB levels in rat plasma could be determined. They've managed to reduce labour and shorten the processing time during their study. In another study, MB concentrations were determined in the plasma and whole blood by methods based on protein precipitation and cation exchange chromatography coupled to electrospray ionisation (ESI) tandem mass spectrometry (MS/MS). These methods have been approved by the FDA (Walter-Sack *et al.,* 2009). A mixed mode column with an aqueous ammonium acetate/acetonitrile gradient was used.

4.3. HPLC results

During this study, various HPLC columns were used in order to determine the most suitable column for the separation of MB and its derivatives (table 4.1).

Mobile phases consisting of 5 mM ammonium acetate dissolved in 10:90 water:methanol (Part A) or acetonitrile or methanol (Part B) in ratios varying from 95:5 to 10:90 were used on each column.

The wavelength of maximum absorption of commercially available MB was determined to be 290 nm and a sample concentration of 20 µg/ml was prepared. A sample volume of 20 µl was injected at ambient temperature (25°C). Flow rates varying between 0.5 – 1.0 ml / minute was used.

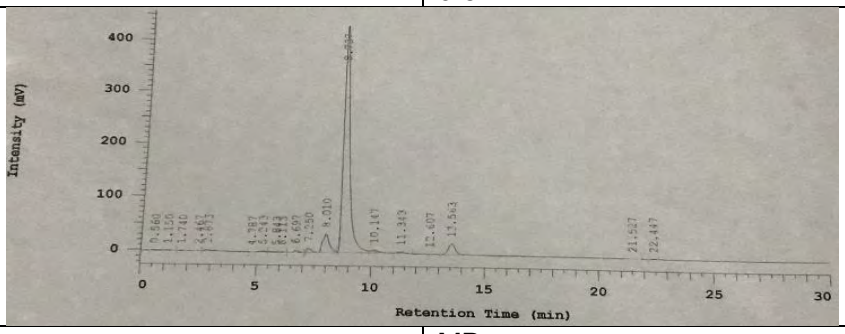
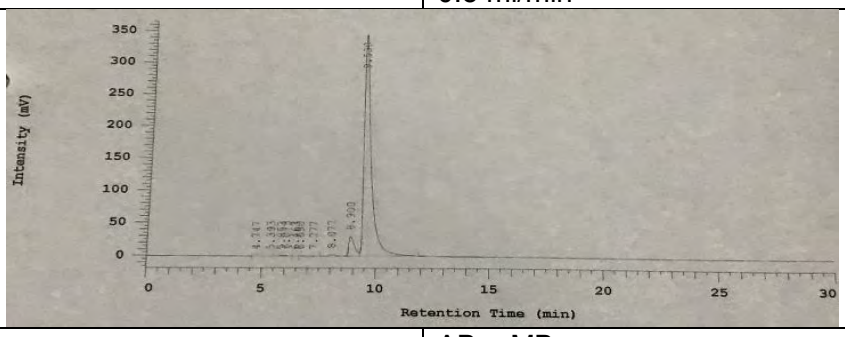
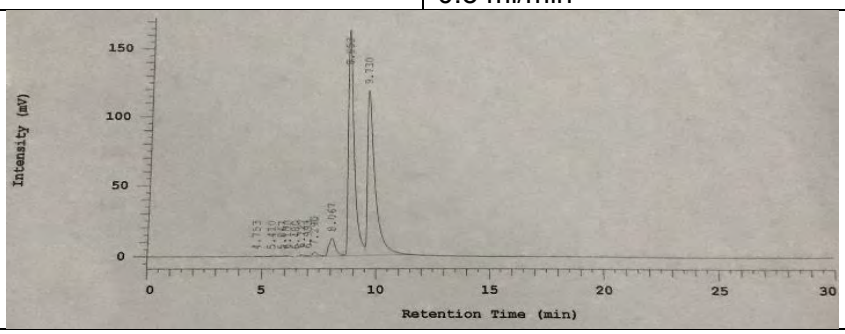
Table 4.1: HPLC columns used in this study.

Manufacturer	**Type**	**Dimensions**
Phenomenex	Luna C_8 (2)	• 250 x 4.6 mm • 5 µ • 100 Å
Phenomenex	Luna C_{18} (2)	• 250 x 4.6 mm • 5 µ • 100 Å
Phenomenex	Kinetex XB-C_{18}	• 75 x 4.6 mm • 2.6 µ • 100 Å
Phenomenex	Luna phenyl-hexyl	• 250 x 4.6 mm • 5 µ • 100 Å
Waters	Spherisorb ODS	• 250 x 4.6 mm • 5 µ • 80 Å
Macherey-Nagel	CC Nucleosil C_6H_5	• 250 x 4 mm • 5 µ • 100 Å
Macherey-Nagel	ET Nucleosil C_2	• 250 x 4 mm • 7 µ • 100 Å
Thermo Fisher Scientific	Hypersil ODS-C_{18}	• 150 x 4.6 mm • 5 µ • 120 Å
Merck	Chromolith ® Performance RP-18e	• 100 x 4.6 mm • 2 µ • 130 Å
GL Sciences	Intersil ODS-2	• 150 x 4.6 mm • 5 µ • 150 Å

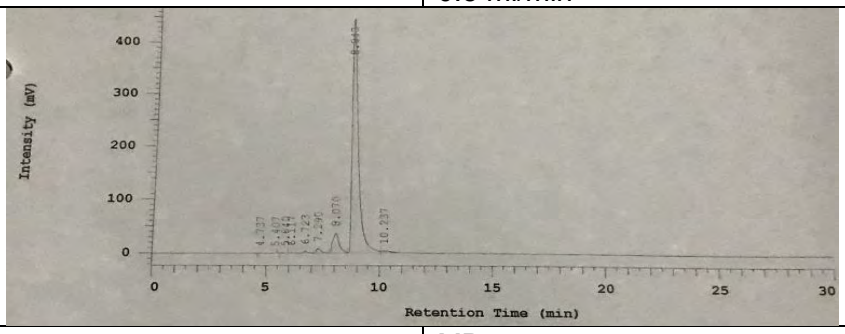
Agela technologies	Venusil XBP-CN	• 250 x 4.6 mm • 5 µ • 100 Å
Phenomenex	Gemini C_{18}	• 150 x 4.6 mm • 5 µ • 110 Å
Agela technologies	Durashell-RP	• 150 x 4.6 mm • 3 µ • 150 Å
Phenomenex	Synergi polar-RP	• 150 x 4.6 mm • 4 µ • 80 Å

All the chromatograms generated during this study are available, but only results indicating successful separation are included in this dissertation (Tables 4.2 and 4.3).

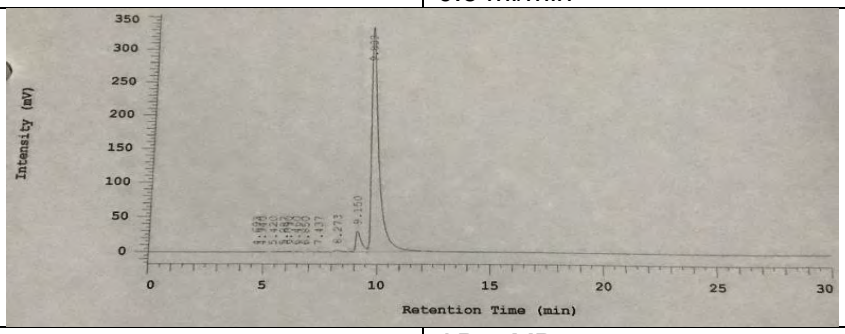
Table 4.2: Chromatograms generated on the Luna Phenyl-hexyl column.

Sample	AB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min
	
Sample	MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min
	
Sample	AB + MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min
	

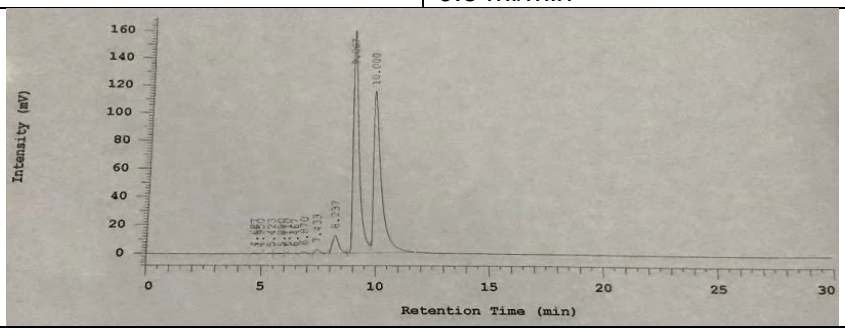
Sample	AB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



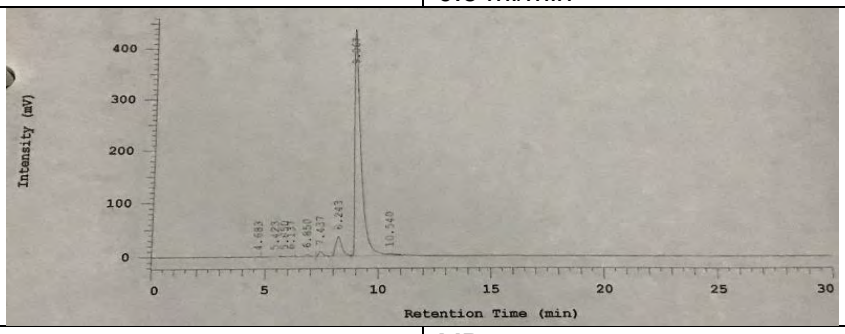
Sample	MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



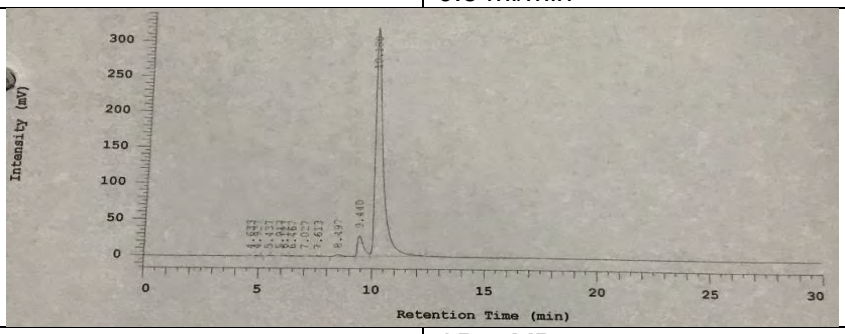
Sample	AB + MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



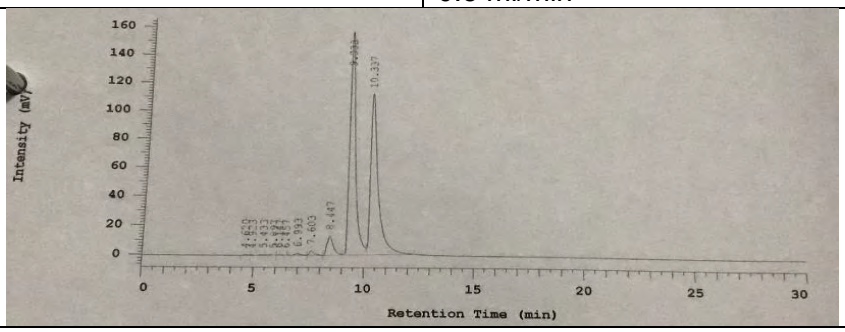
Sample	AB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



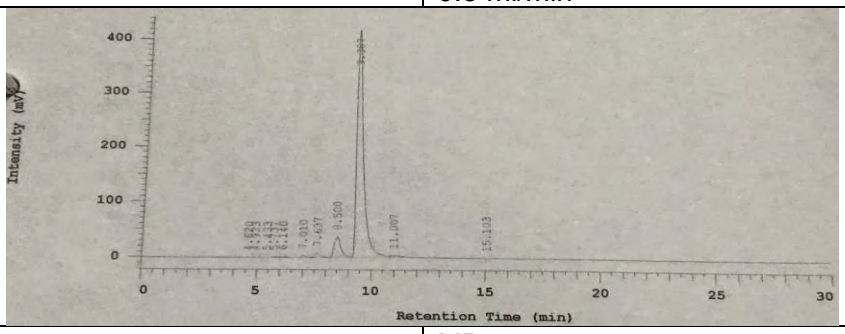
Sample	MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



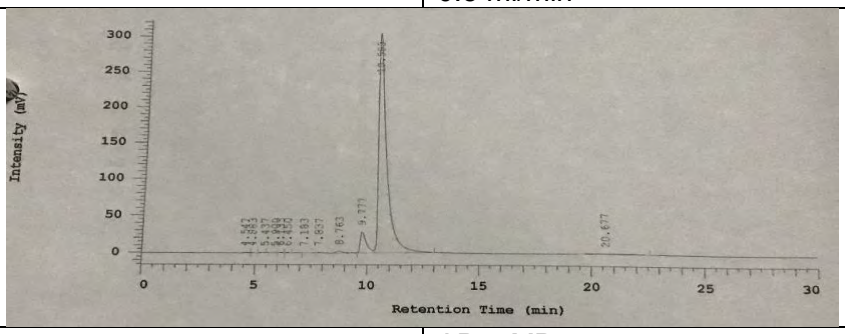
Sample	AB + MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



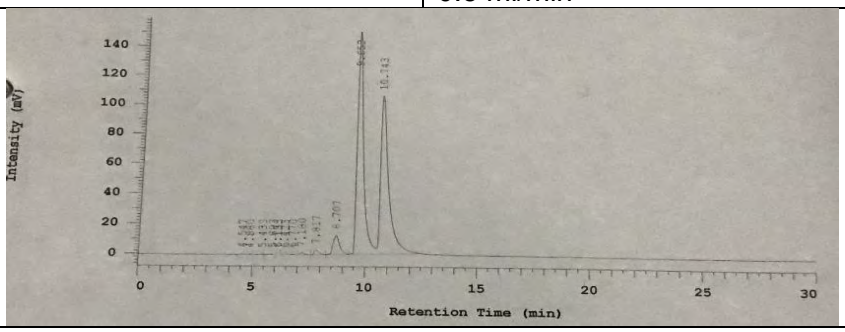
Sample	AB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



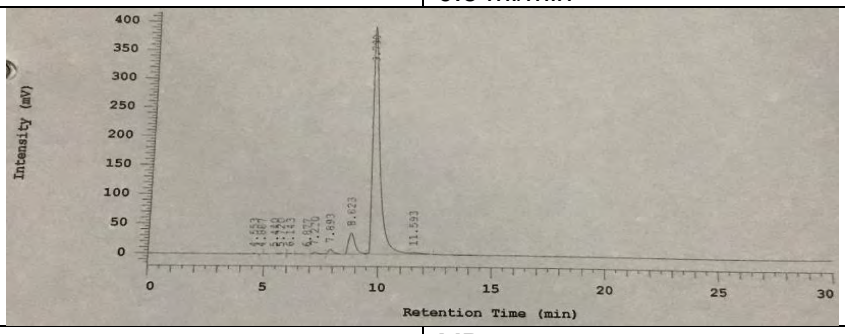
Sample	MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



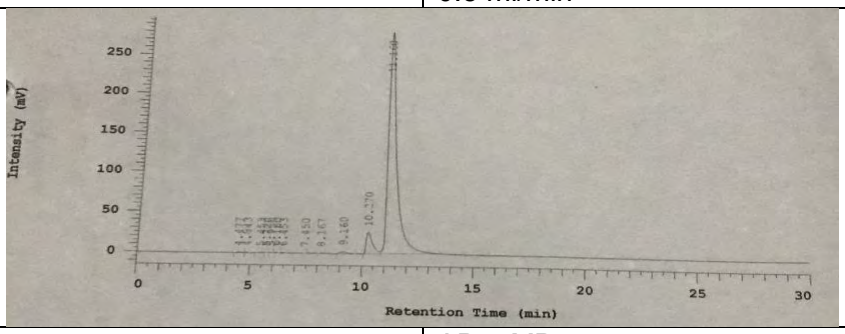
Sample	AB + MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



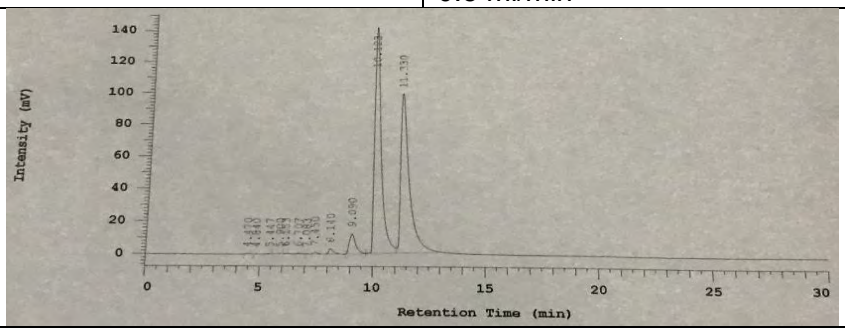
Sample	AB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



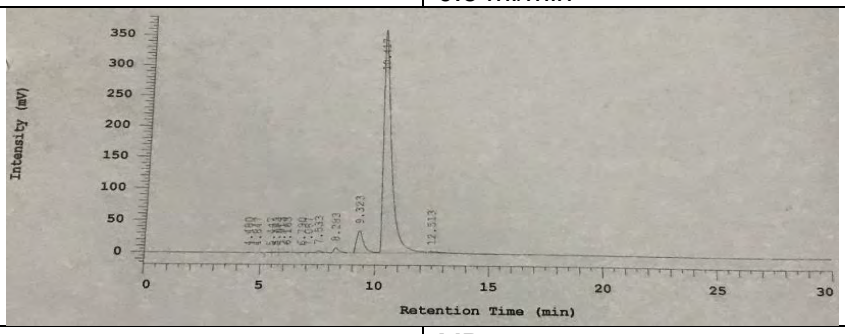
Sample	MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



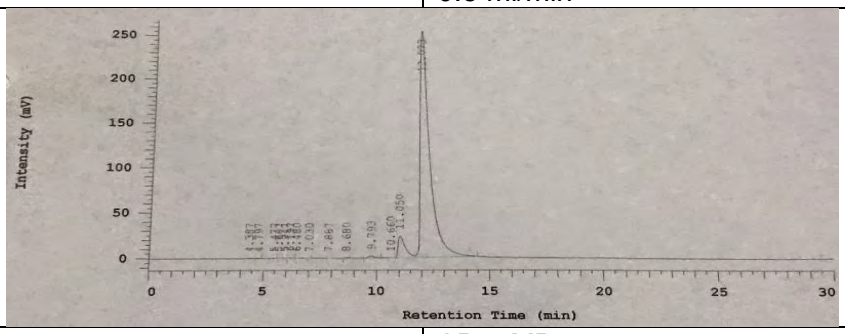
Sample	AB + MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



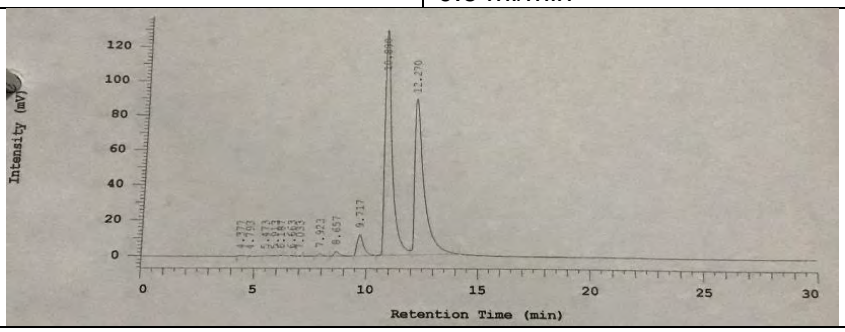
Sample	AB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



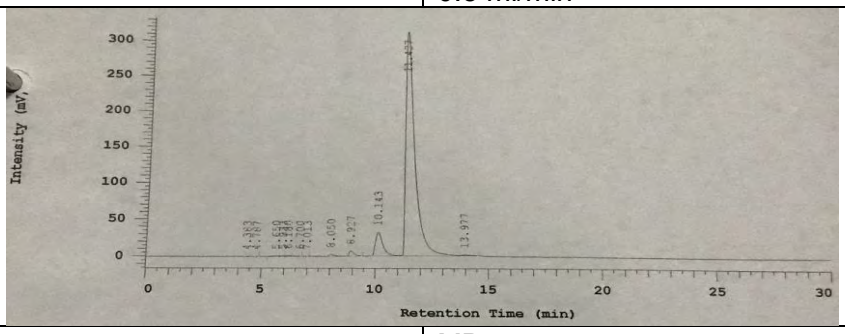
Sample	MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



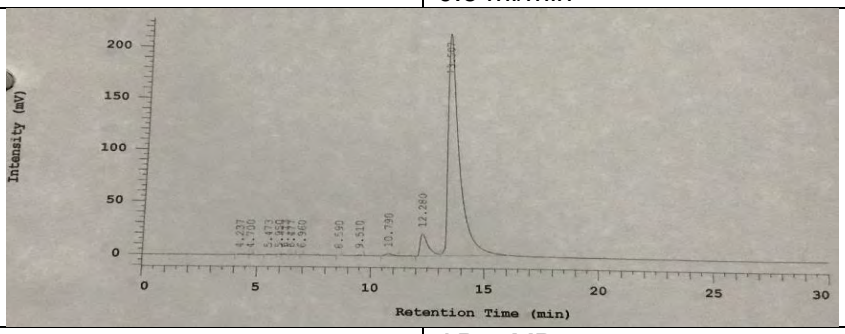
Sample	AB + MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



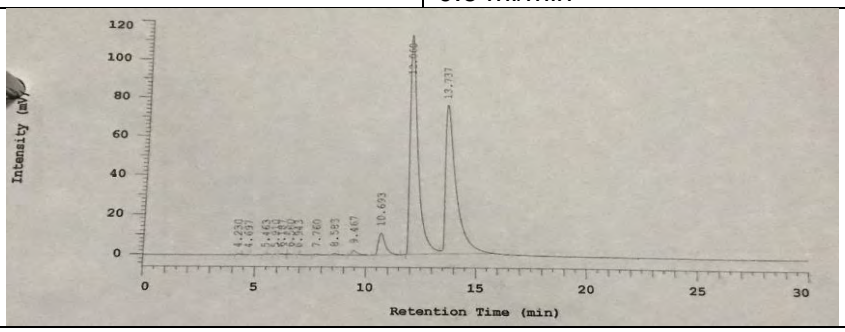
Sample	AB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



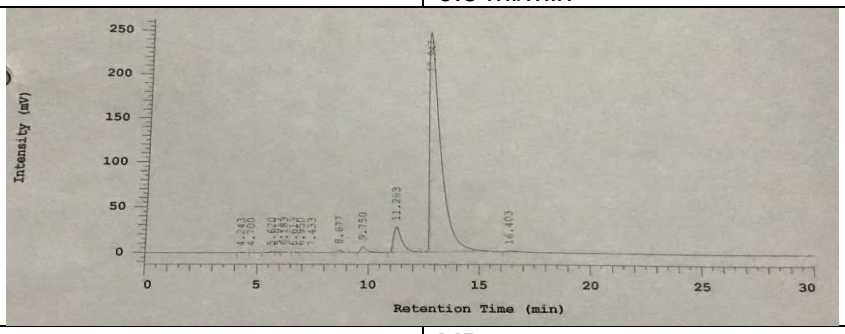
Sample	MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



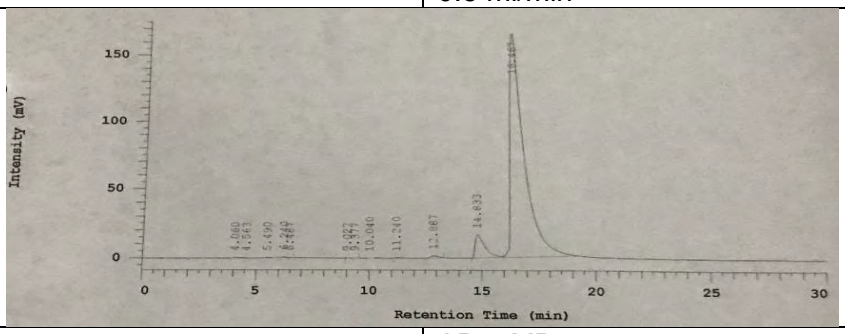
Sample	AB + MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



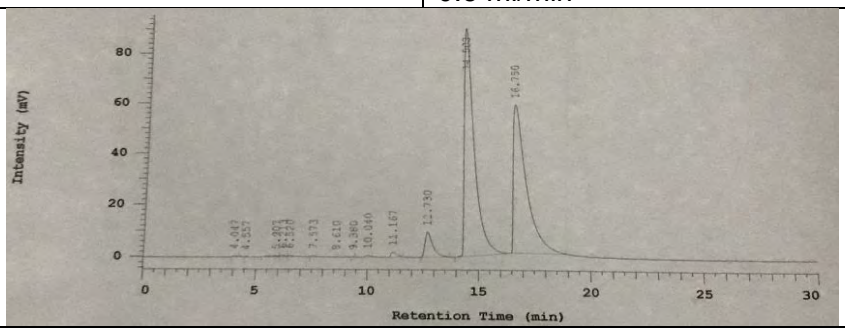
Sample	AB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



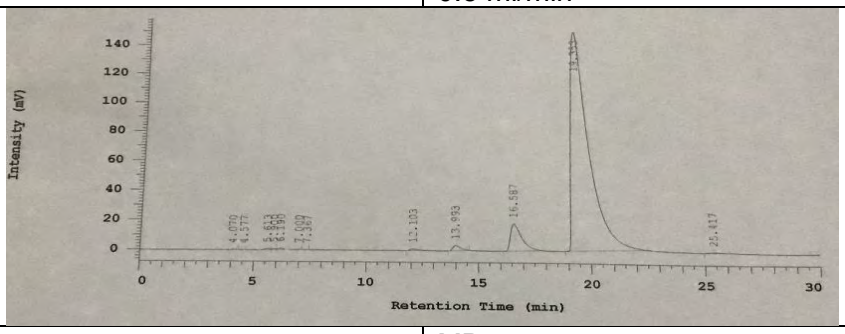
Sample	MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



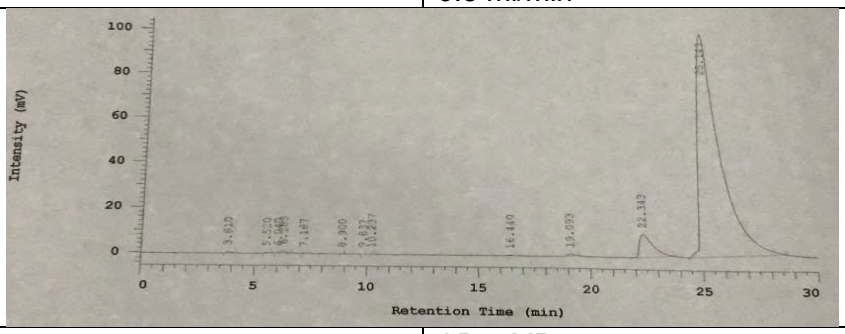
Sample	AB + MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



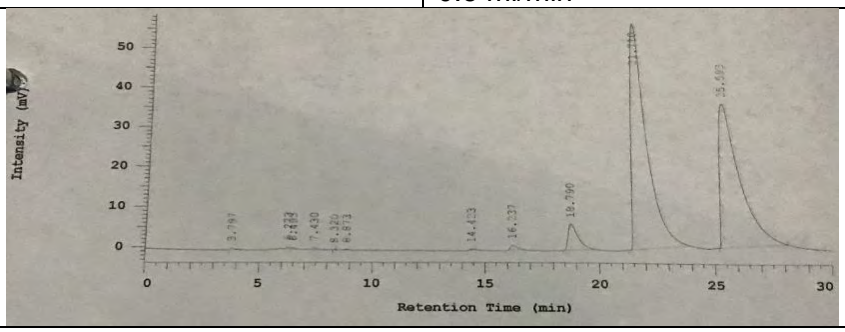
Sample	AB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



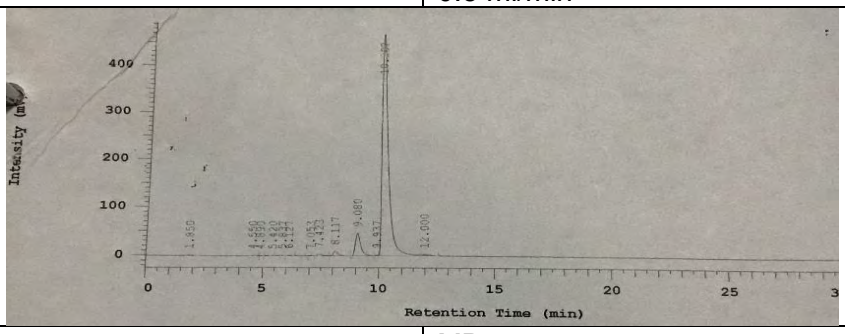
Sample	MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



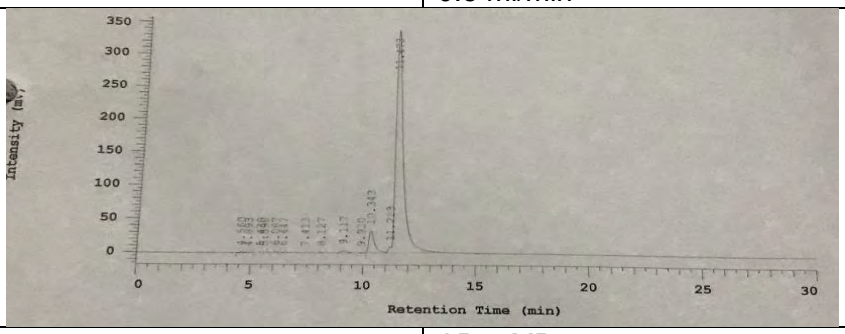
Sample	AB + MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	0.5 ml/min



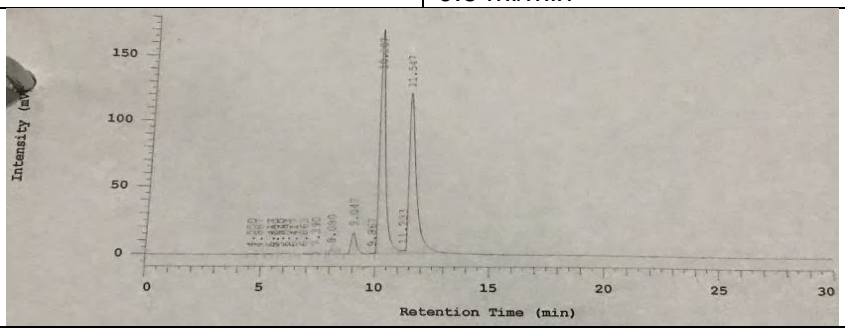
Sample	AB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



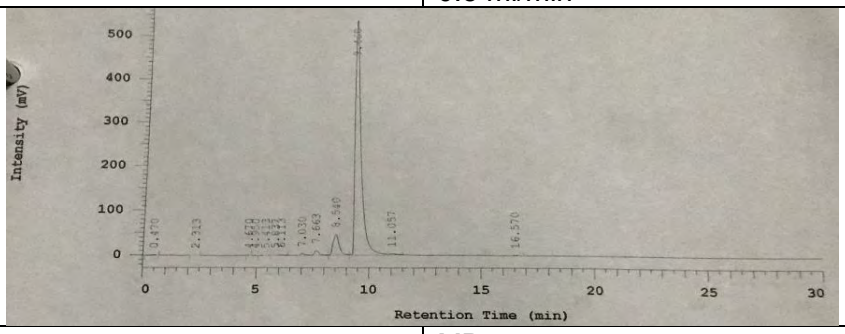
Sample	MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



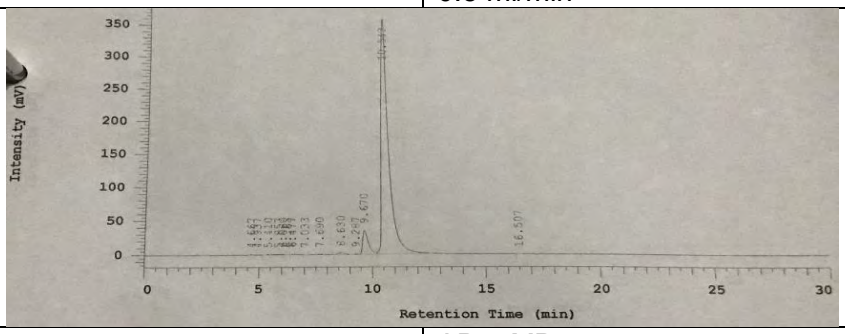
Sample	AB + MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



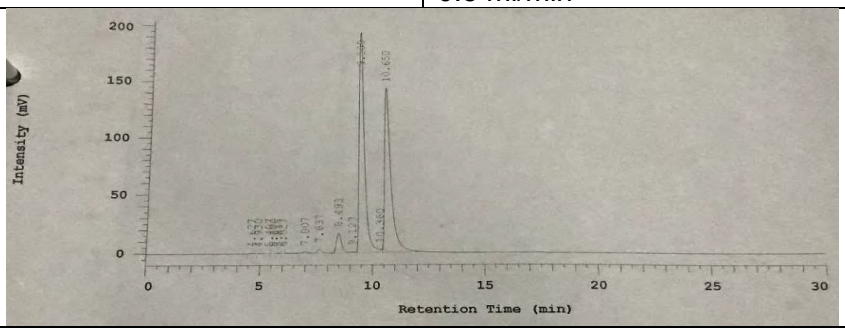
Sample	AB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



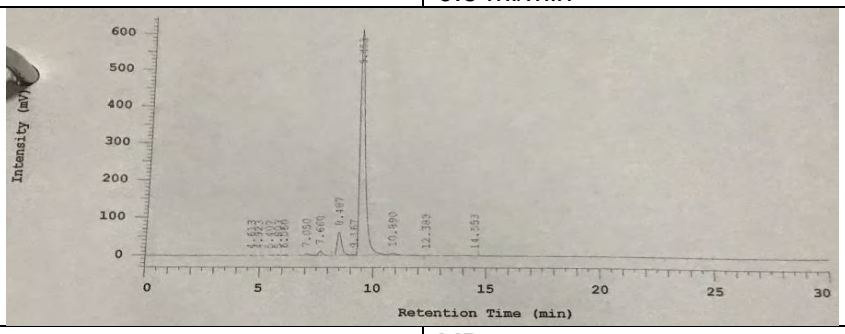
Sample	MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



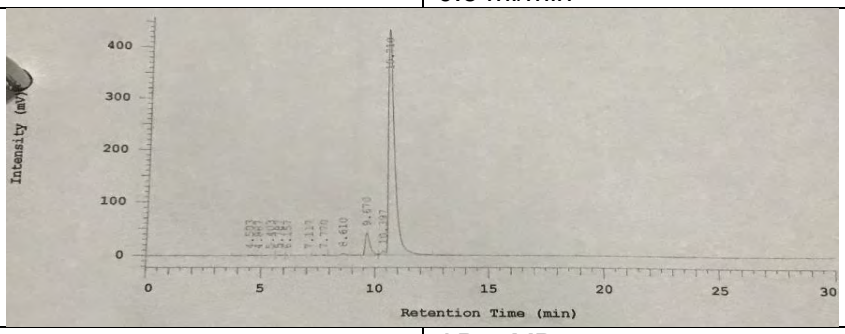
Sample	AB + MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



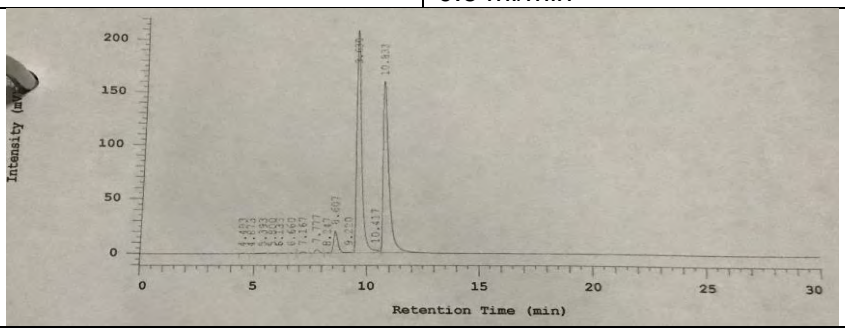
Sample	AB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



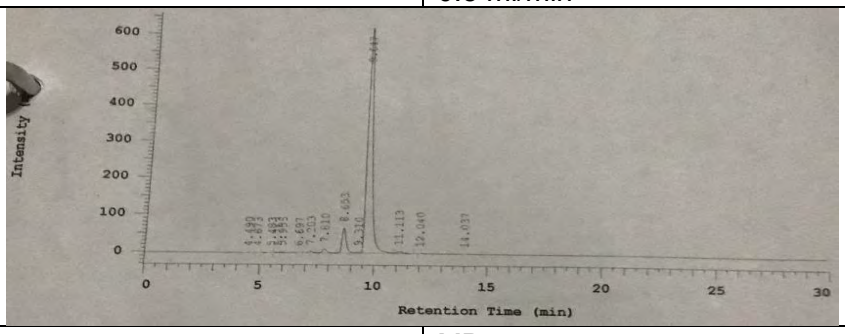
Sample	MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



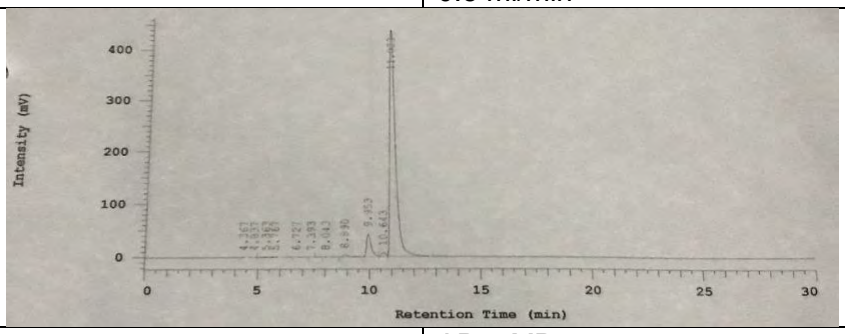
Sample	AB + MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



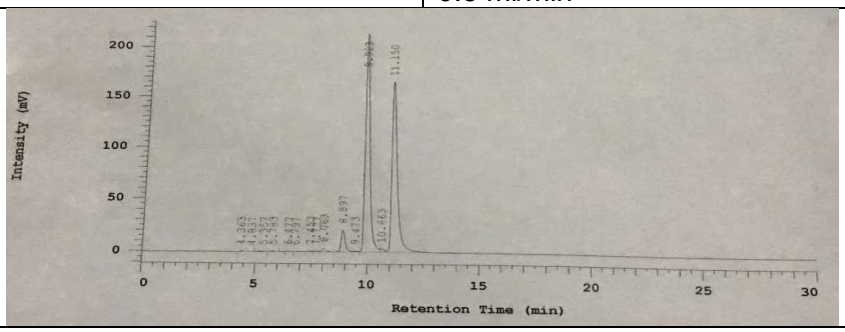
Sample	AB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



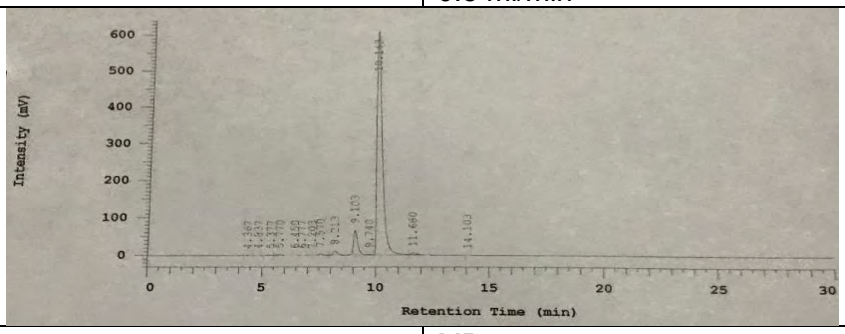
Sample	MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



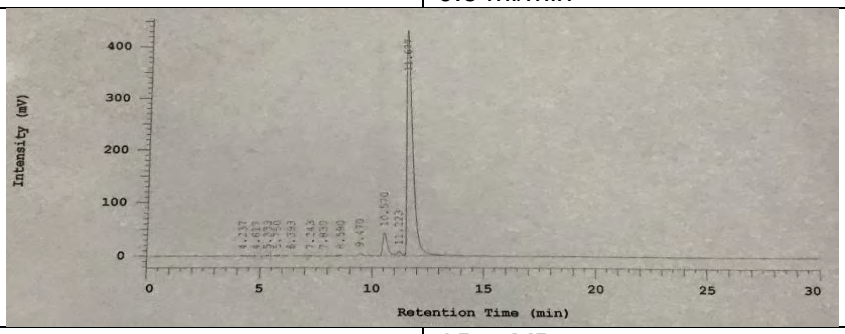
Sample	AB + MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



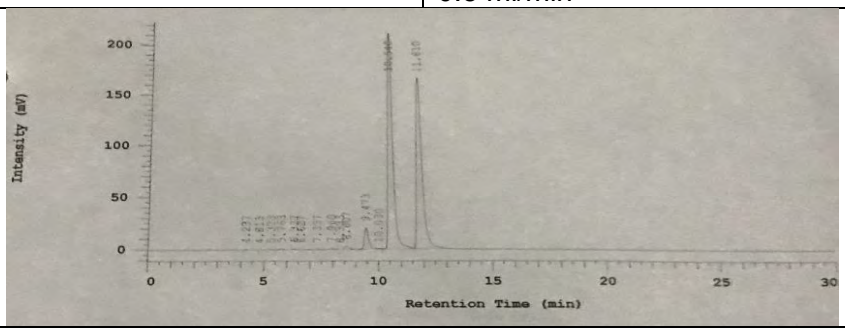
Sample	AB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



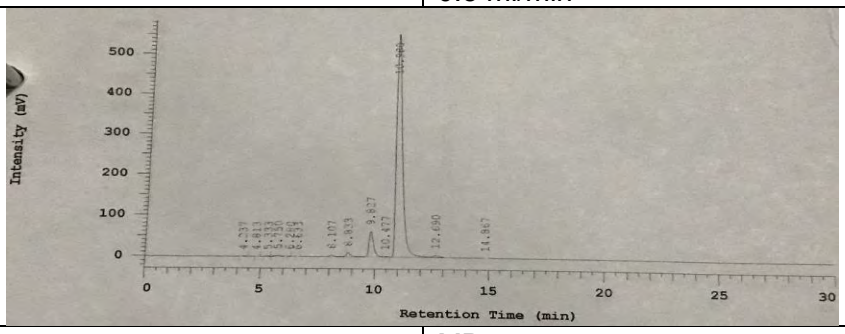
Sample	MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



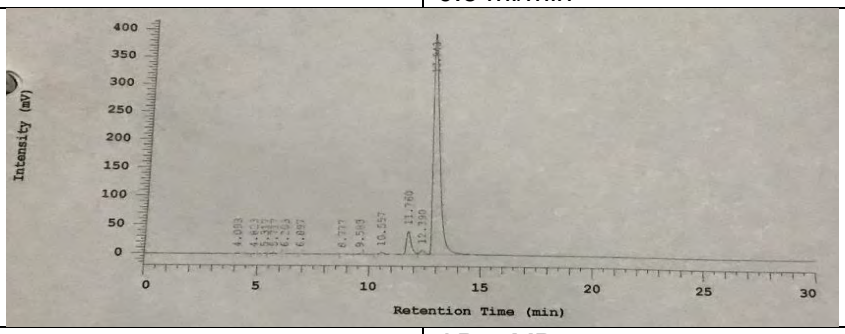
Sample	AB + MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



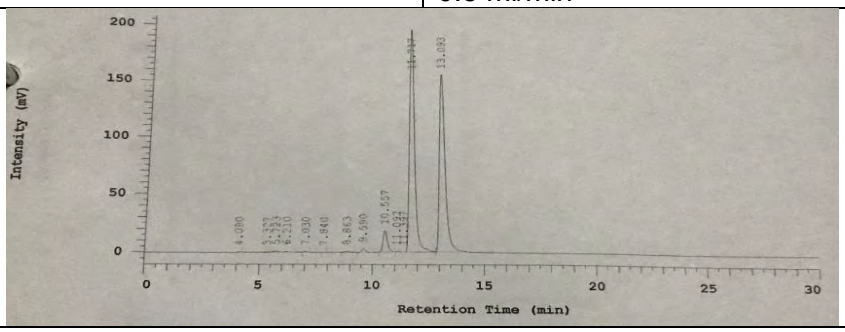
Sample	AB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



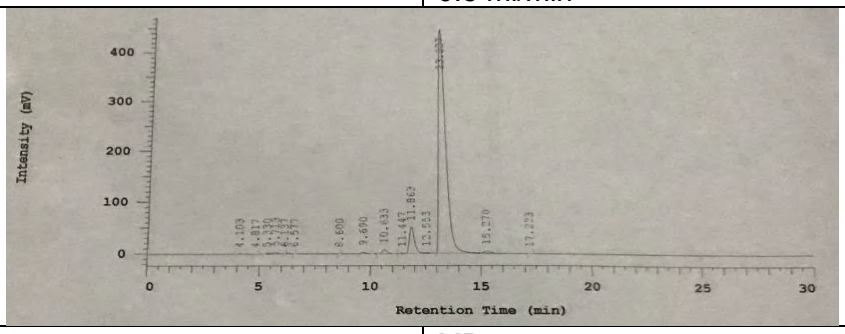
Sample	MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



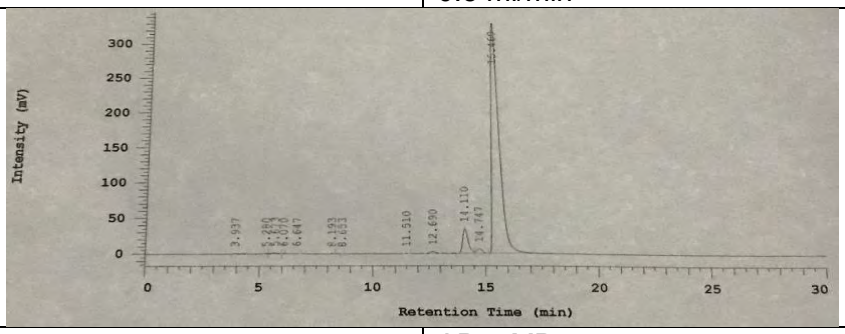
Sample	AB + MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



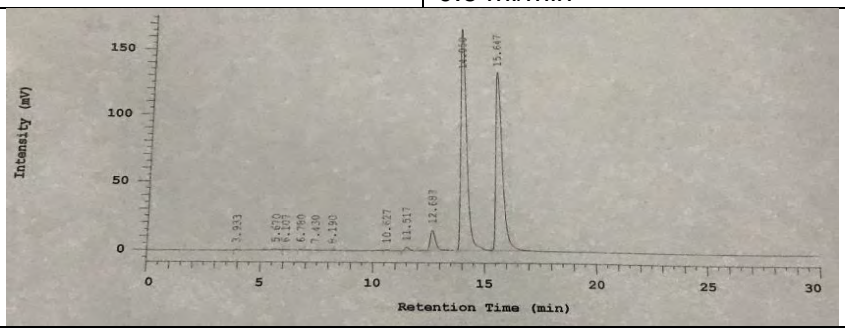
Sample	AB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



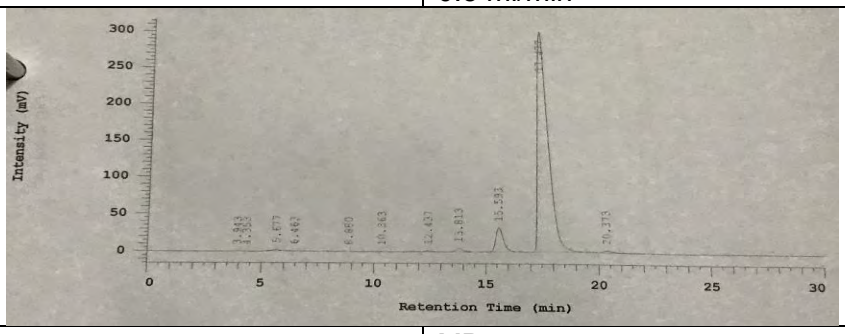
Sample	MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



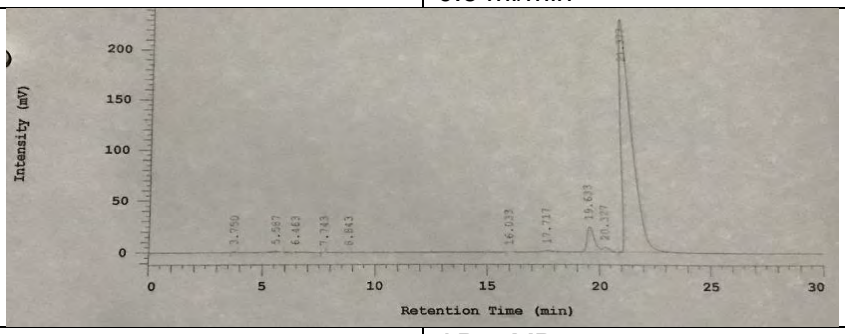
Sample	AB + MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



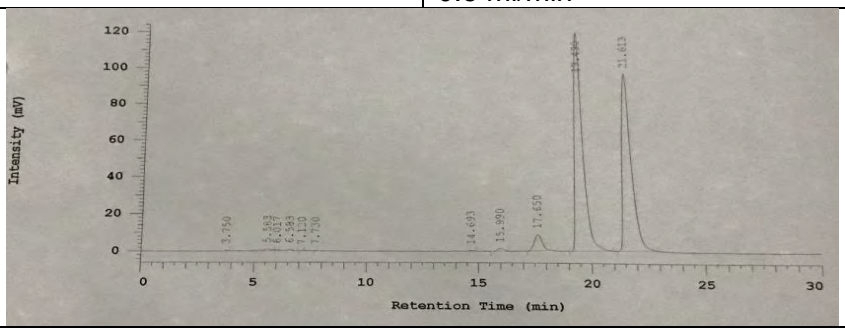
Sample	AB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



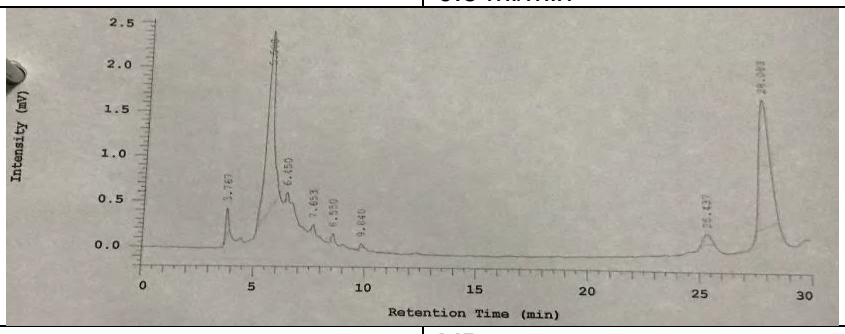
Sample	MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



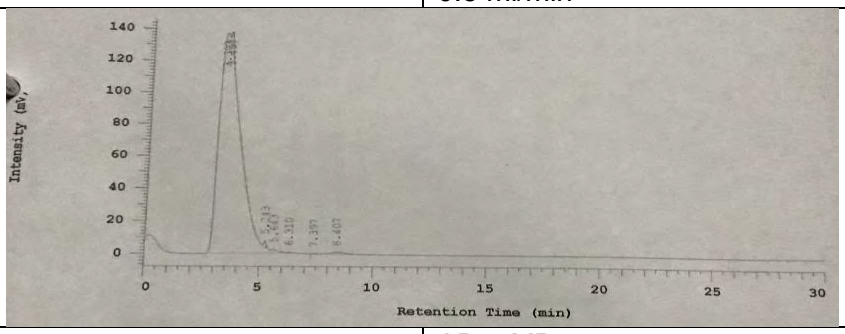
Sample	AB + MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



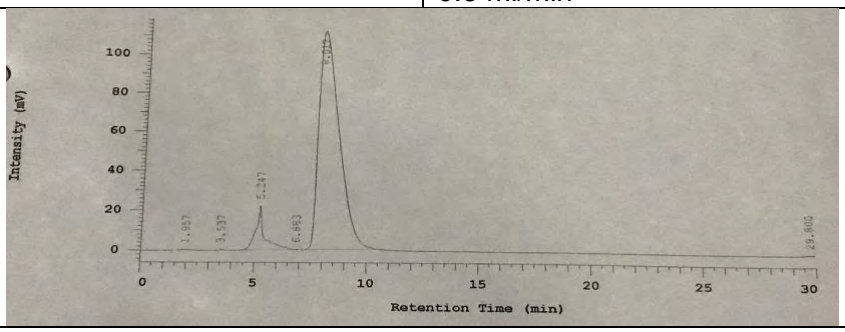
Sample	AB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



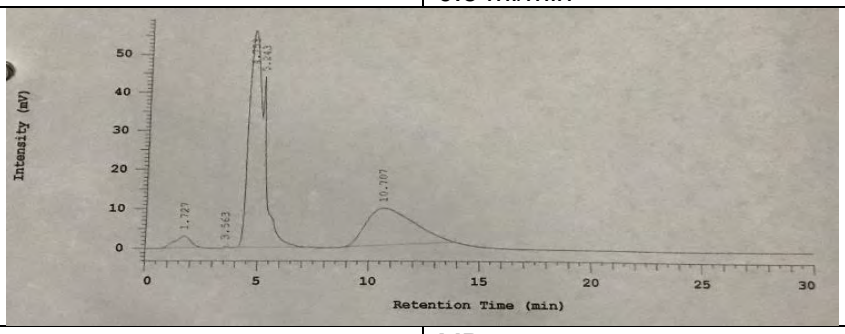
Sample	MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



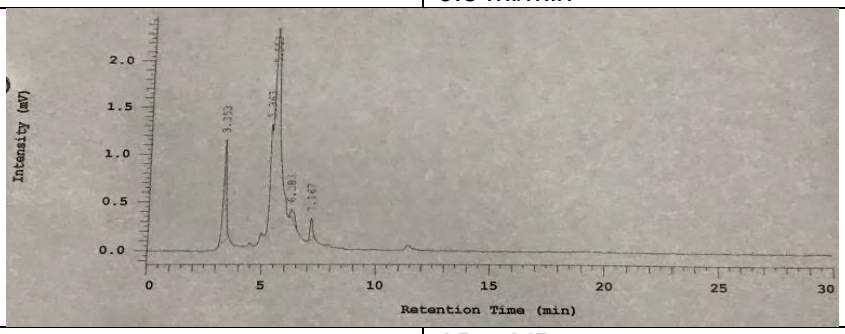
Sample	AB + MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



Sample	AB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



Sample	MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min



Sample	AB + MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.5 ml/min

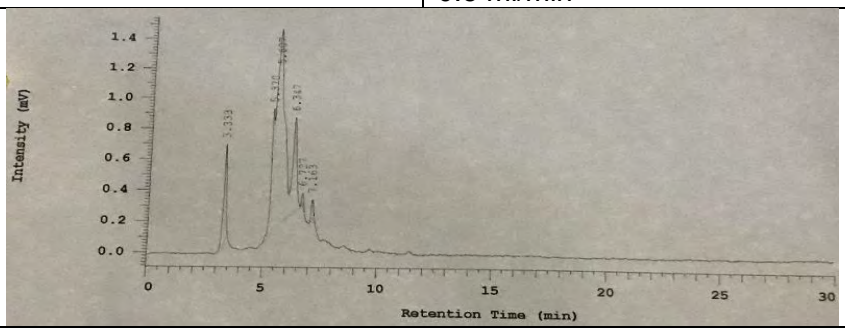
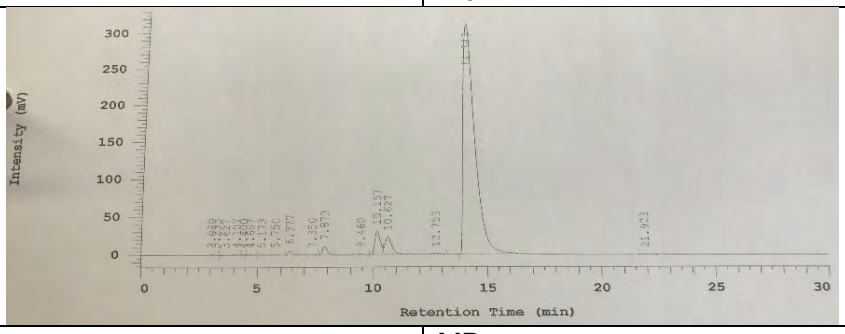
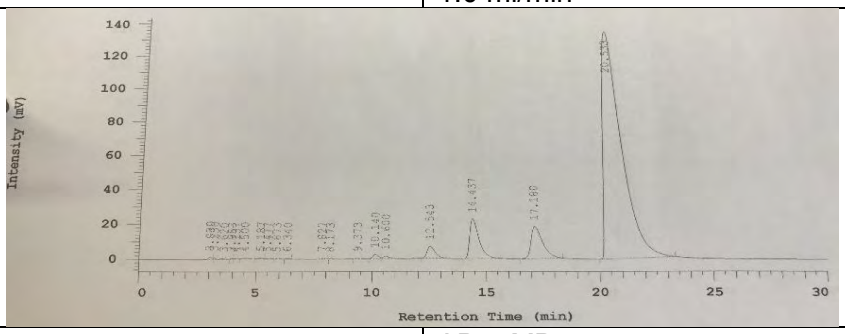
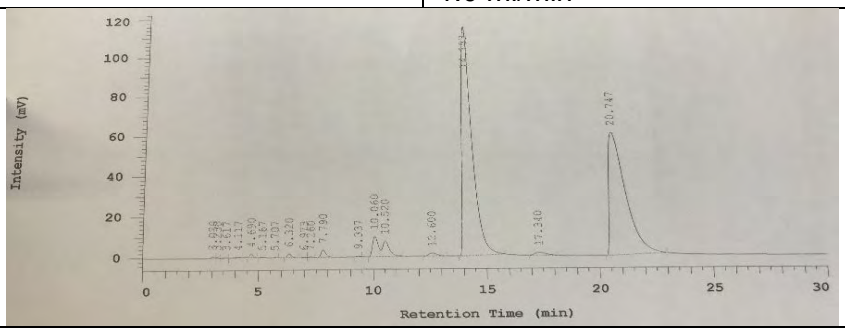
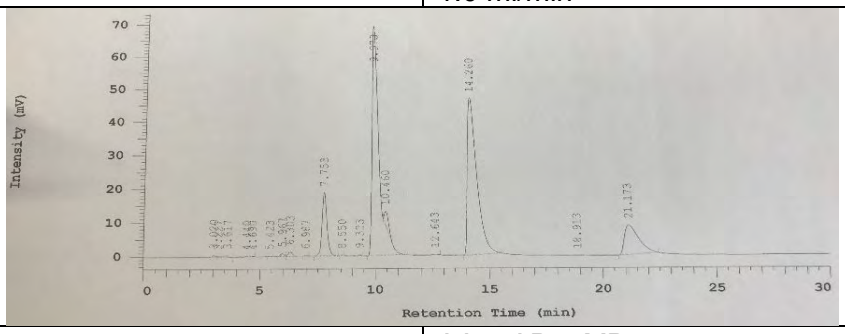


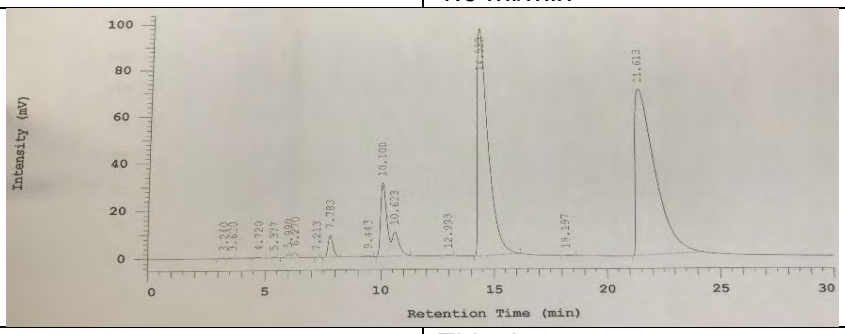
Table 4.3: Chromatograms generated on the Synergi polar-RP column.

Sample	AB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	AB + MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	

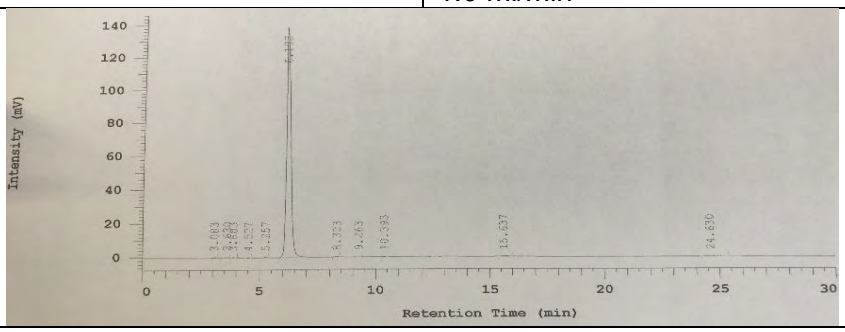
Sample	AA
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



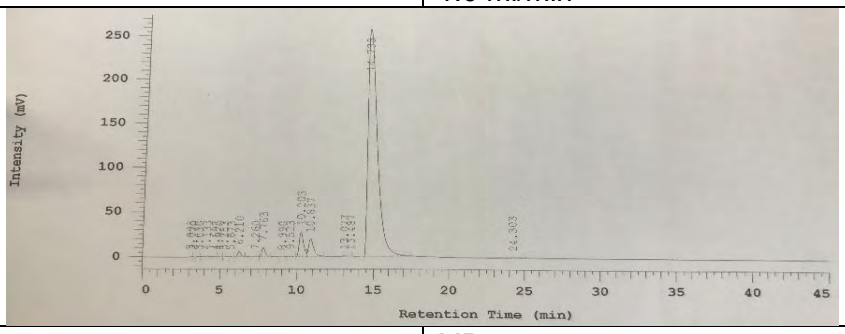
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



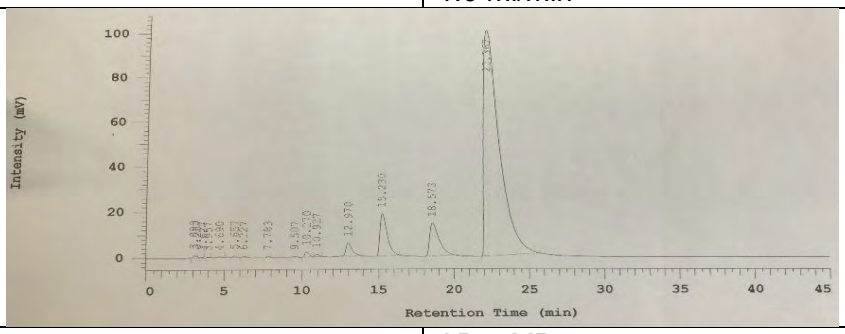
Sample	Thionin
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



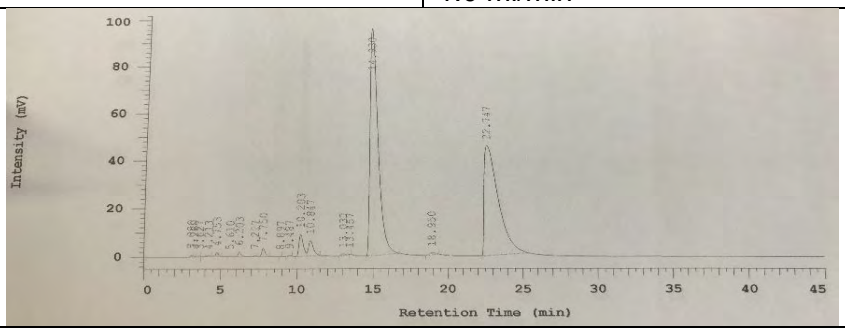
Sample	AB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



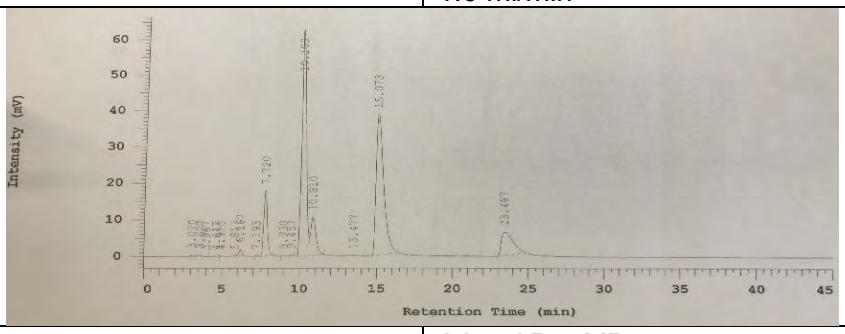
Sample	MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



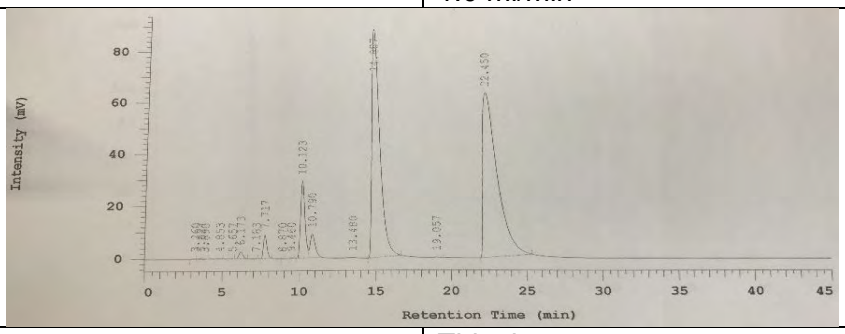
Sample	AB + MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



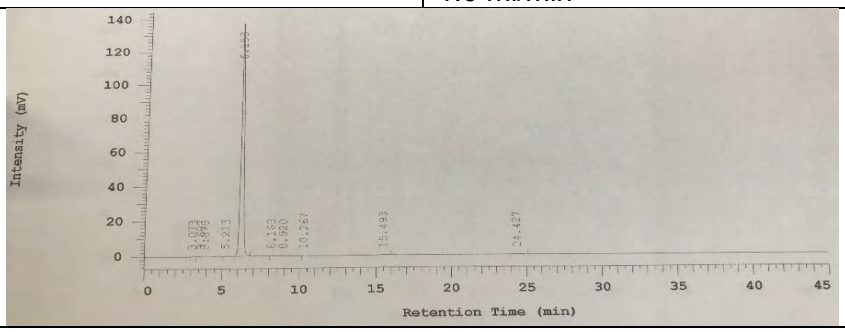
Sample	AA
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



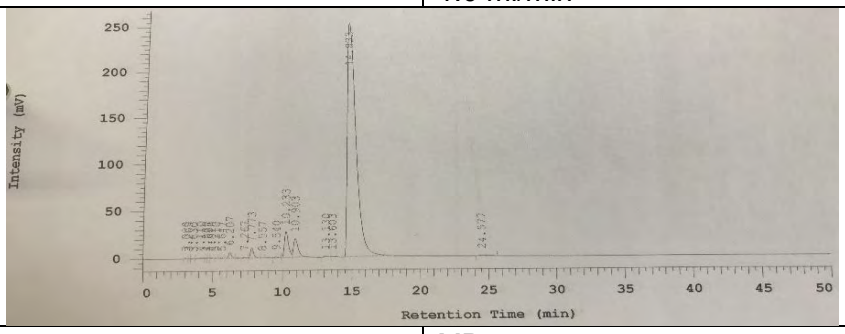
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



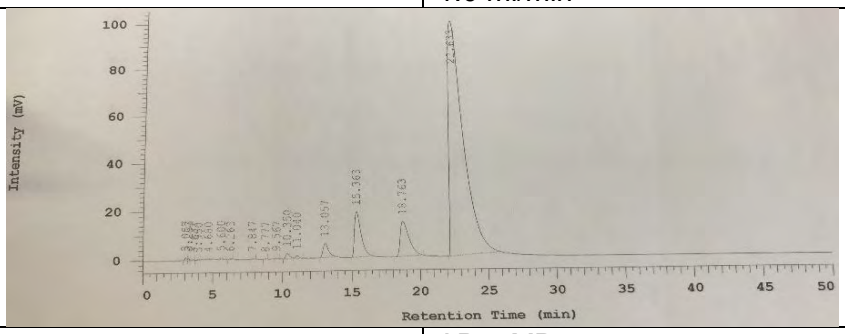
Sample	Thionin
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



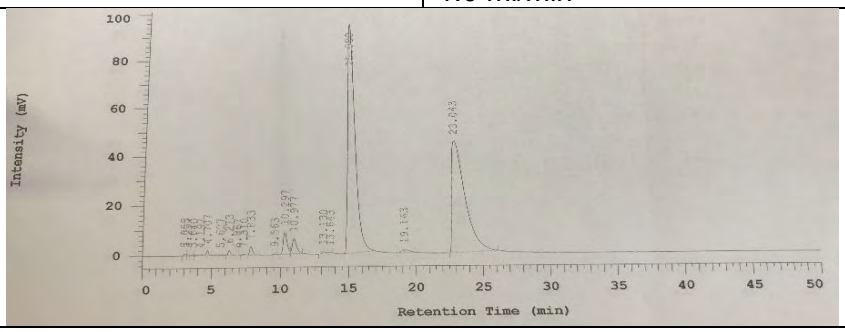
Sample	AB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



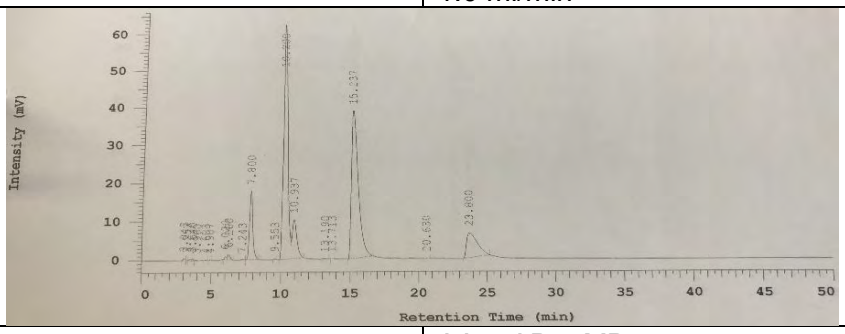
Sample	MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



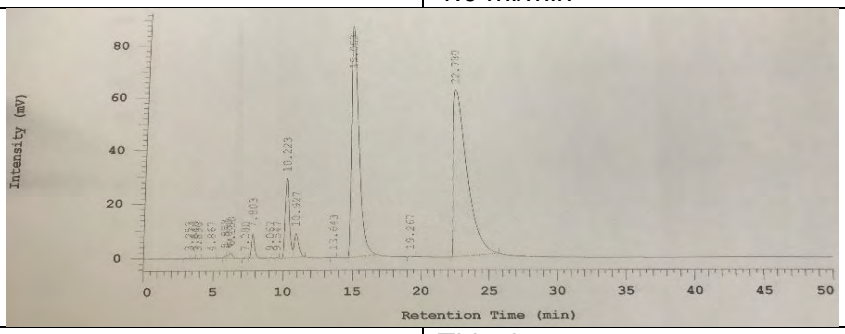
Sample	AB + MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



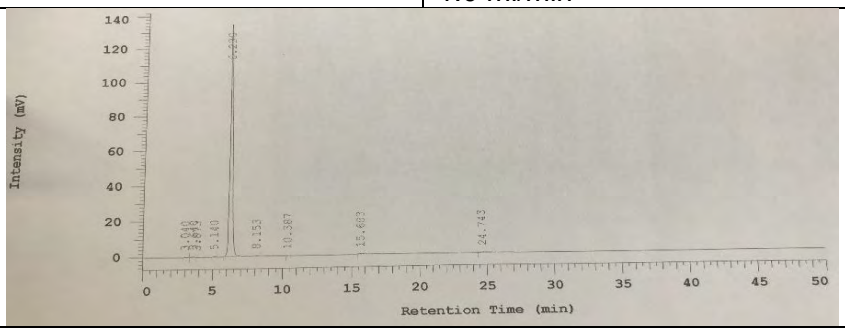
Sample	AA
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

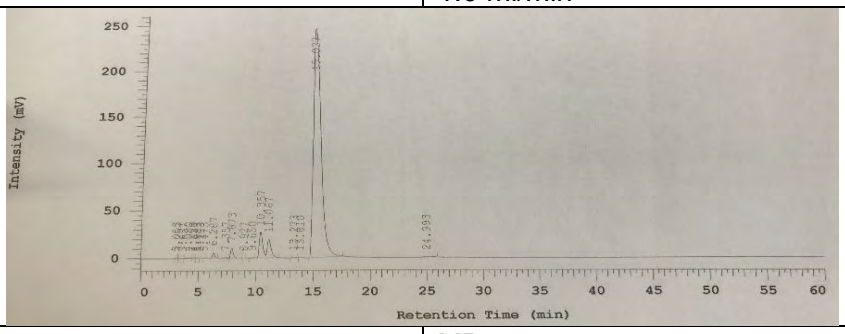
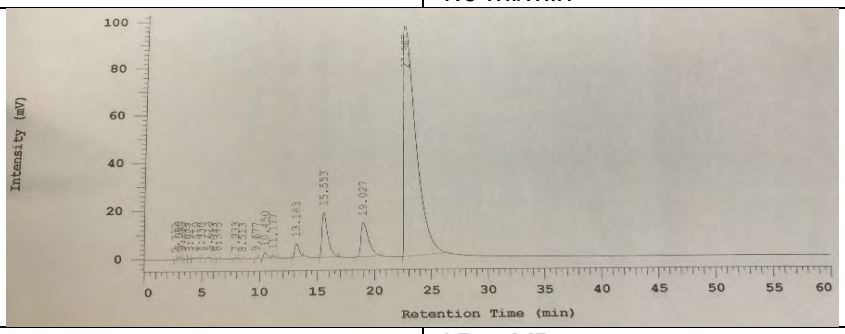
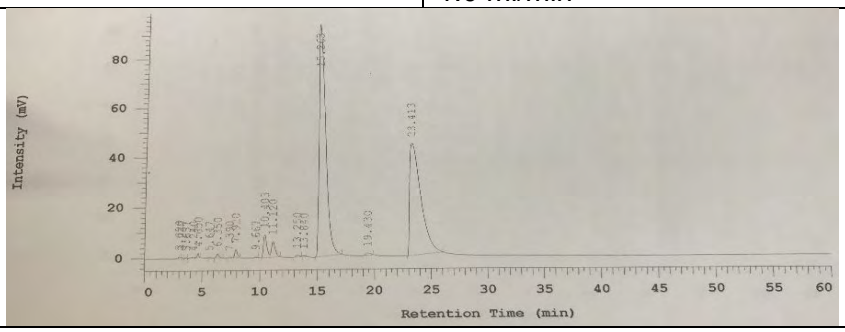


Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

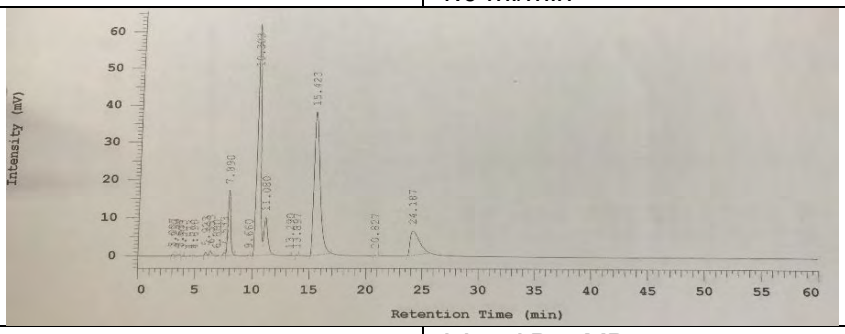


Sample	Thionin
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

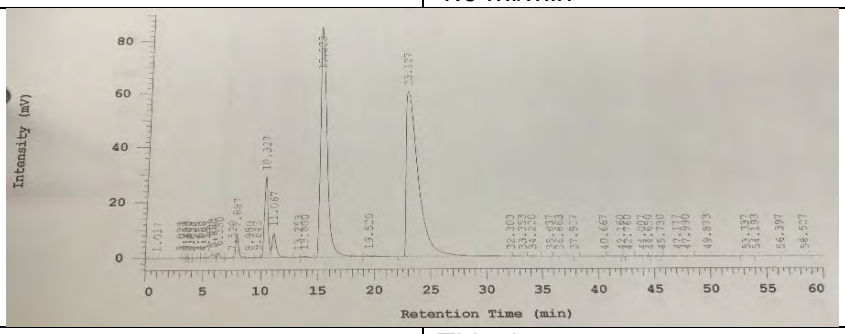


Sample	AB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	AB + MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	

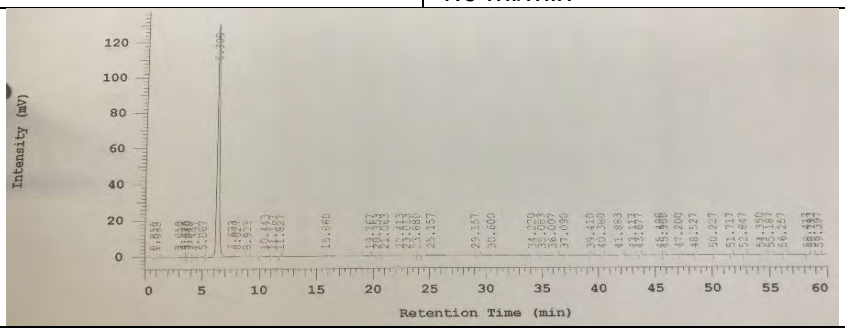
Sample	AA
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



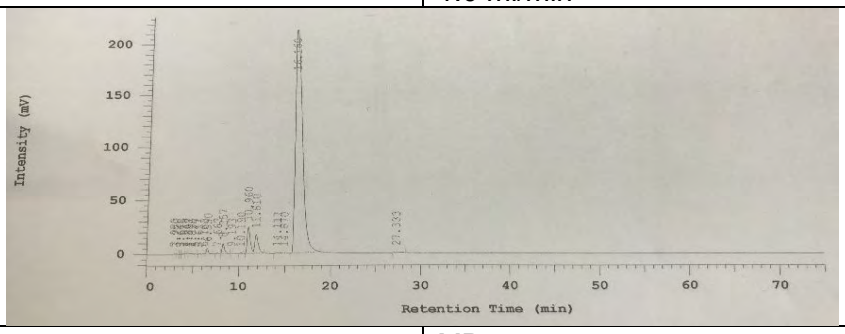
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



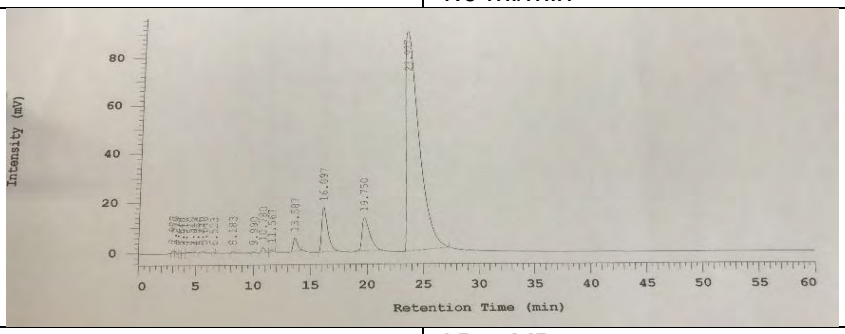
Sample	Thionin
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



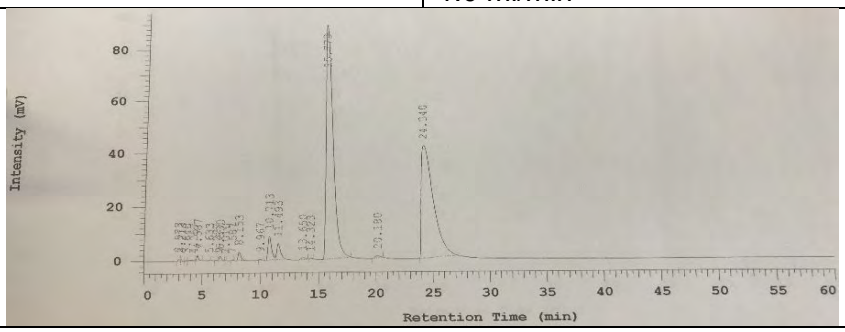
Sample	AB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



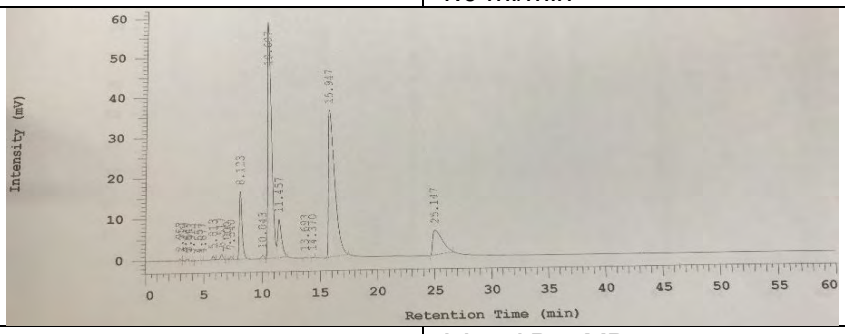
Sample	MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



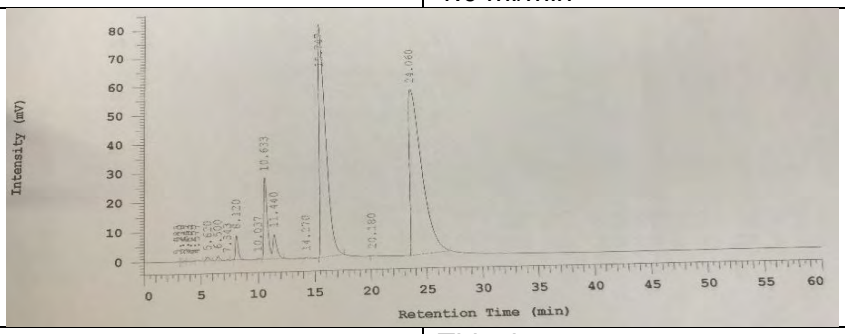
Sample	AB + MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



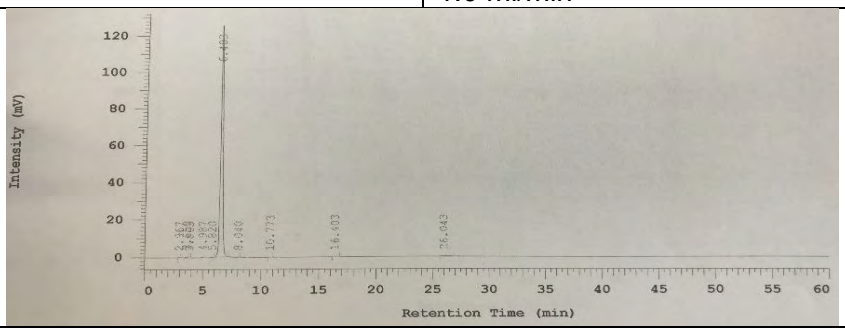
Sample	AA
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



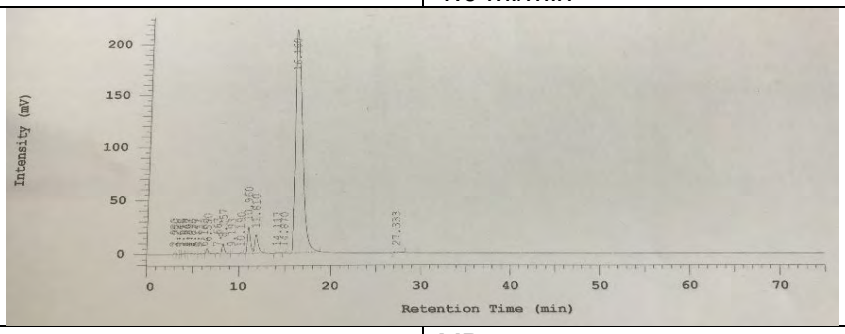
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



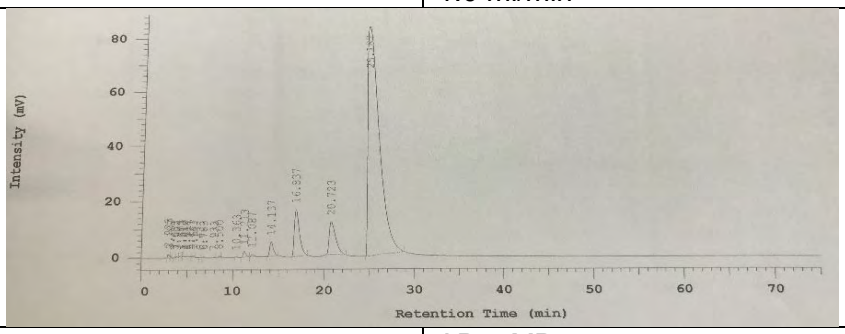
Sample	Thionin
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min



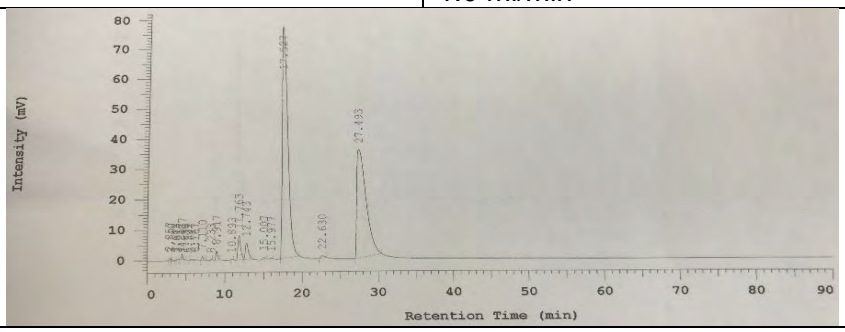
Sample	AB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

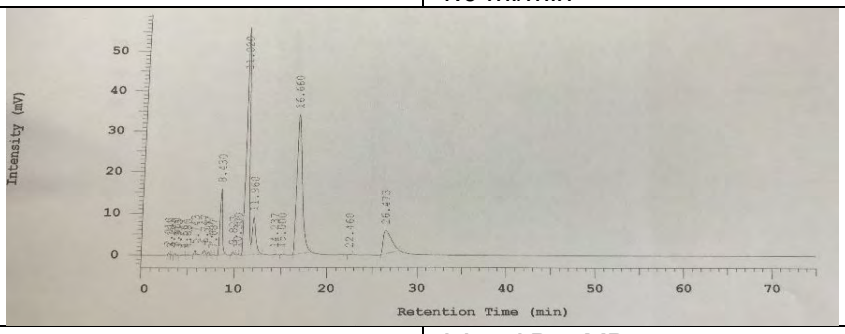
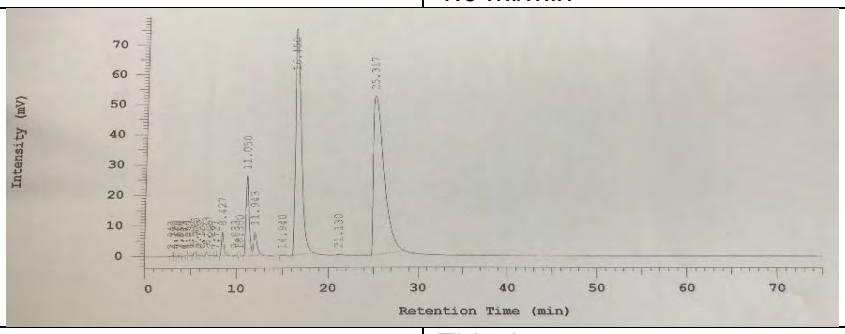
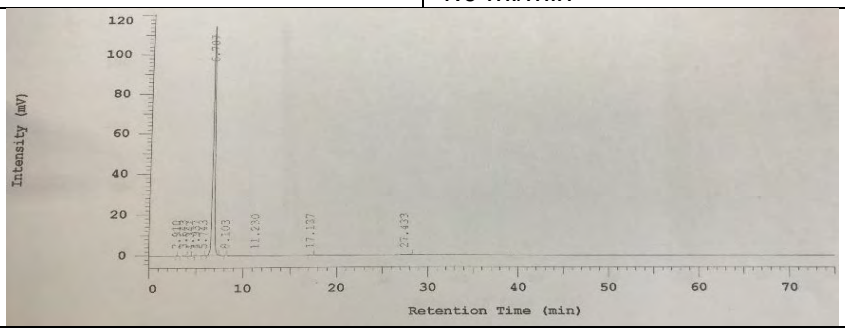


Sample	MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

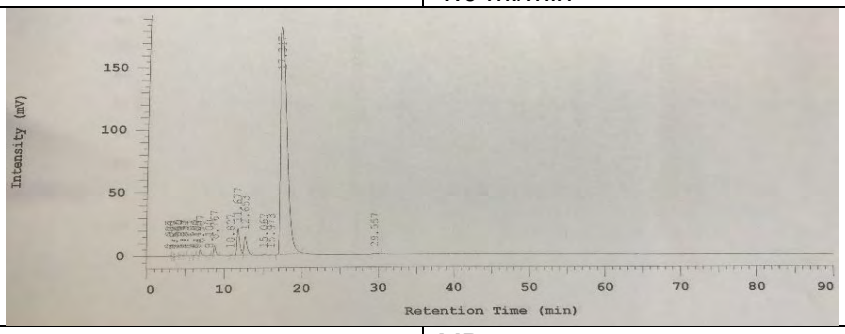


Sample	AB + MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

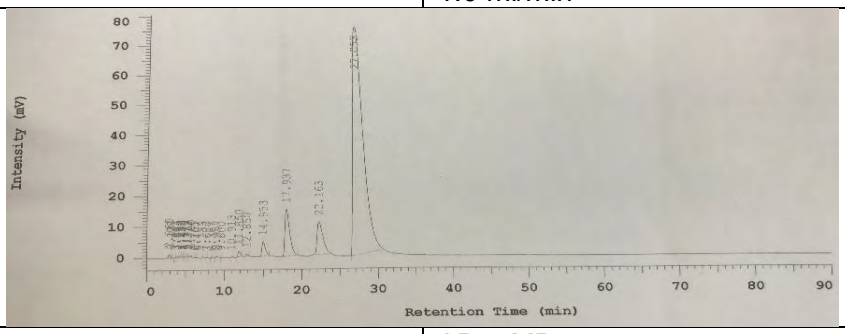


Sample	AA
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	Thionin
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	

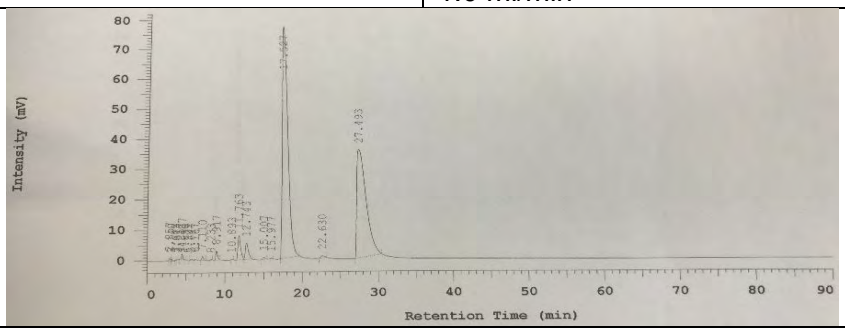
Sample	AB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

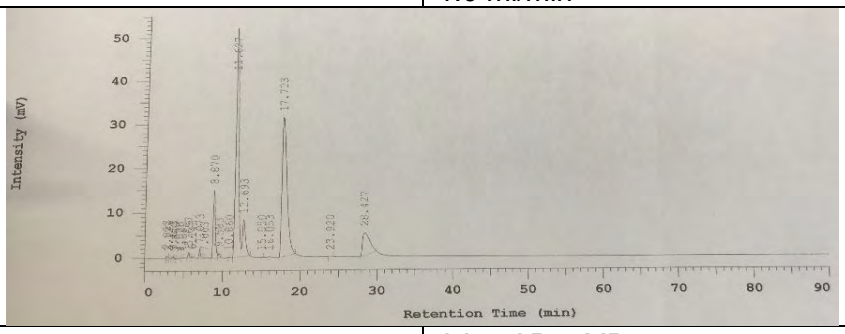
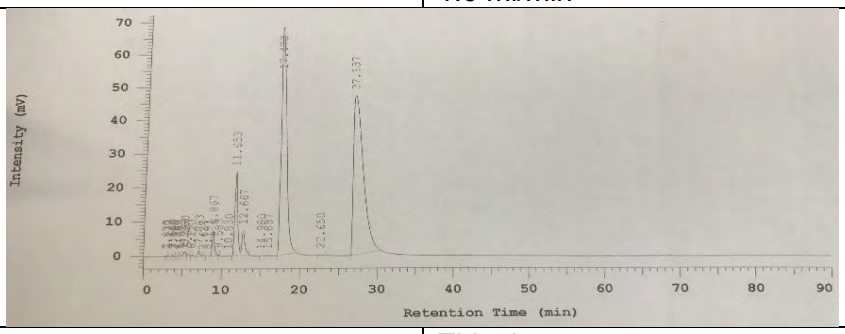
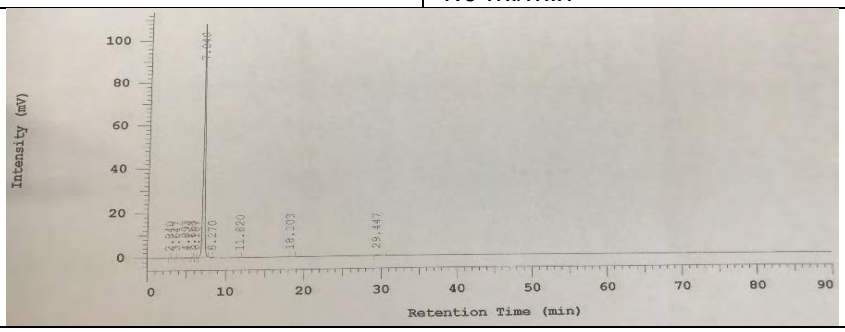


Sample	MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

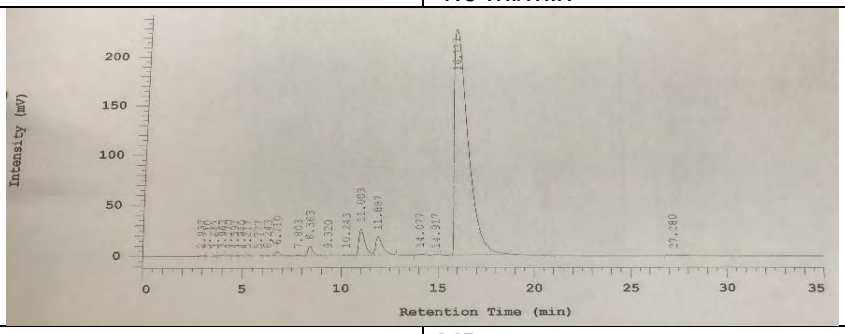


Sample	AB + MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min

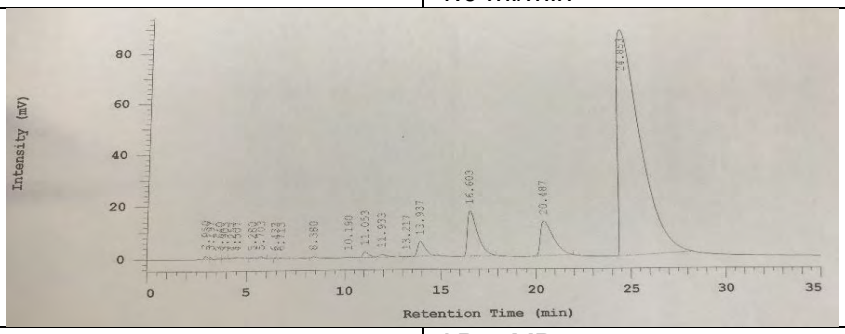


Sample	AA
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	
Sample	Thionin
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol
Flow rate	1.0 ml/min
	

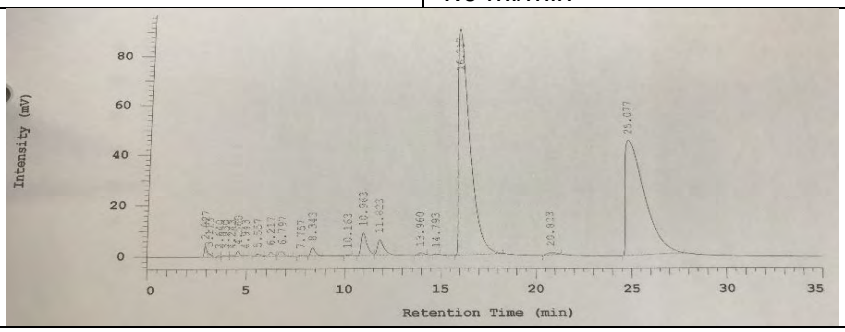
Sample	AB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



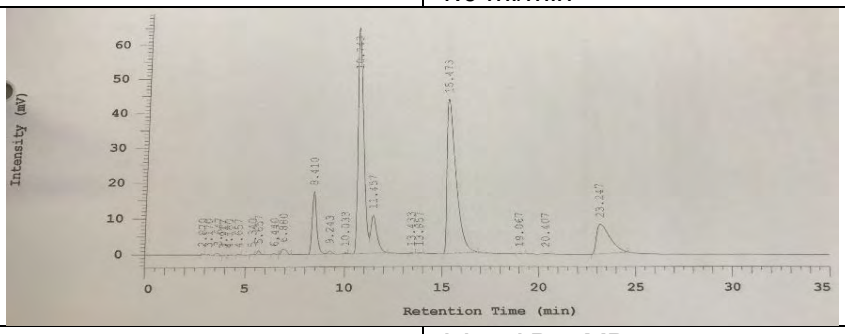
Sample	MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



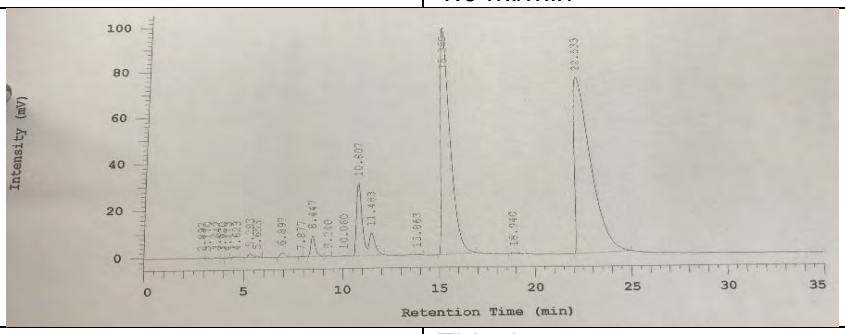
Sample	AB + MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



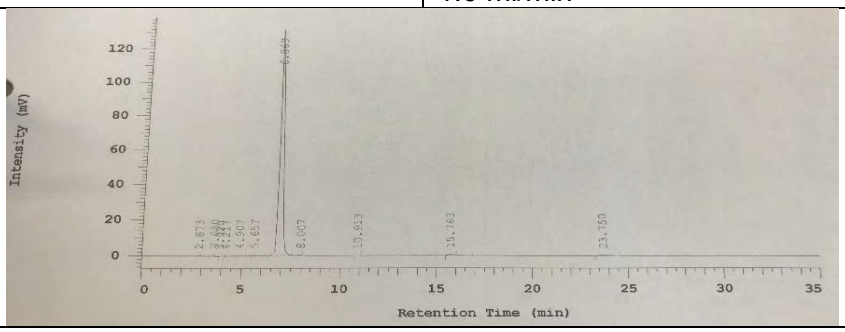
Sample	AA
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



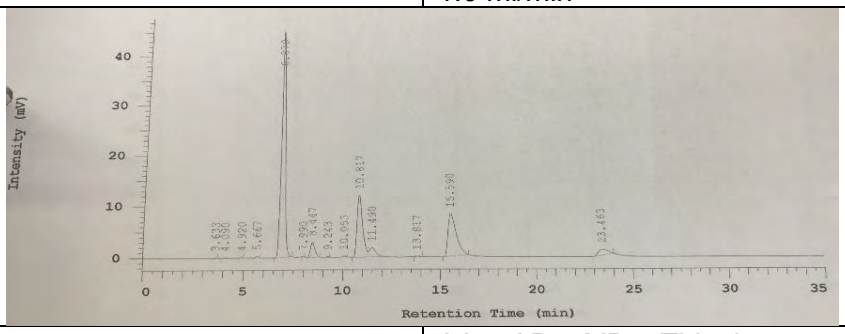
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



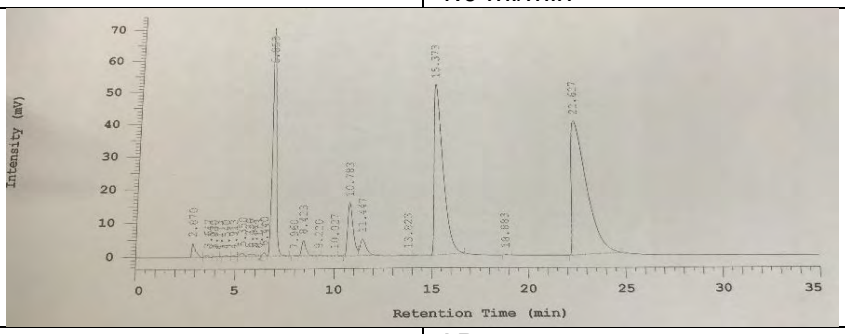
Sample	Thionin
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



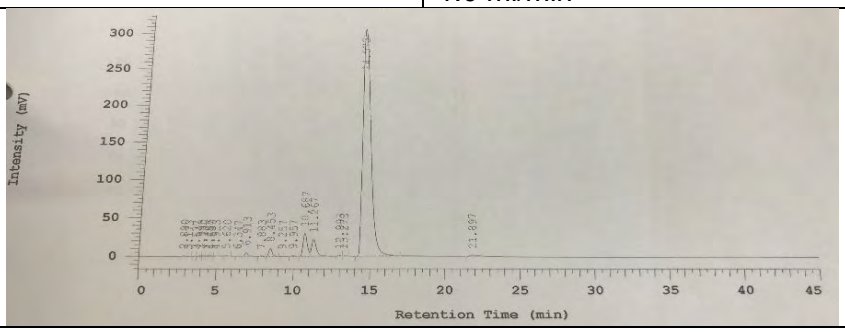
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



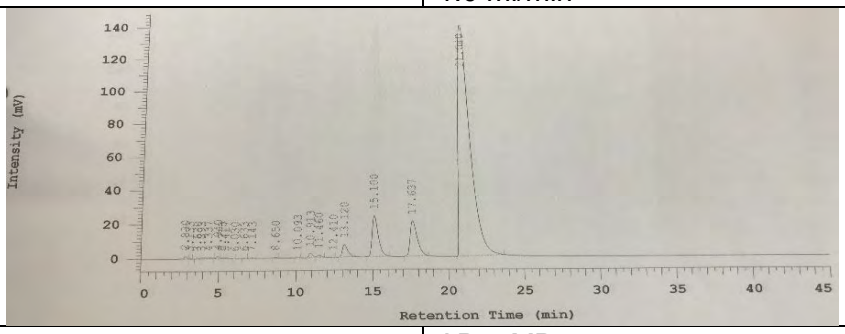
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	95:5 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



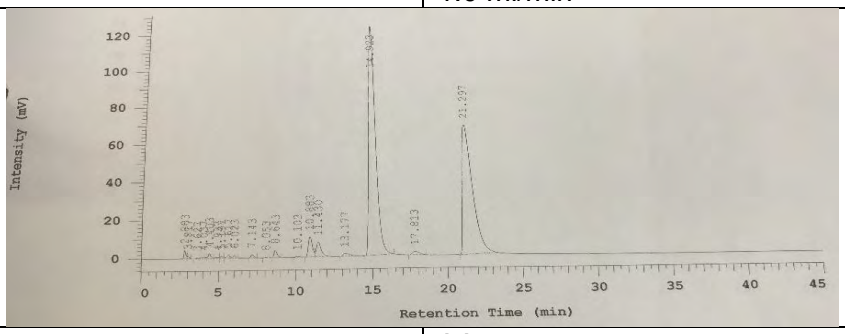
Sample	AB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



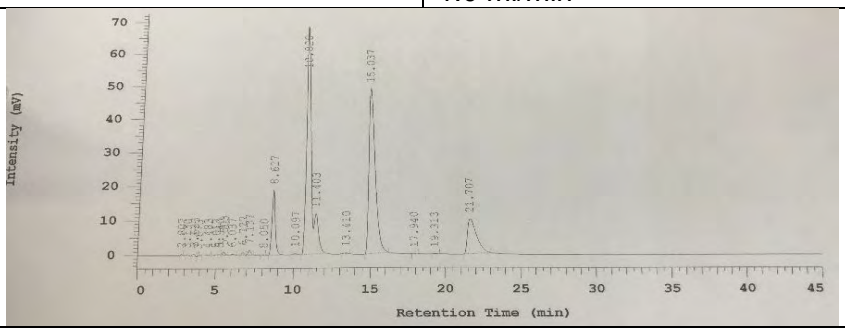
Sample	MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



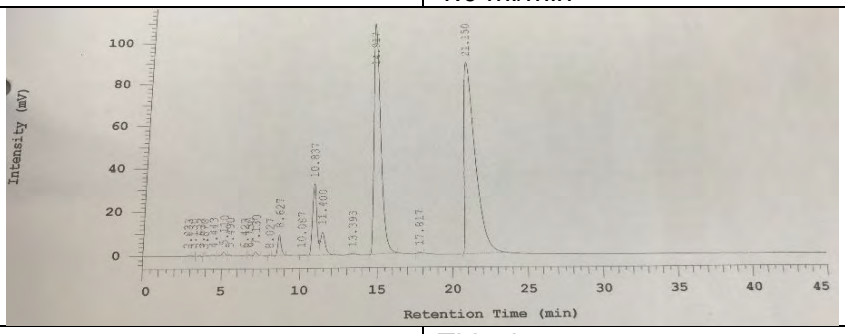
Sample	AB + MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



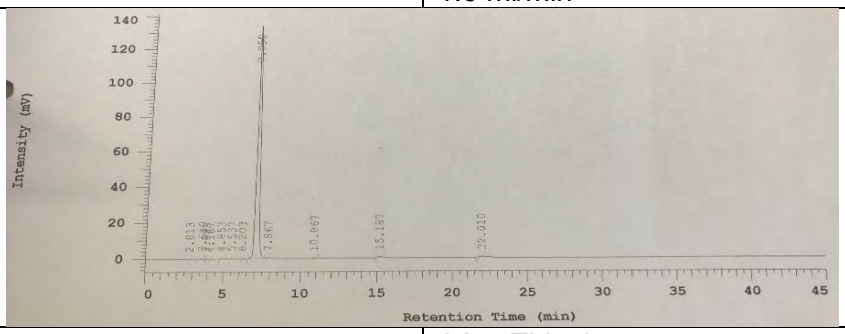
Sample	AA
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



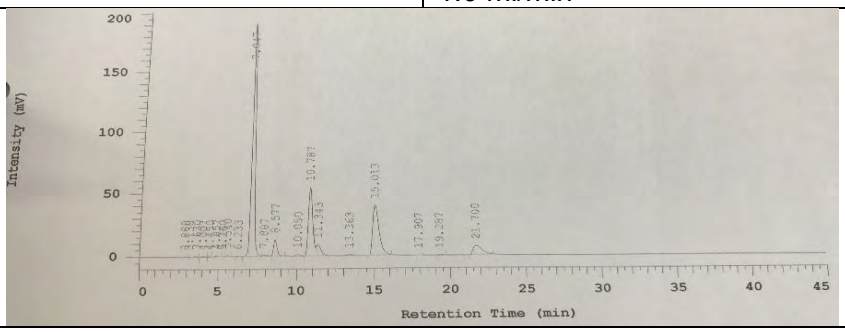
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



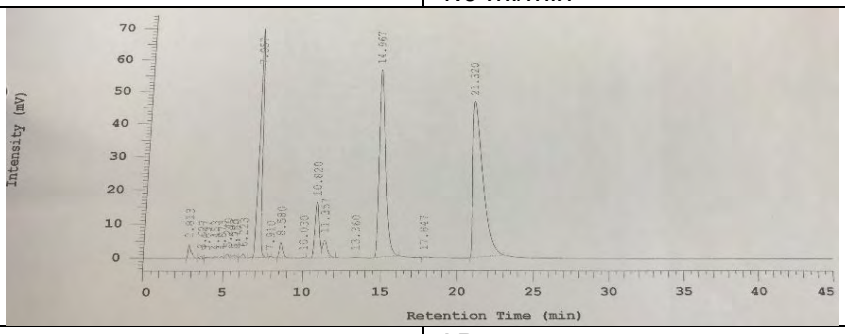
Sample	Thionin
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



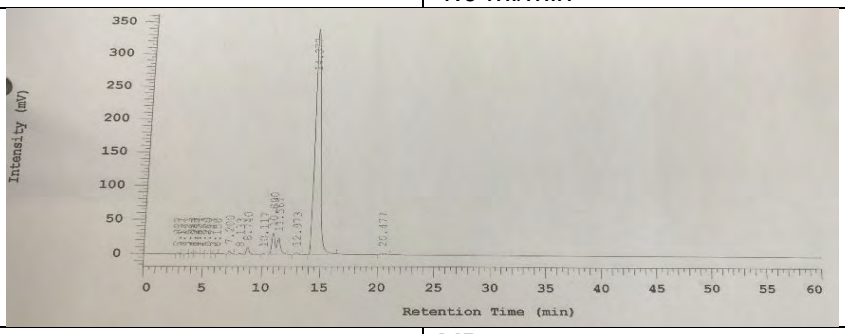
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



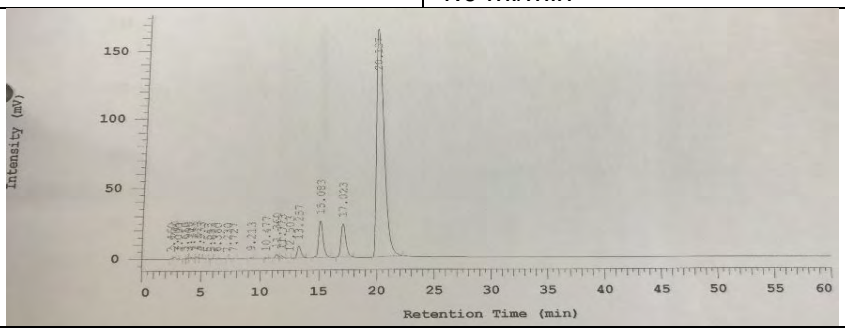
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	90:10 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



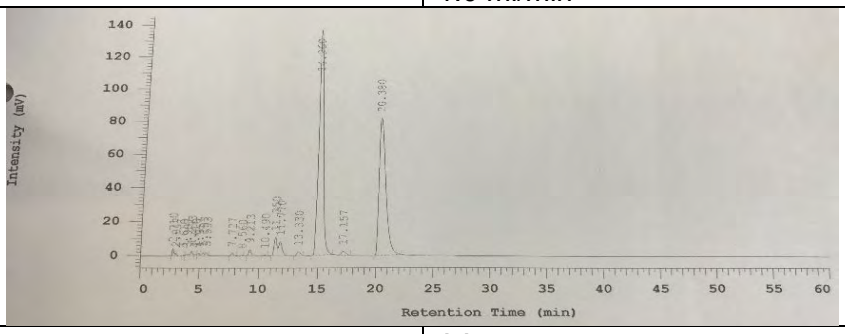
Sample	AB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



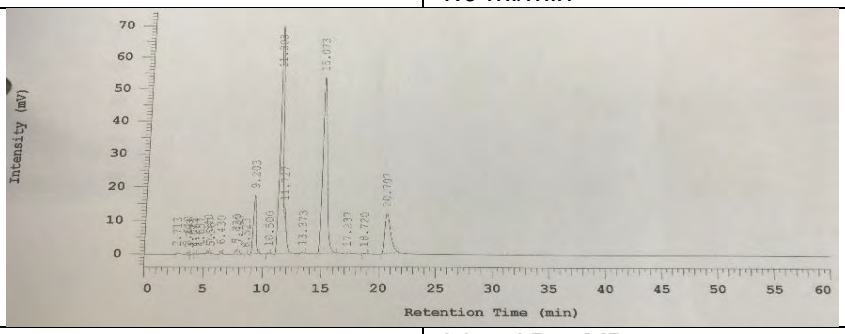
Sample	MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



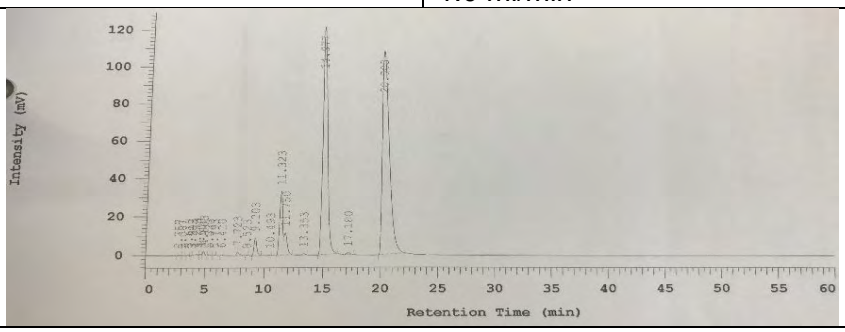
Sample	AB + MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



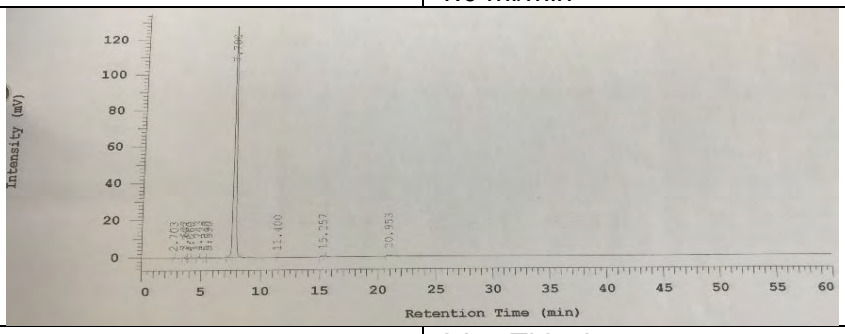
Sample	AA
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



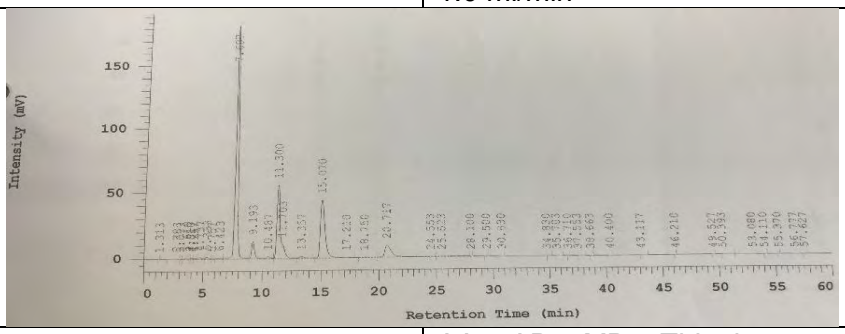
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



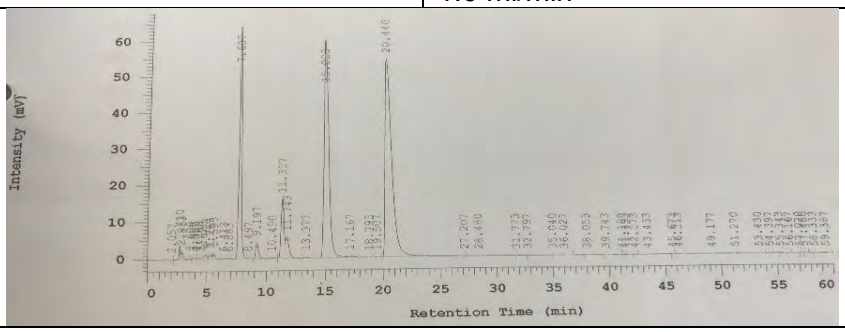
Sample	Thionin
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



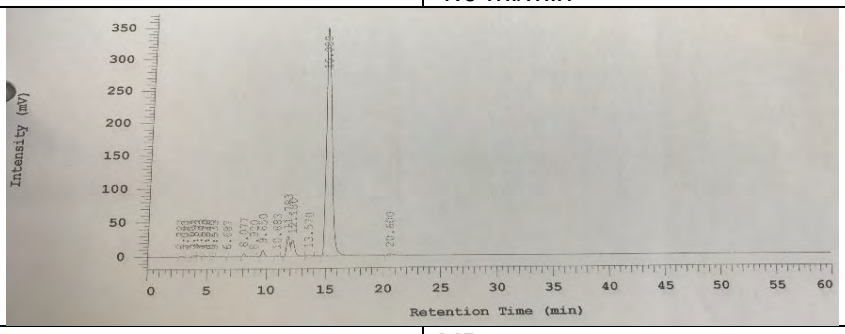
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



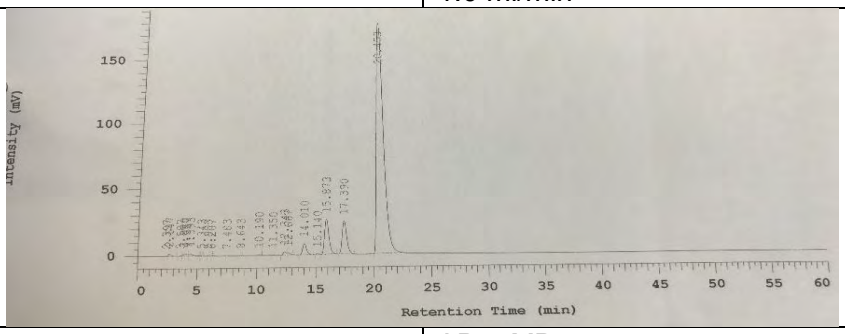
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	80:20 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



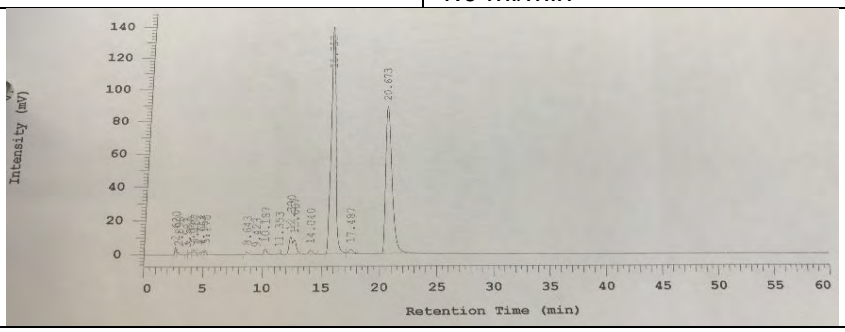
Sample	AB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



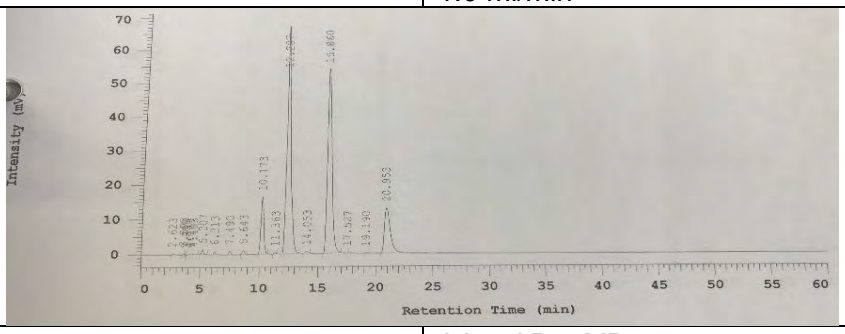
Sample	MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



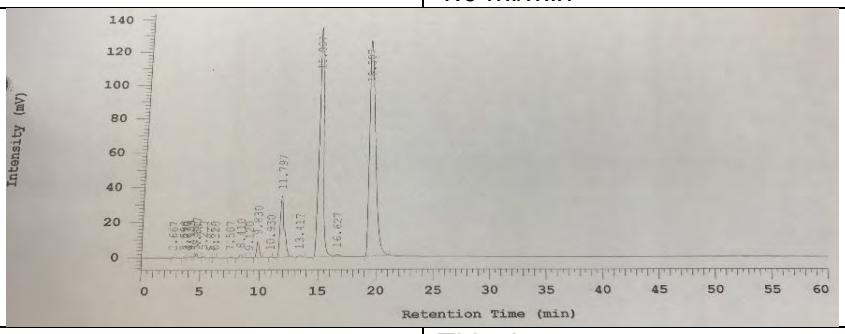
Sample	AB + MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



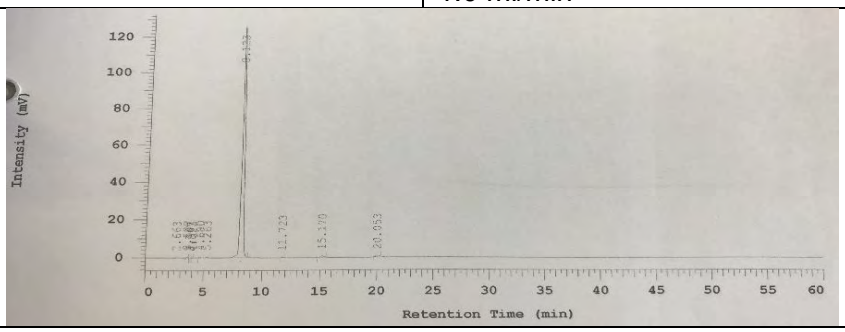
Sample	AA
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min

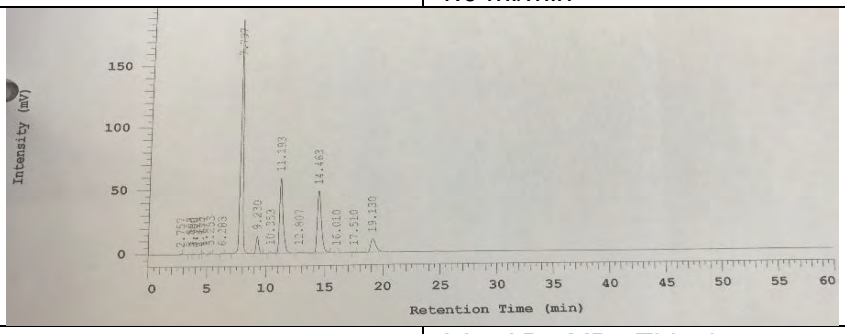
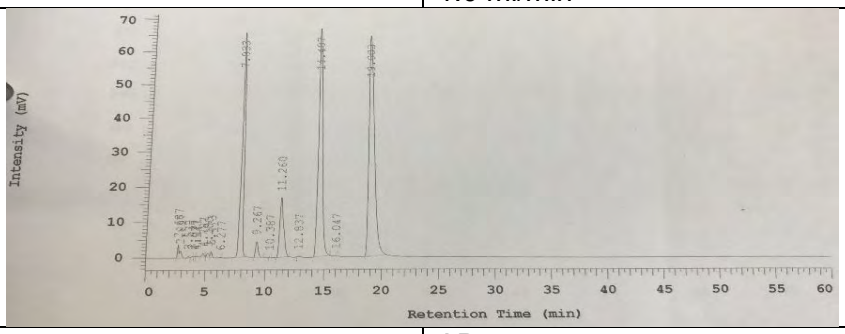
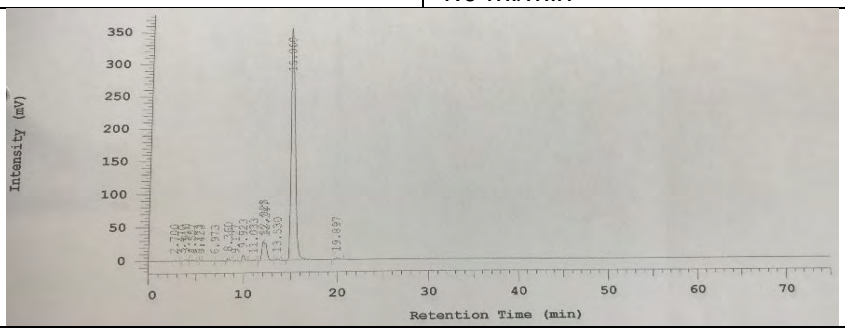


Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min

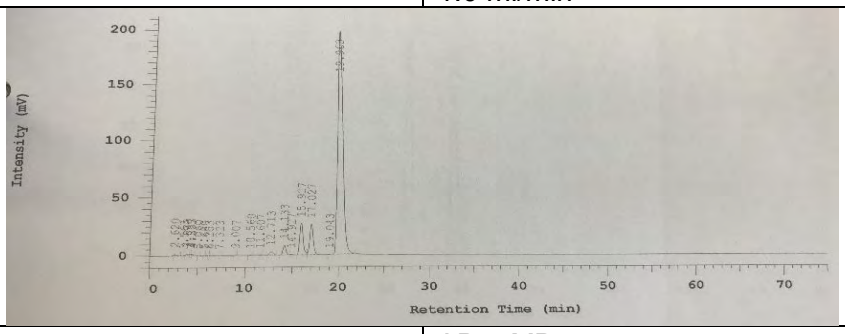


Sample	Thionin
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min

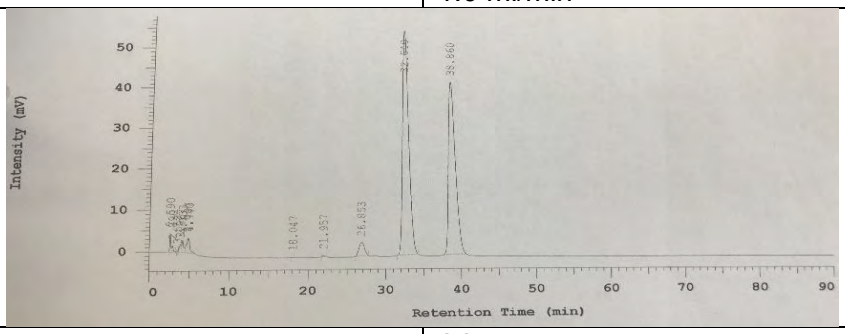


Sample	AA + Thionin
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min
	
Sample	AA +AB +MB +Thionin
Solvent	Methanol
Mobile phase	70:30 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min
	
Sample	AB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min
	

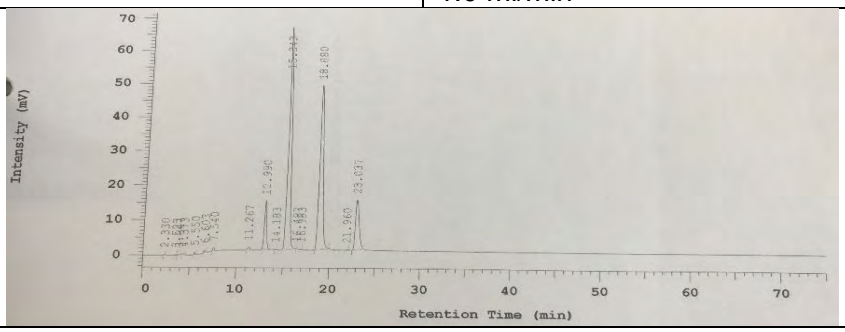
Sample	MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



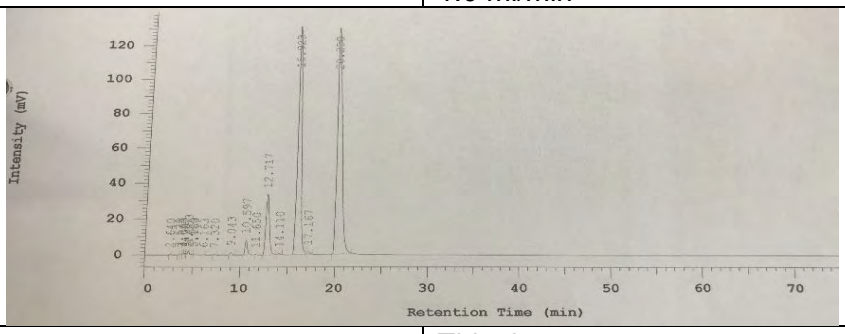
Sample	AB + MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



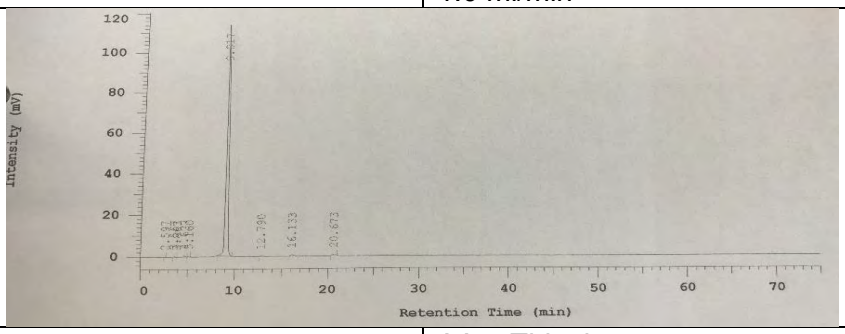
Sample	AA
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



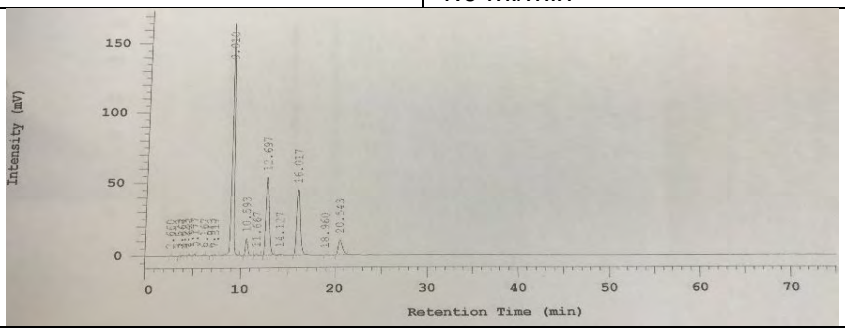
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min

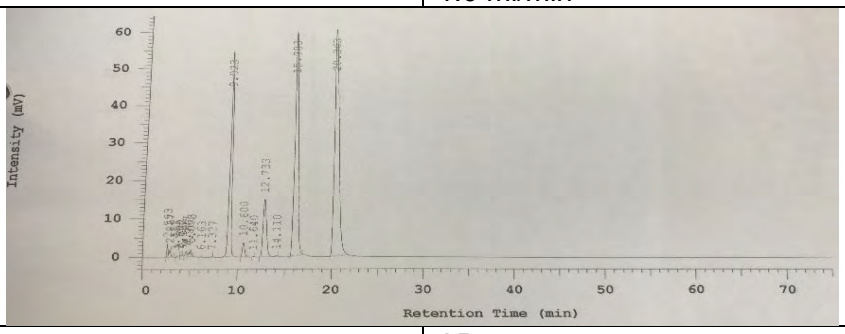
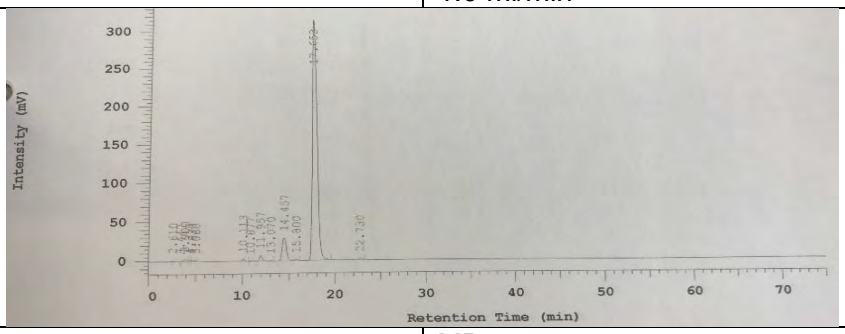
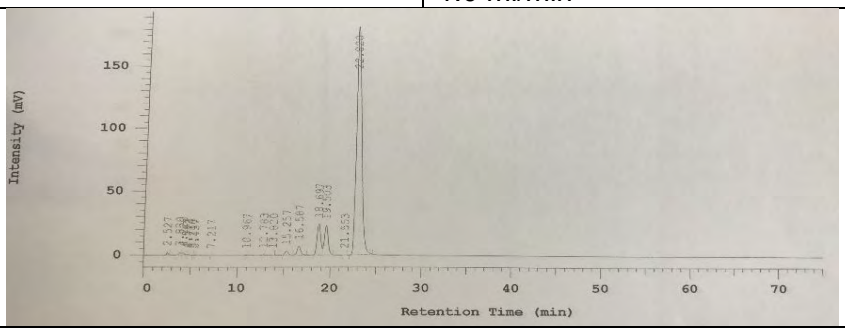


Sample	Thionin
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min

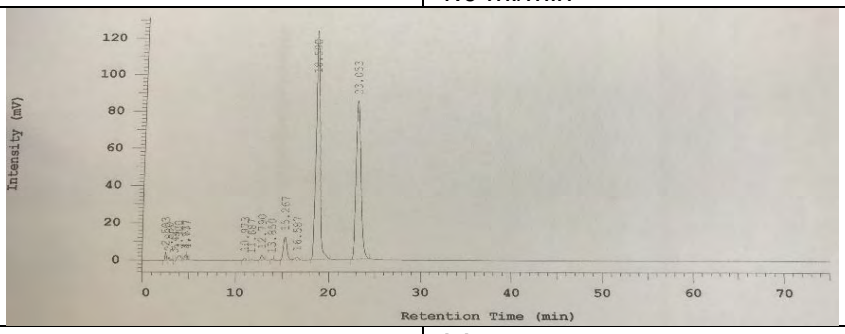


Sample	AA + Thionin
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min

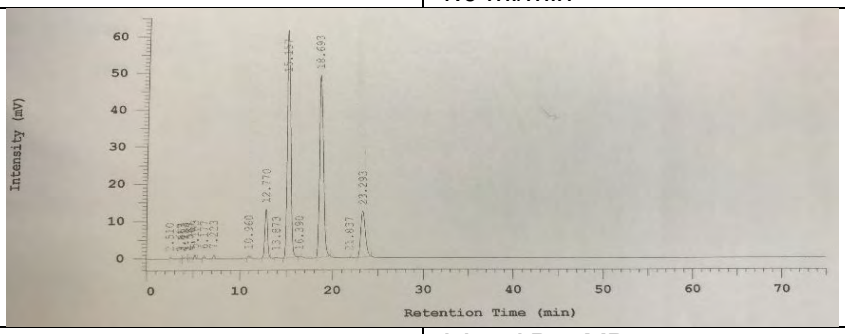


Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min
	
Sample	AB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min
	
Sample	MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min
	

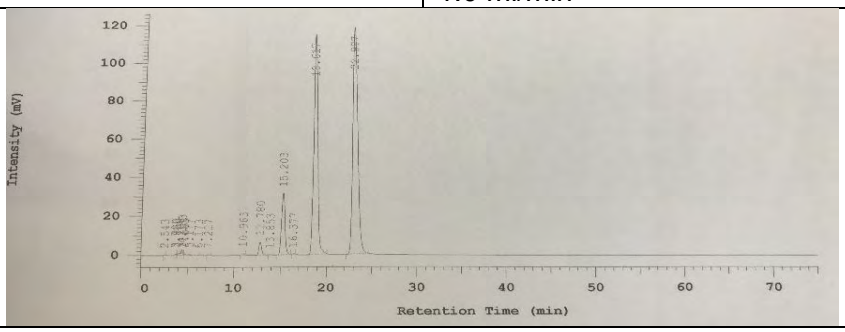
Sample	AB + MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



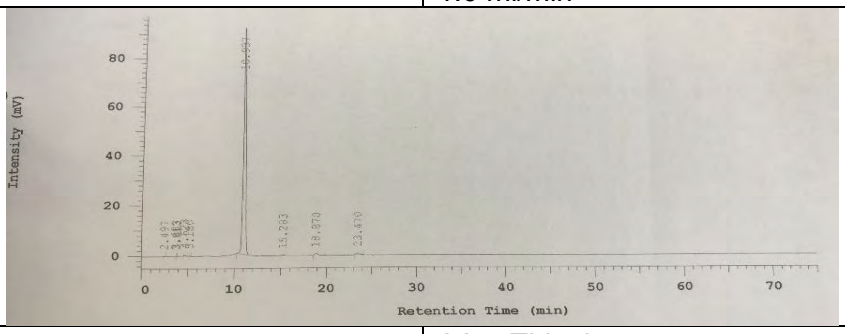
Sample	AA
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



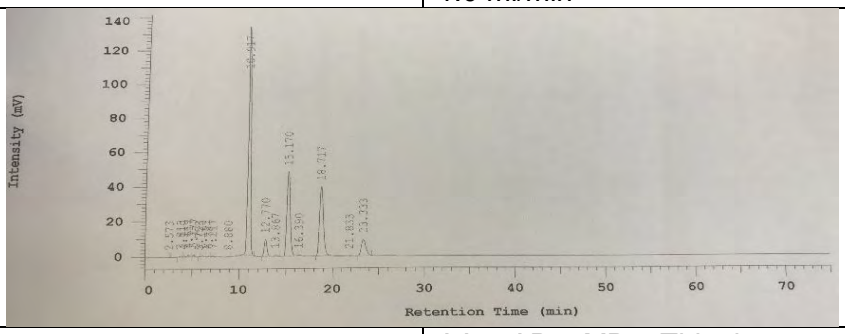
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



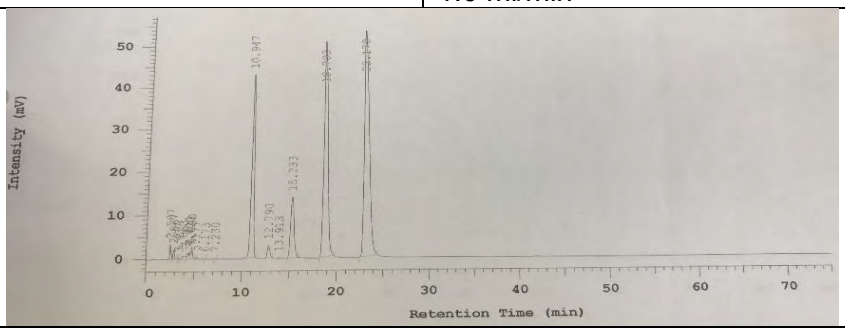
Sample	Thionin
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



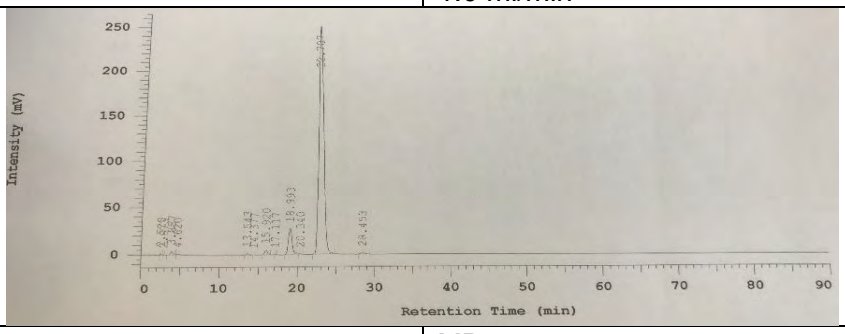
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



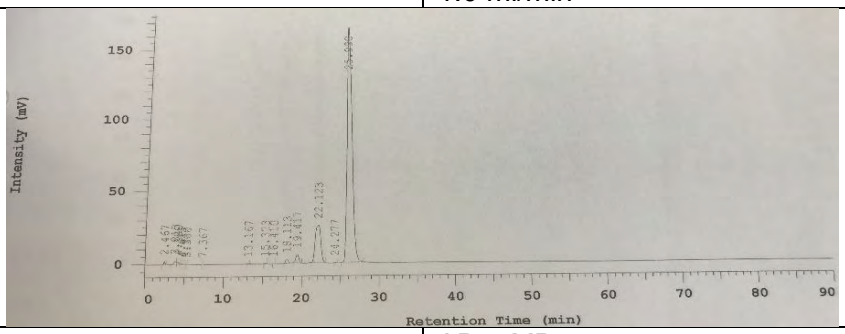
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



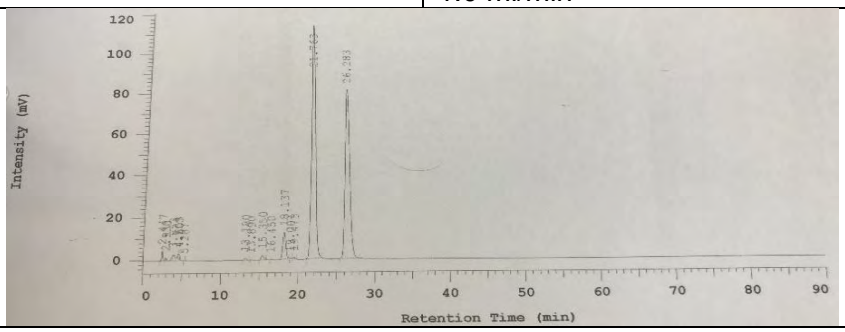
Sample	AB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



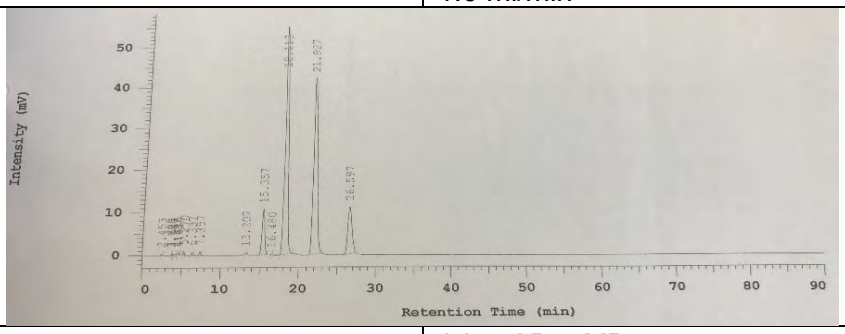
Sample	MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



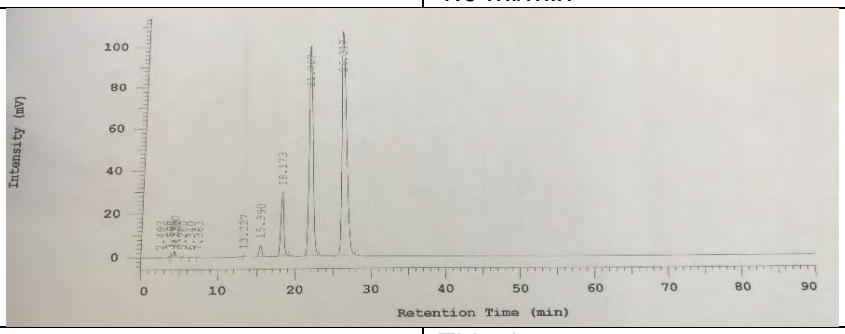
Sample	AB + MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



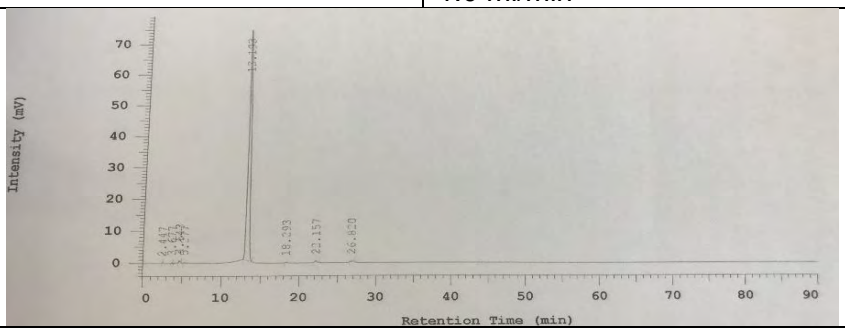
Sample	AA
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



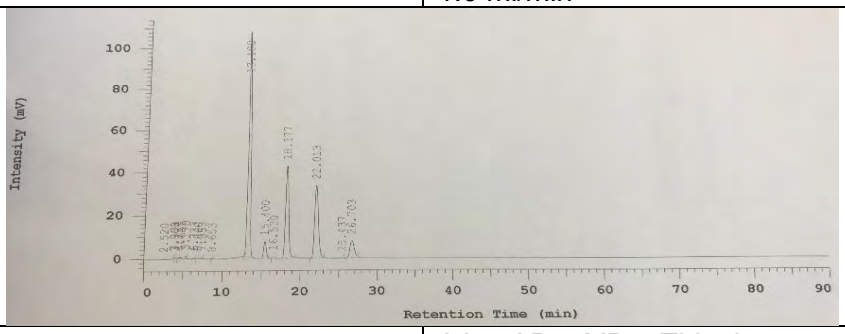
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



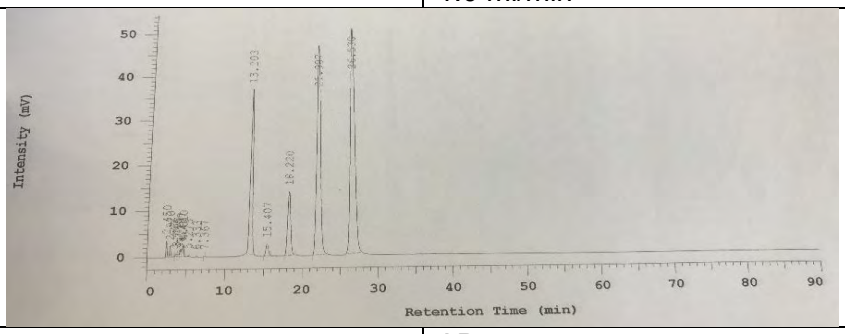
Sample	Thionin
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



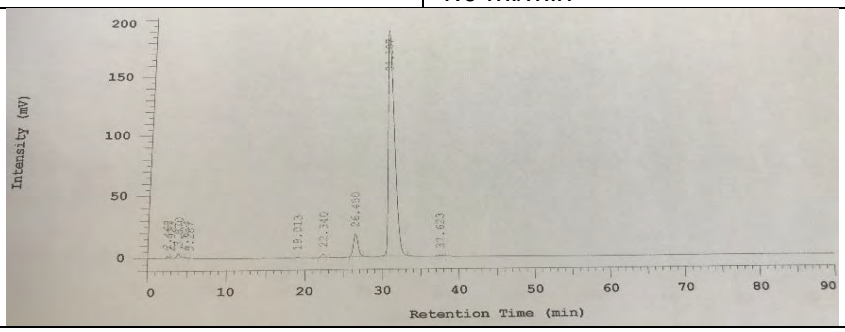
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



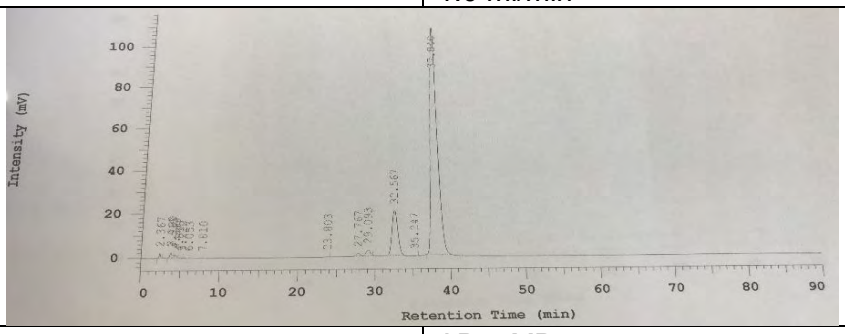
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



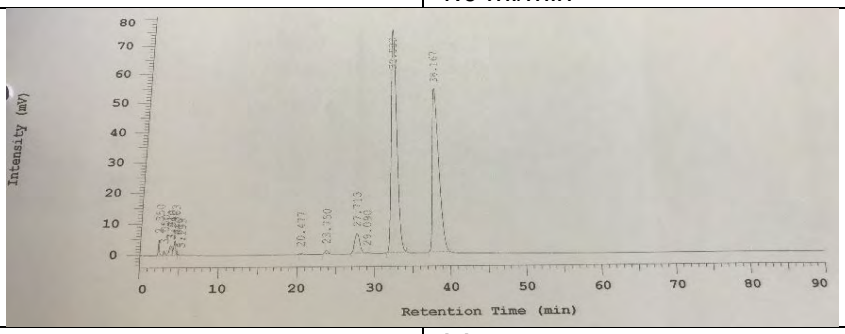
Sample	AB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



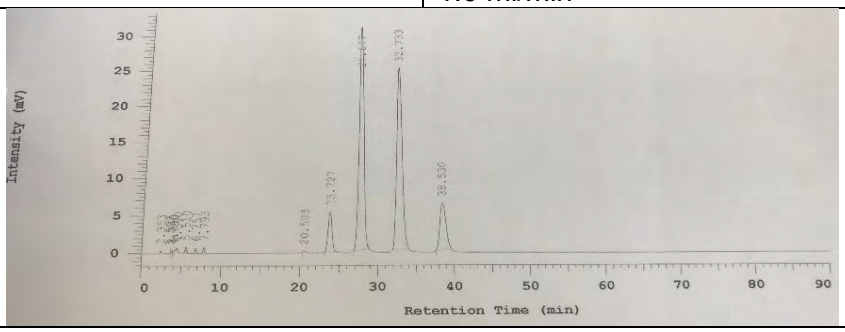
Sample	MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



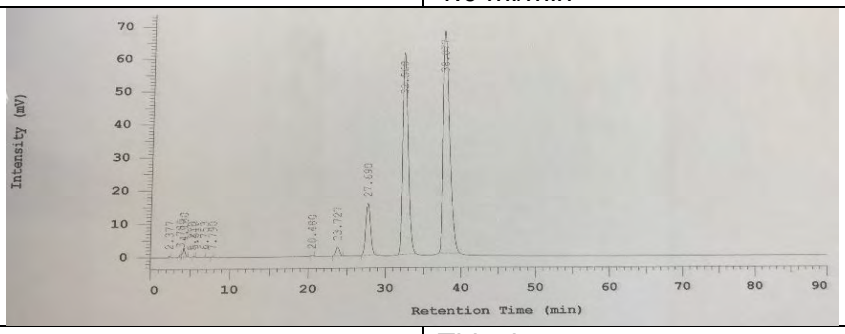
Sample	AB + MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



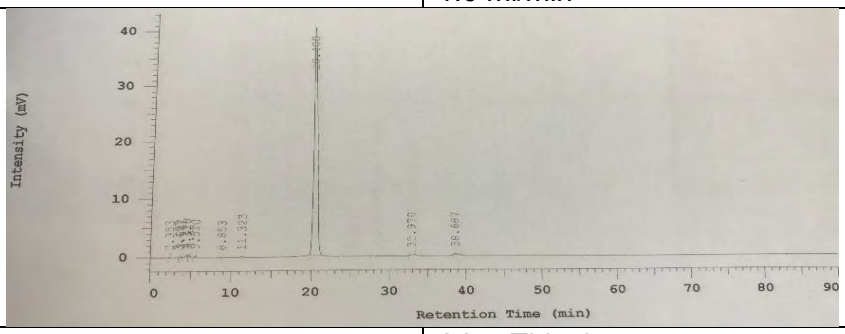
Sample	AA
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



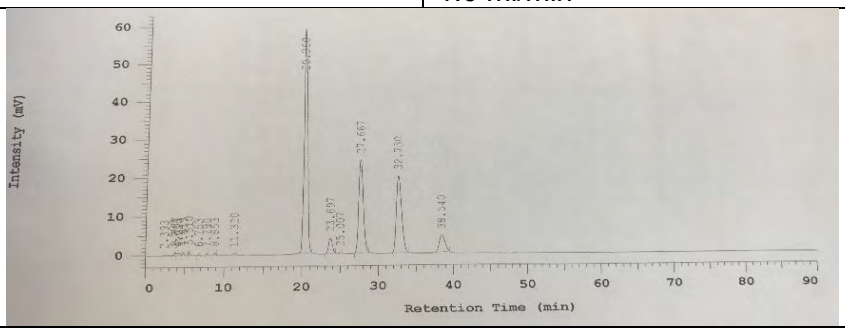
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



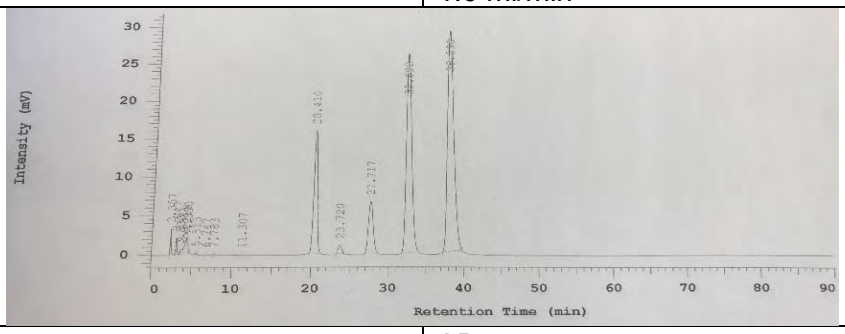
Sample	Thionin
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



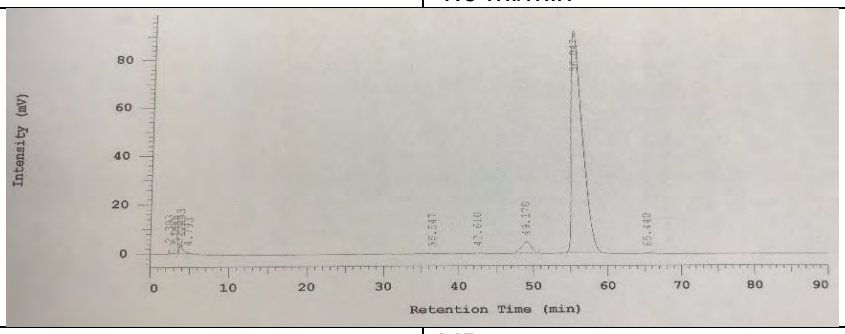
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



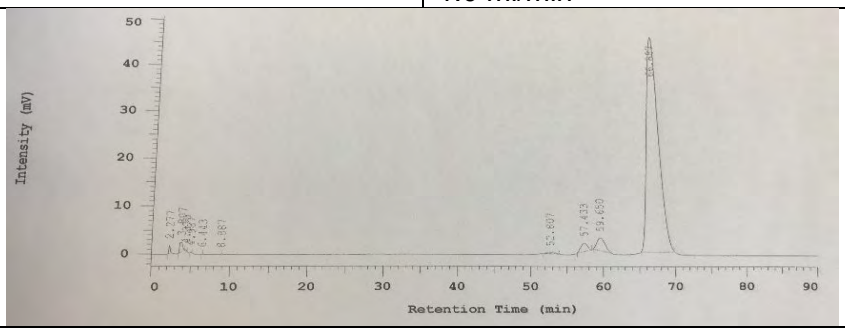
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	30:70 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



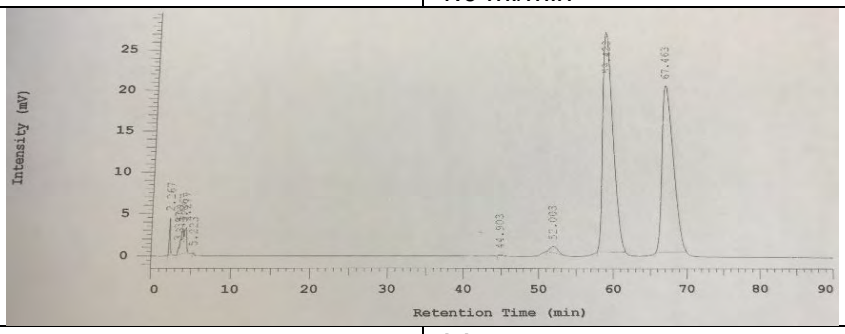
Sample	AB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



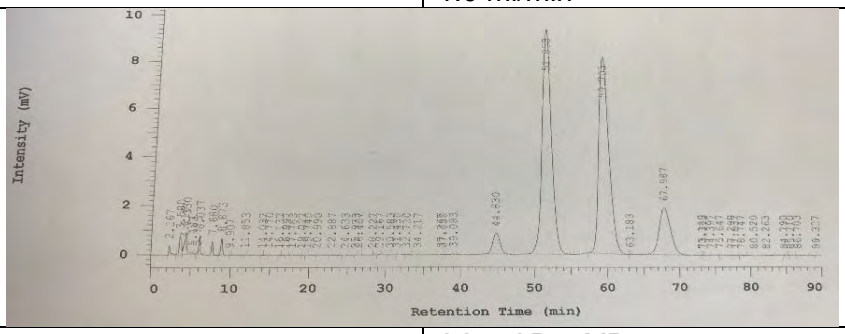
Sample	MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



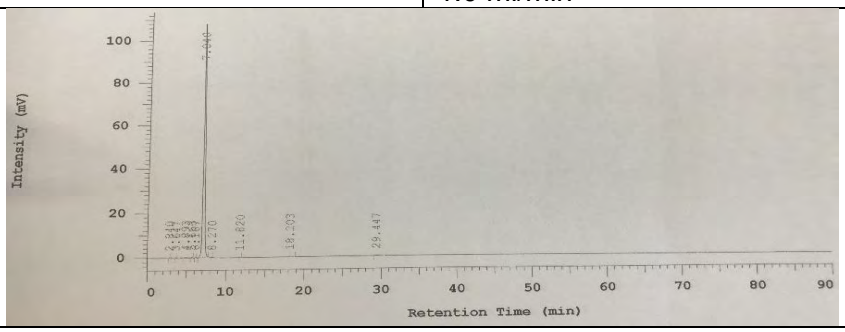
Sample	AB + MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



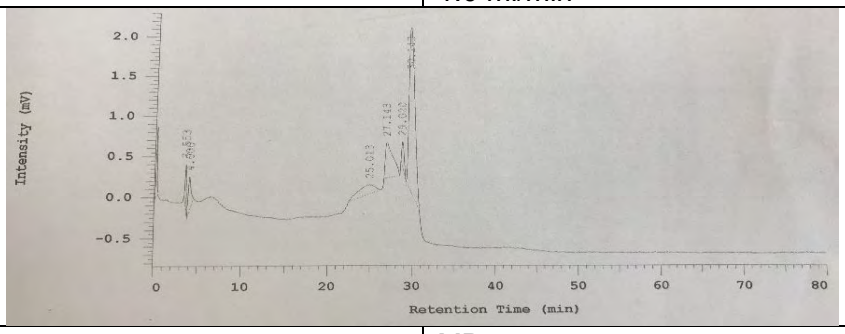
Sample	AA
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



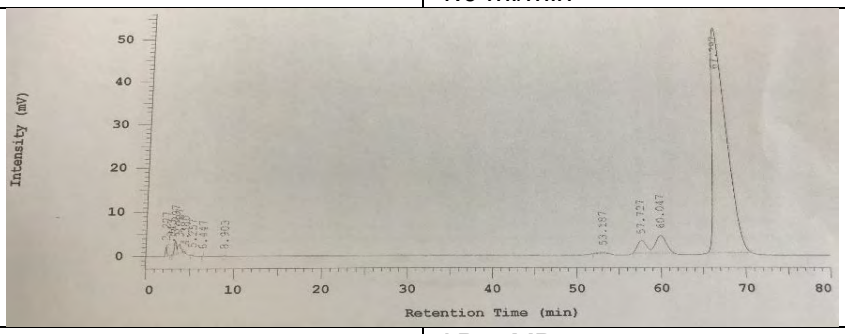
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	20:80 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



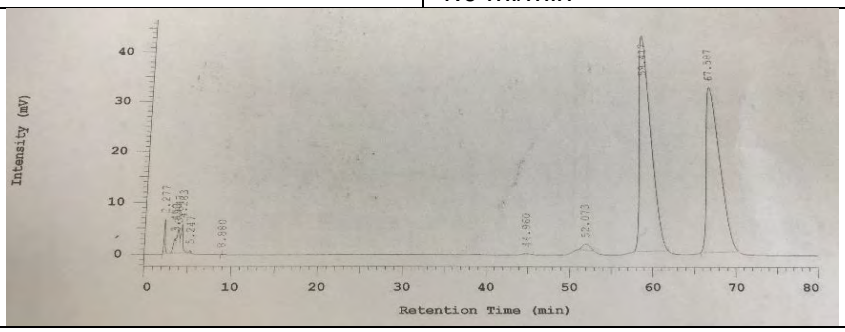
Sample	AB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



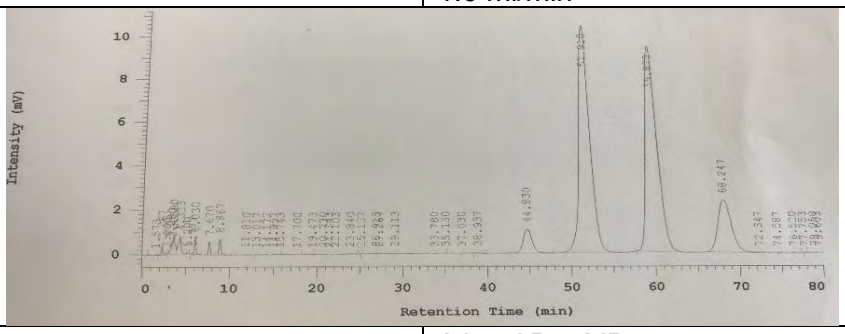
Sample	MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



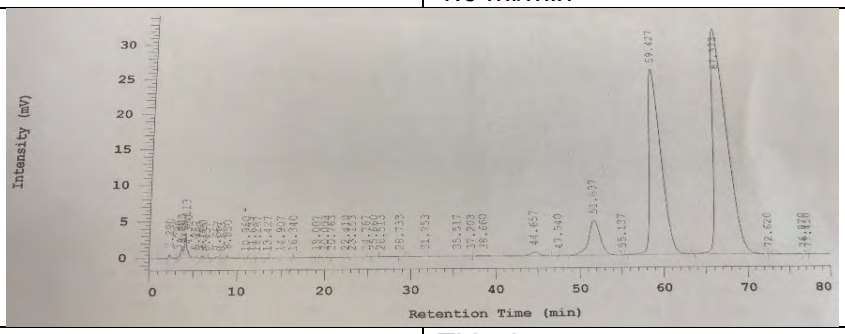
Sample	AB + MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



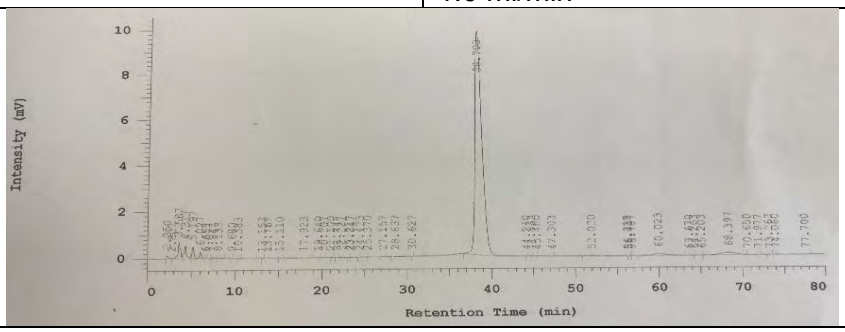
Sample	AA
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



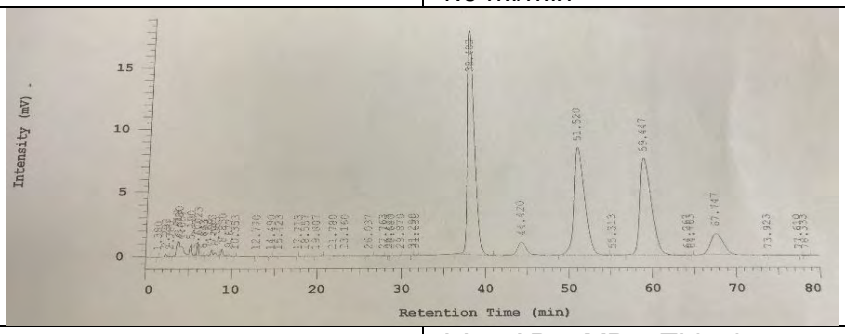
Sample	AA + AB + MB
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



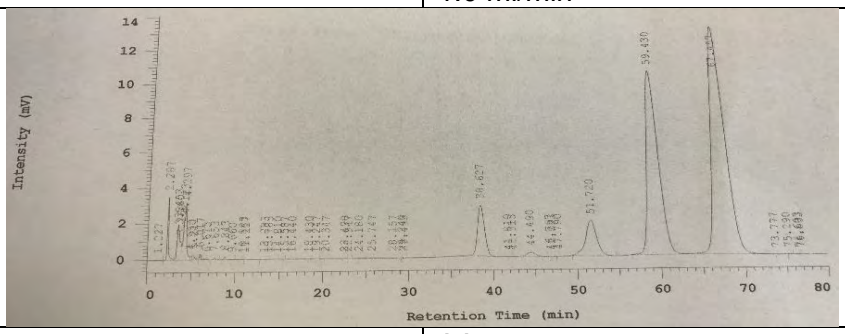
Sample	Thionin
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



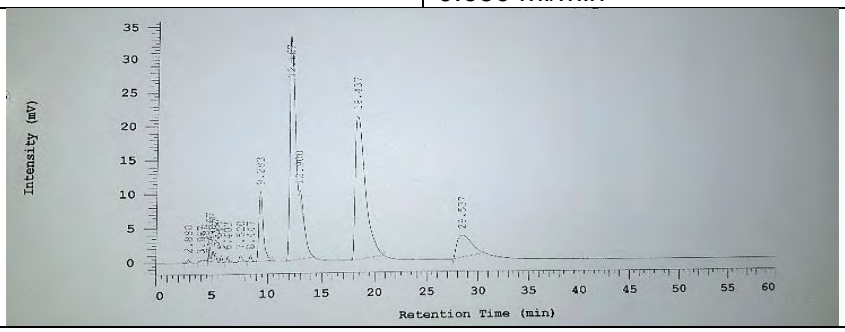
Sample	AA + Thionin
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



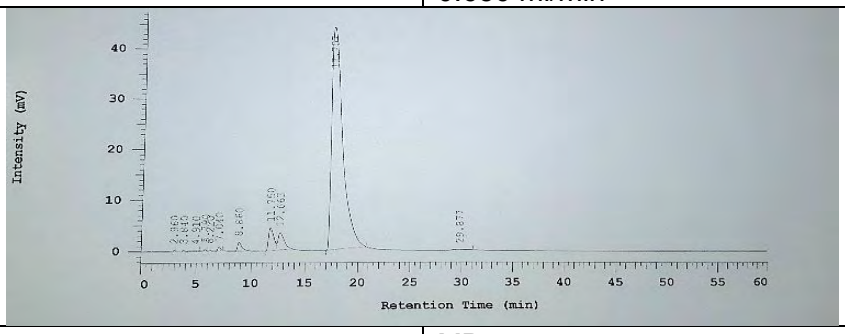
Sample	AA + AB + MB + Thionin
Solvent	Methanol
Mobile phase	10:90 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	1.0 ml/min



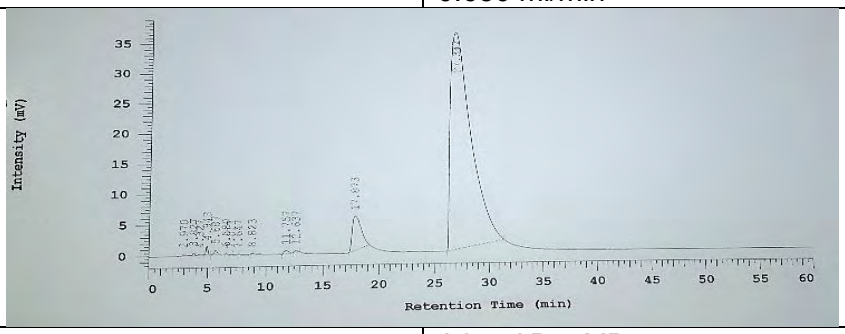
Sample	AA
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



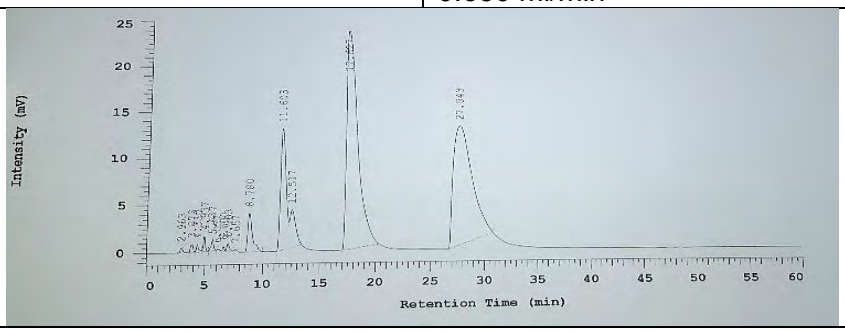
Sample	AB
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



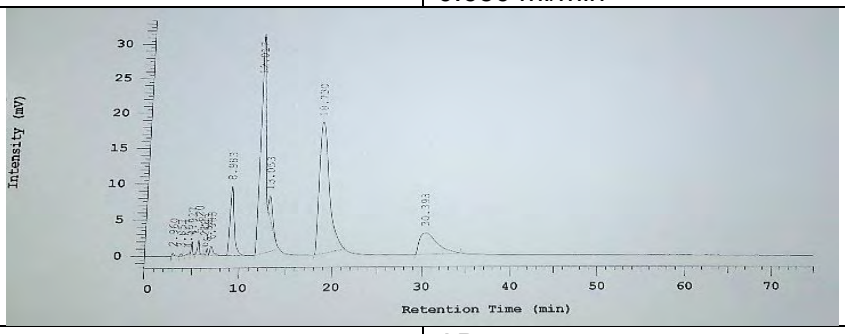
Sample	MB
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



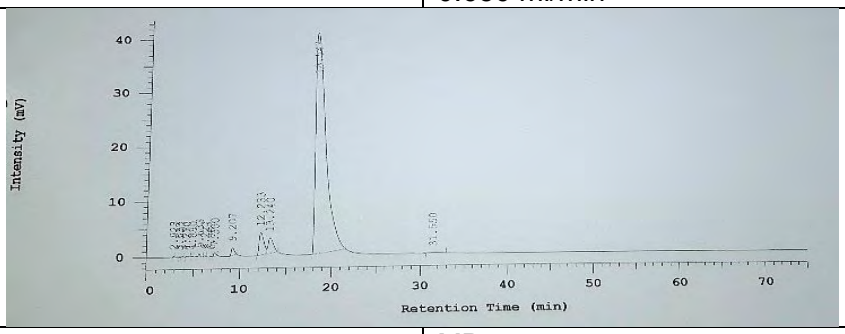
Sample	AA + AB + MB
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



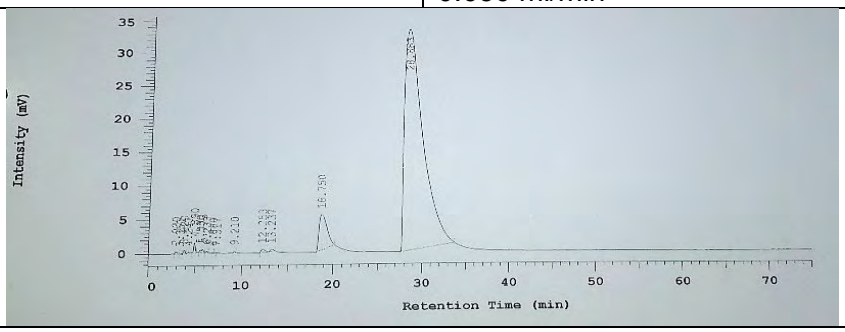
Sample	AA
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



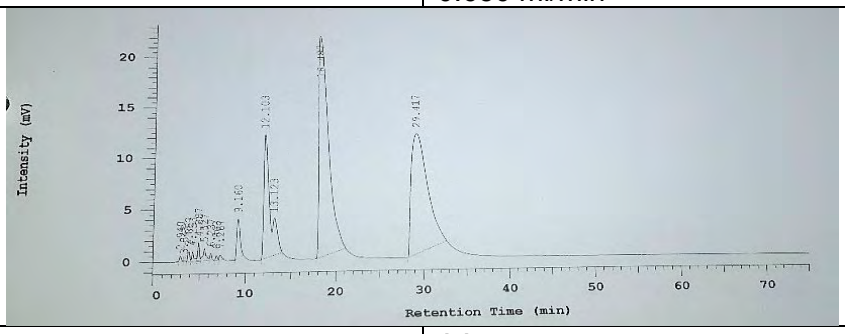
Sample	AB
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



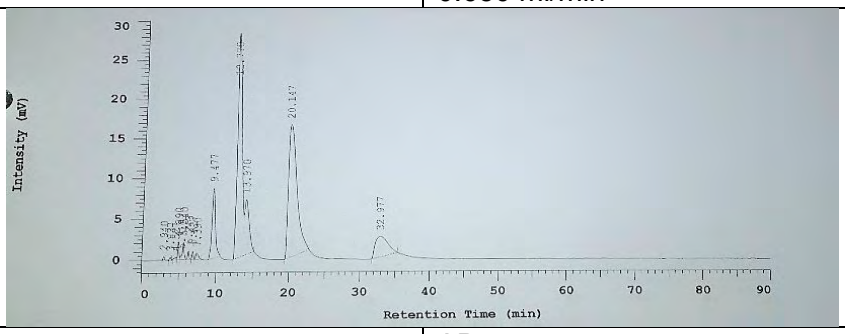
Sample	MB
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min



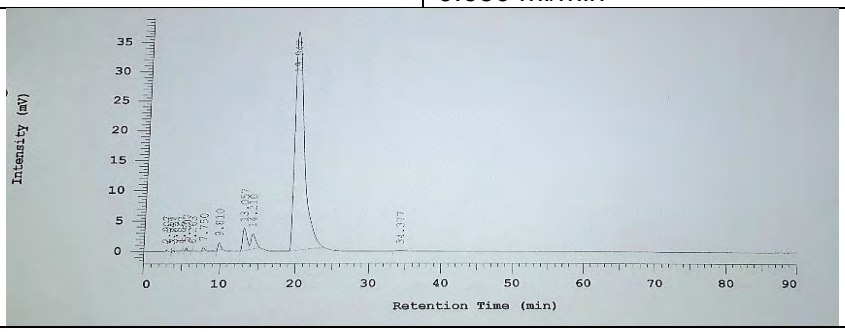
Sample	AA + AB + MB
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min

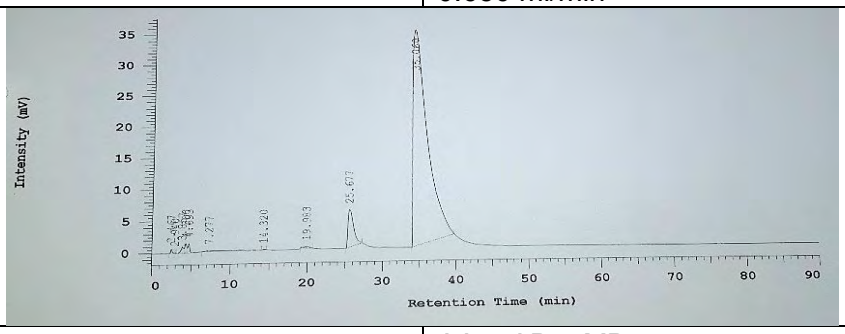
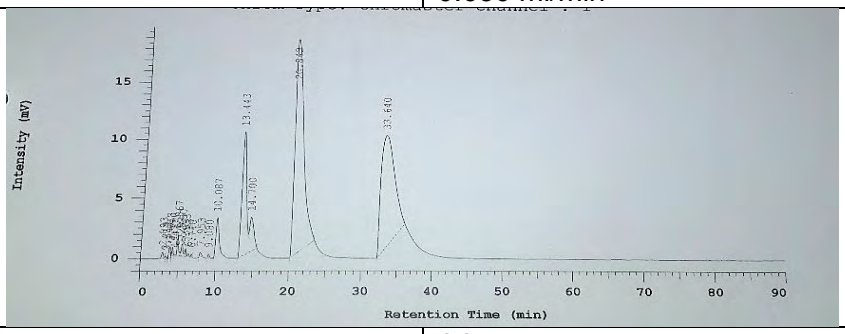
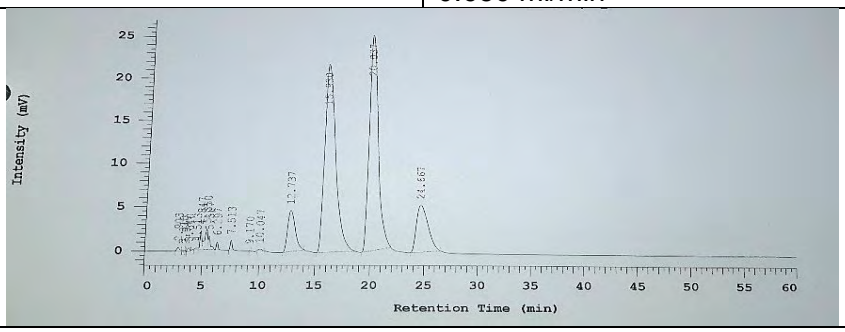


Sample	AA
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min

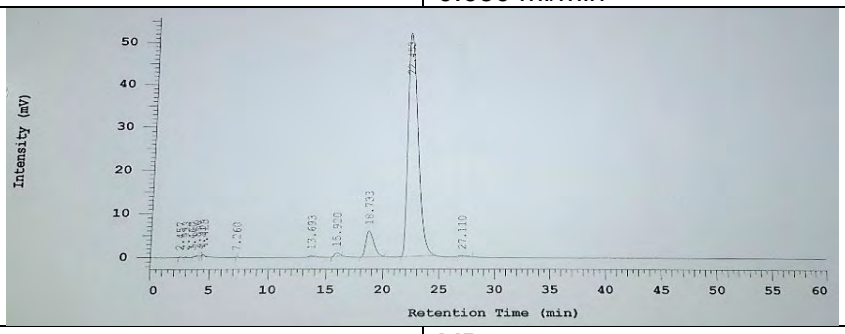


Sample	AB
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min

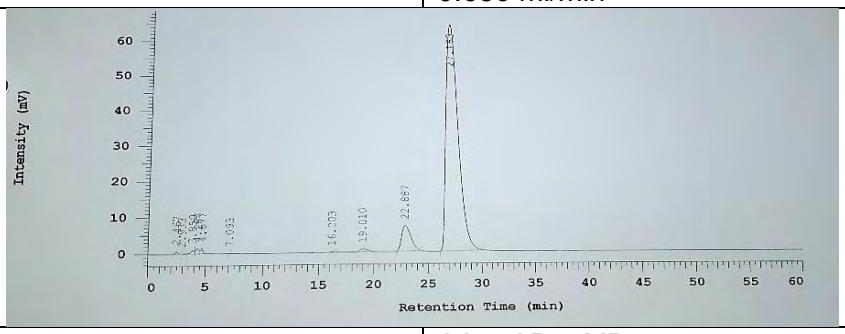


Sample	MB
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min
	
Sample	AA + AB + MB
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: methanol.
Flow rate	0.650 ml/min
	
Sample	AA
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min
	

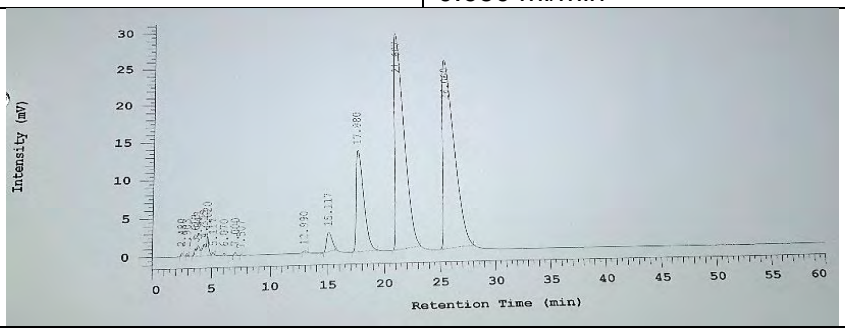
Sample	AB
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



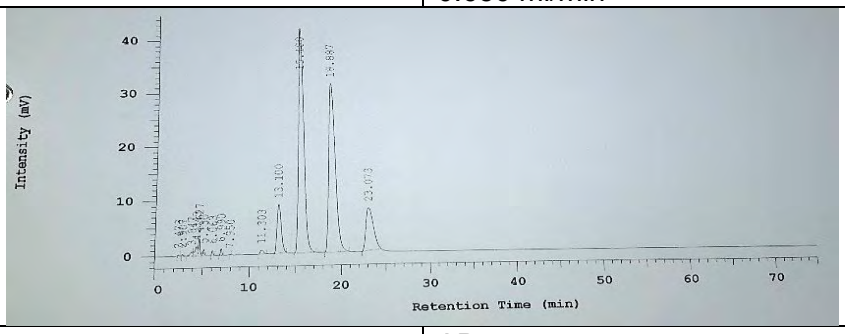
Sample	MB
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



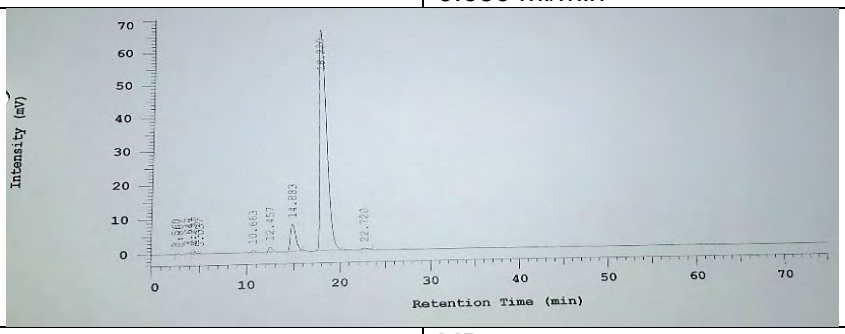
Sample	AA + AB + MB
Solvent	Mobile phase
Mobile phase	60:40 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



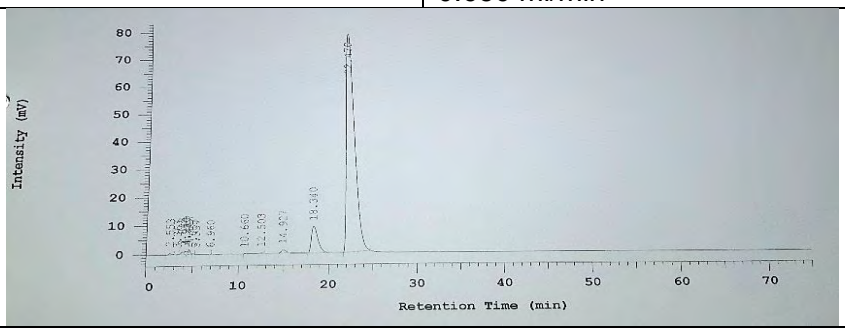
Sample	AA
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



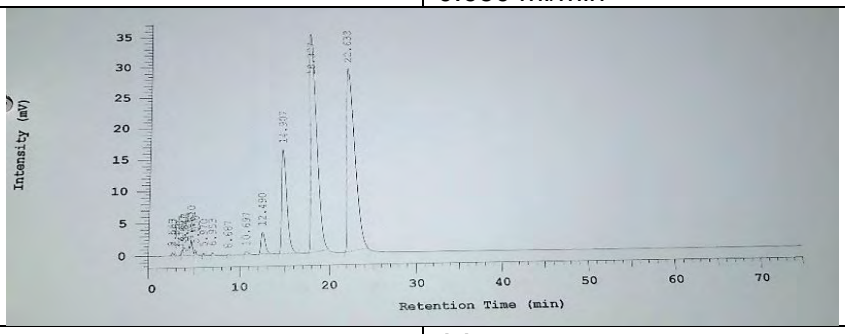
Sample	AB
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



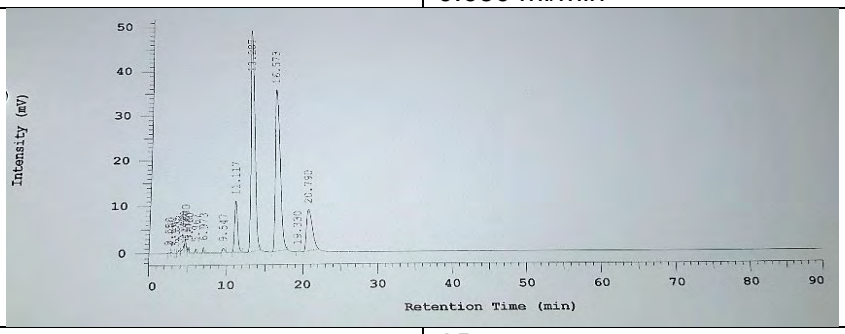
Sample	MB
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



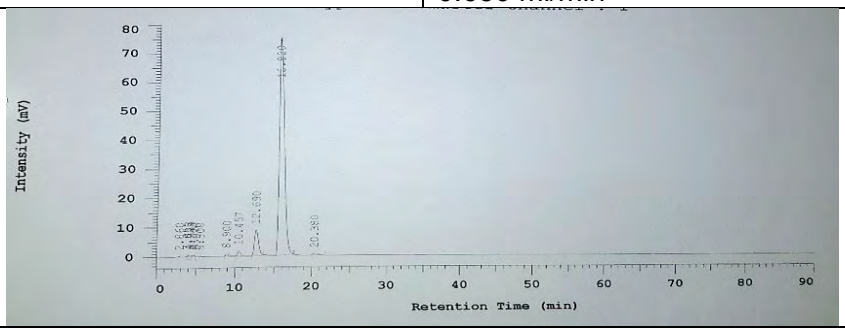
Sample	AA + AB + MB
Solvent	Mobile phase
Mobile phase	50:50 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



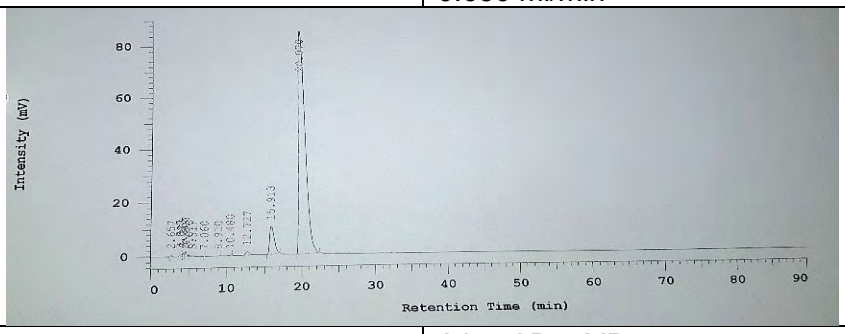
Sample	AA
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



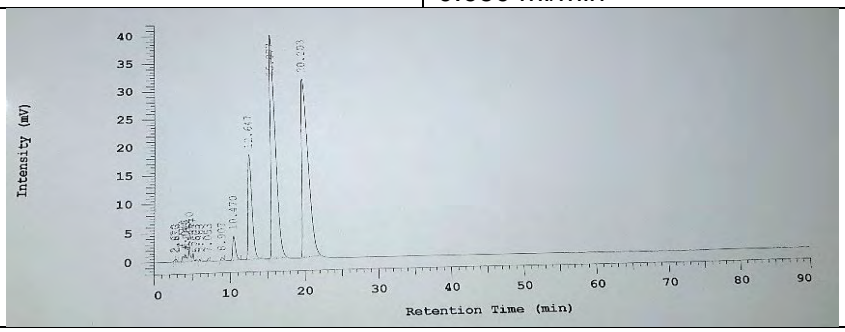
Sample	AB
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



Sample	MB
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



Sample	AA + AB + MB
Solvent	Mobile phase
Mobile phase	40:60 Part A: 5 mM ammonium acetate in 10:90 water-methanol. Part B: acetonitrile.
Flow rate	0.650 ml/min



4.4. Discussion

The best separation was seen when the Synergi Polar-RP column was used. The reason for this can be explained by looking at the chemical properties of the column, the MP and the analytes.

- *The column*:

This column's packing material (which serves as our SP), is polar endcapped and has ether-groups linked to the phenyl groups (figure 4.11).

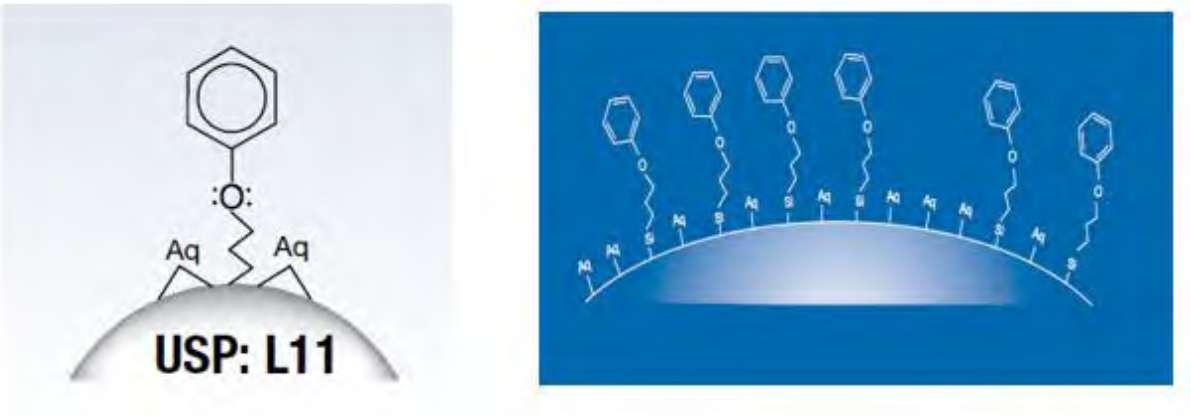


Figure 4.11: The chemical composition of the packing material inside the Synergi polar-RP column (Phenomenex, 2013, 2015).

This column is especially designed to optimise the selectivity and retention of aromatic and polar analytes such as MB (figure 4.12). And also for the selective separation of compounds with similar structures (such as MB and its metabolites). It offers high cationic retention. Being electron rich due to the phenyl groups, the SP will rather act as an electron donor. MB is an electron acceptor (chapter 2). This explains why this column specifically enhances the retention of cationic analytes. In addition to the hydrophobic interaction (the basis of RPC), the phenyl groups allow the SP to form unique π-π interactions with the aromatic rings of the analytes (Long & Mack, 2009; Yang *et al.,* 2005). This interaction is a type of donor-electron-acceptor interaction, originating from π-electron systems in two unsaturated functional groups through either intermolecular or intramolecular interactions (Long & Mack, 2009; Yang *et al.,* 2005). These π-π interactions will improve the separation (selectivity) of analytes with very similar structures such as metabolites (Long & Mack, 2009).

Figure 4.12: The selectivity profile of the Synergi Polar-RP column (Phenomenex, 2013, 2015).

- *The MP:*

The addition of methanol as part of the MP in combination with the Synergi Polar-RP SP, will maximise the selectivity and retention of aromatic and ionised analytes (Long & Mack, 2009). The rationale behind this lies in the ability of methanol to facilitate the π-π interactions between the phenyl group of the SP and the aromatic rings of the analyte (Long & Mack, 2009). Acetonitrile will partially suppress the π-π interaction due to the triple bond on its nitrile group that competes with the nitro-substituted analyte for available binding spaces on the SP or MP, thus causing lower selectivity and sub-optimal retention (Long & Mack, 2009; Yang *et al.,* 2005) (figures 4.13, 4.14 and 4.15).

Advantages associated with the Synergi Polar-RP column are the sharp peak shapes generated on the chromatograms, and improved stability and resolution when used in combination with highly aqueous MP's, due to the ether-linkage.

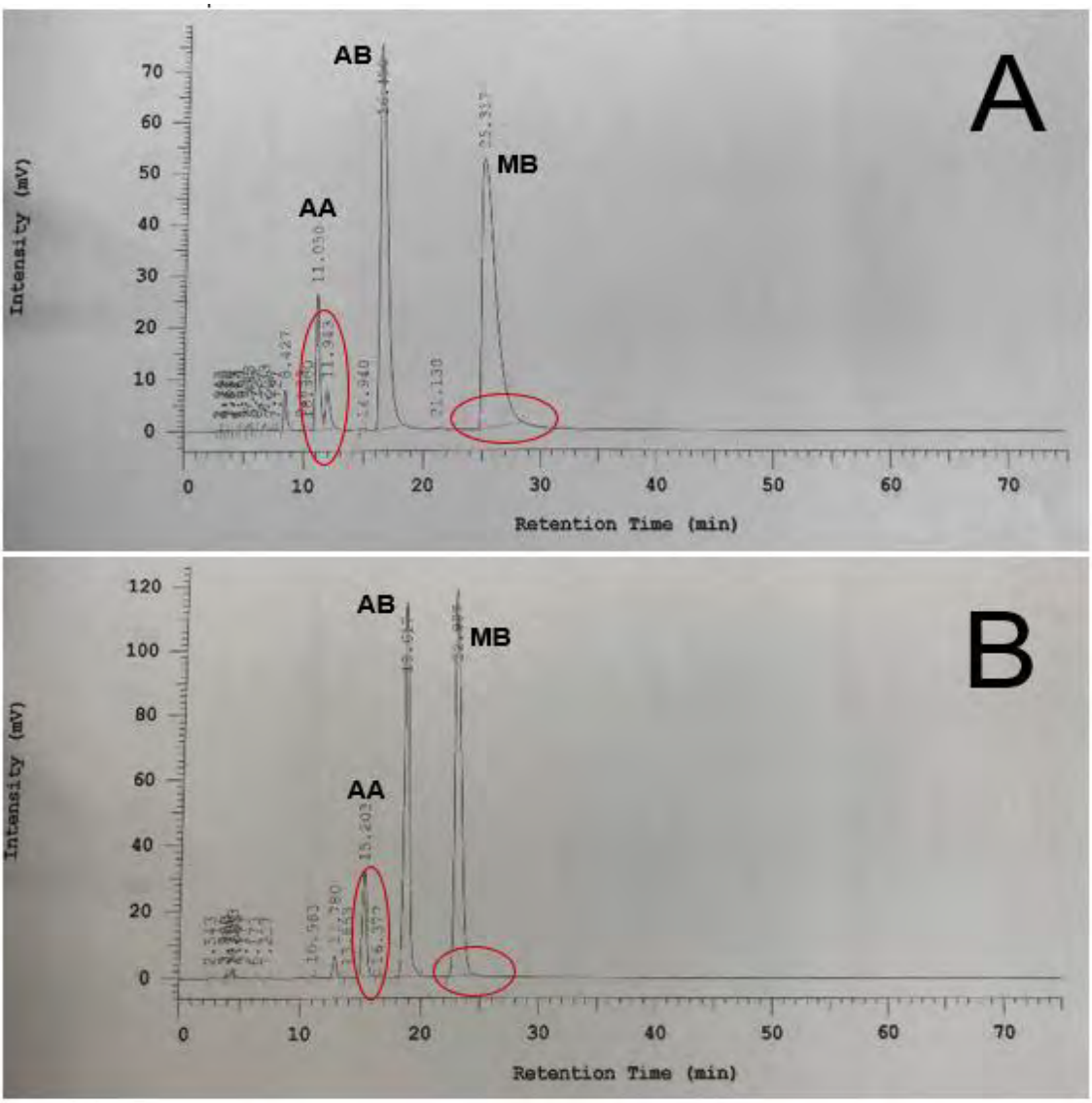


Figure 4.13: Comparison between two chromatograms done on the Synergi Polar-RP column. A) The MP consisted of 50% part A and 50% methanol. B) The MP consisted of 50% part A and 50% acetonitrile. All samples where dissolved in methanol.

In figure 4.13, the small unkown peak has a greater area when methanol is used (A) as part of the MP in comparrison to when acetonitrile is used (B). Also, the AA peak seems to have an impurity that is not separated completely, but can be seen when methanol is used, but not when acetonitrile is used, making the method that involves methanol, more selective. The peaks of AB and MB are better separated, but the peaks shows significant band broadening and tailing. When acetonitrile is used, the AB and MB peaks are closer to each other, but there are much less tailing, thus making the method that involves acetonitrile more efficient.

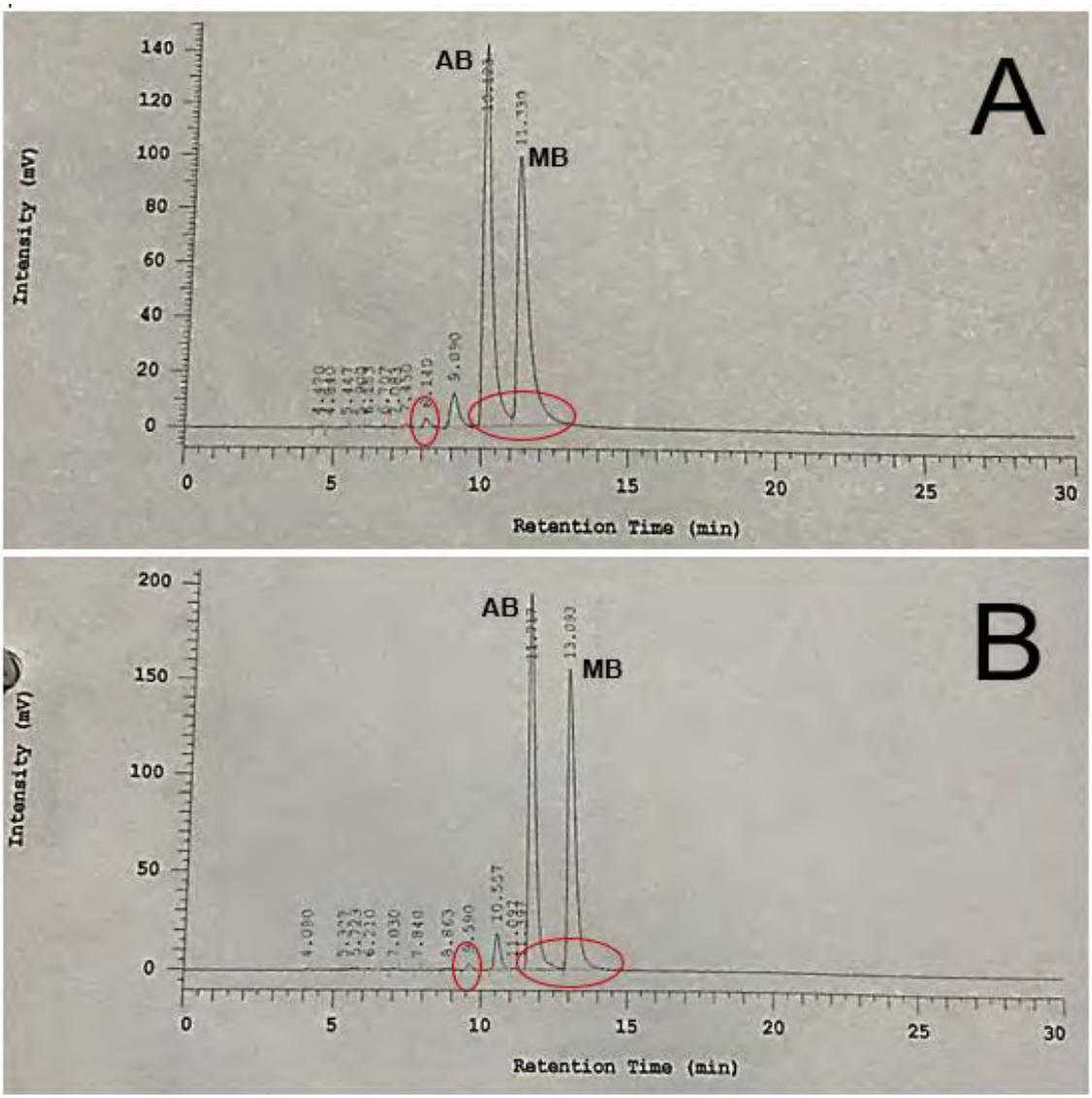


Figure 4.14: Comparison between two chromatograms done on the Phenyl-hexyl column. A) The MP consisted of 50% part A and 50% methanol. B) The MP consisted of 50% part A and 50% acetonitrile. All samples were dissolved in methanol.

In figure 4.14, better separation is seen when acetonitrile (B) is used instead of methanol (A). However, when methanol is used, the small peak that elutes first, is more prominent (with a bigger area) in comparison to when acetonitrile is used. Also, the peaks of A show much more tailing than those of B and their bases are not separated from each other well enough, meaning that the efficiency and selectivity is slightly better when acetonitrile is used.

When the chromatograms of the Synergi Polar-RP and the Phenyl-hexyl columns were compared, the Synergi Polar-RP (figure 4.13) was more selective and efficient, thus it was the chosen column for further method development. Like described in figure 4.13, both methanol and acetonitrile respectively, had some advantages. Therefore, further experimental studies were done to optimise the MP composition (chapter 5).

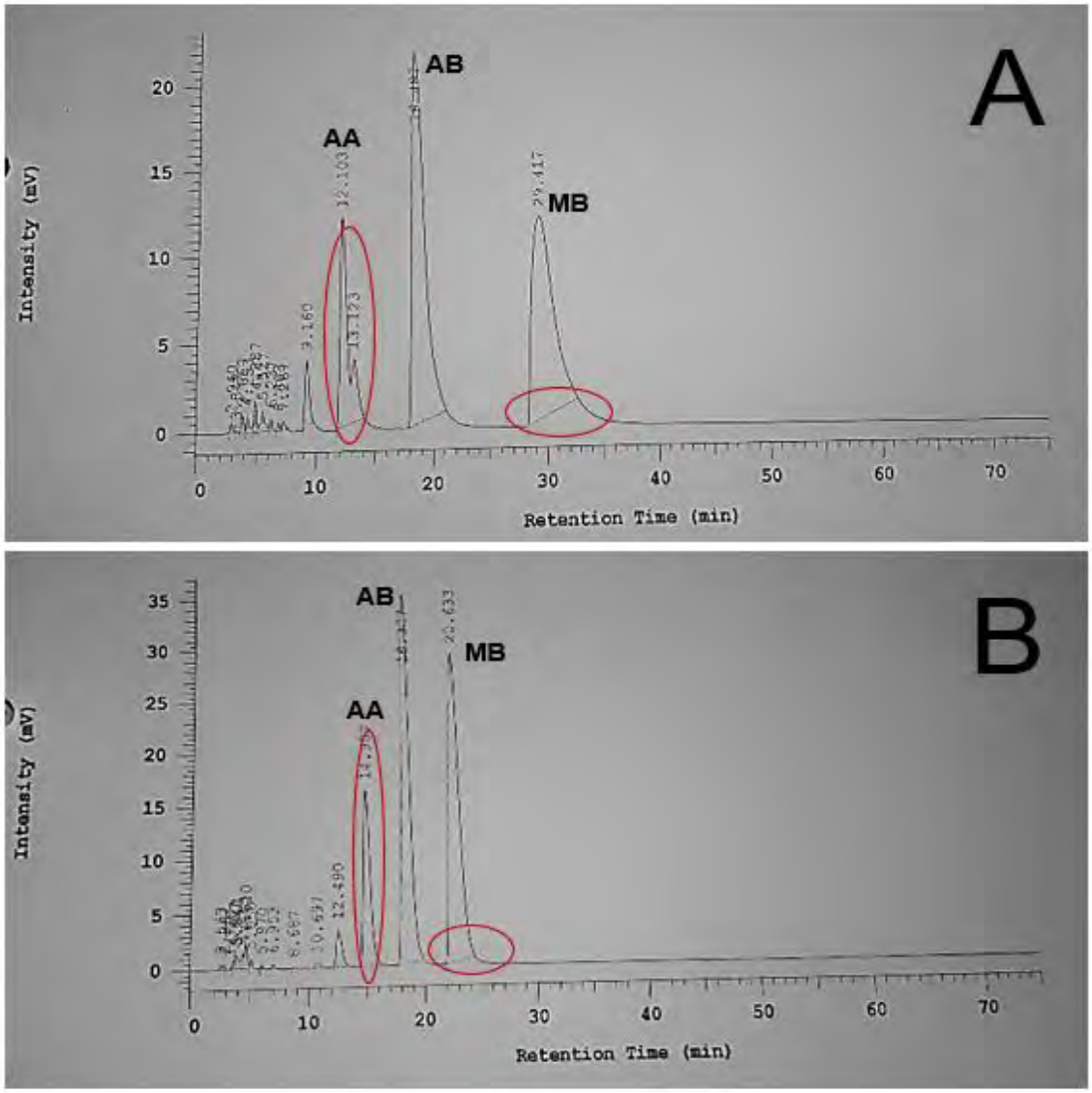


Figure 4.15: Comparison between two chromatograms done on the Synergi Polar-RP column. A) The MP consisted of 50% part A and 50% methanol. B) The MP consisted of 50% part A and 50% acetonitrile. All samples were dissolved in MP.

In figure 4.15, the influence of the MP was studied by dissolving the samples in the MP. A) The use of methanol lead to more selective separation, causing the peaks to elute further from each other, however, significant tailing is visible (less efficient). The AA peak still has the second peak that is not separated completely. B) The use of acetonitrile lead to more efficient peaks without tailing, however, the peaks did not elute as far from each other like observed in A, but selectivity is still acceptable. Also, the AA peak showed no sign of a second peak that might be separated from it like in A.

Even though the recommendation is to use methanol as part of the MP, the chromatographs where methanol was used compared to acetonitrile, were very similar and both of these solvents had unique advantages. Thus, it was decided that these two solvents will both be used as part B

of the MP. During the robustness studies, the MP composition that optimises the advantages of both methanol and acetonitrile, were identified.

In contrast to our findings, Kim *et al.* (2014), reported that significant tailing were observed when acetonitrile was used as MP during the development of an LC-MS/MS method. The MP was adapted by completely excluding acetonitrile and rather using a combination of water and methanol to reduce peak tailing.

4.5. Conclusion

During this study it was proven that MB can be separated from its metabolites by choosing the most appropriate column and MP composition. The selectivity for benzene compounds and nitro substituted aromatics such as MB, can thus be improved by closely studying the chemical properties of potential columns and MPs together with the chemical structure of the analytes.

When the chemical structure of each compound is compared, a correlation between the amount of methyl groups that the compound possesses and the retardation of the compound, can easily be recognised. With an increased amount of methyl groups present on the compound, the retention time will also increase (Wainwright & McLean, 2017). In figure 4.16 the chemical structures of MB and its metabolites are given. For example, MB is the most methylated form of these compounds and thus has the longest retention time. The most demethylated form is thionin, which elutes very fast and has the smallest retention time. The small peak that elutes before AA elutes (indicated in red) remains unknown. However, there is a great possibility that it could be azure C (Wainwright & McLean, 2017). Due to the fact that azure C has been removed from the market, it could not be purchased and identified with experimental work during this study. However, if the theoretical principle that have just been explained is correct, the chances that the peak indicated in red is azure C, is a real possibility. Azure C has one less methyl group than azure B, and one more than thionin, thus it justifies the hypothesis that it would elute between azure B and thionin.

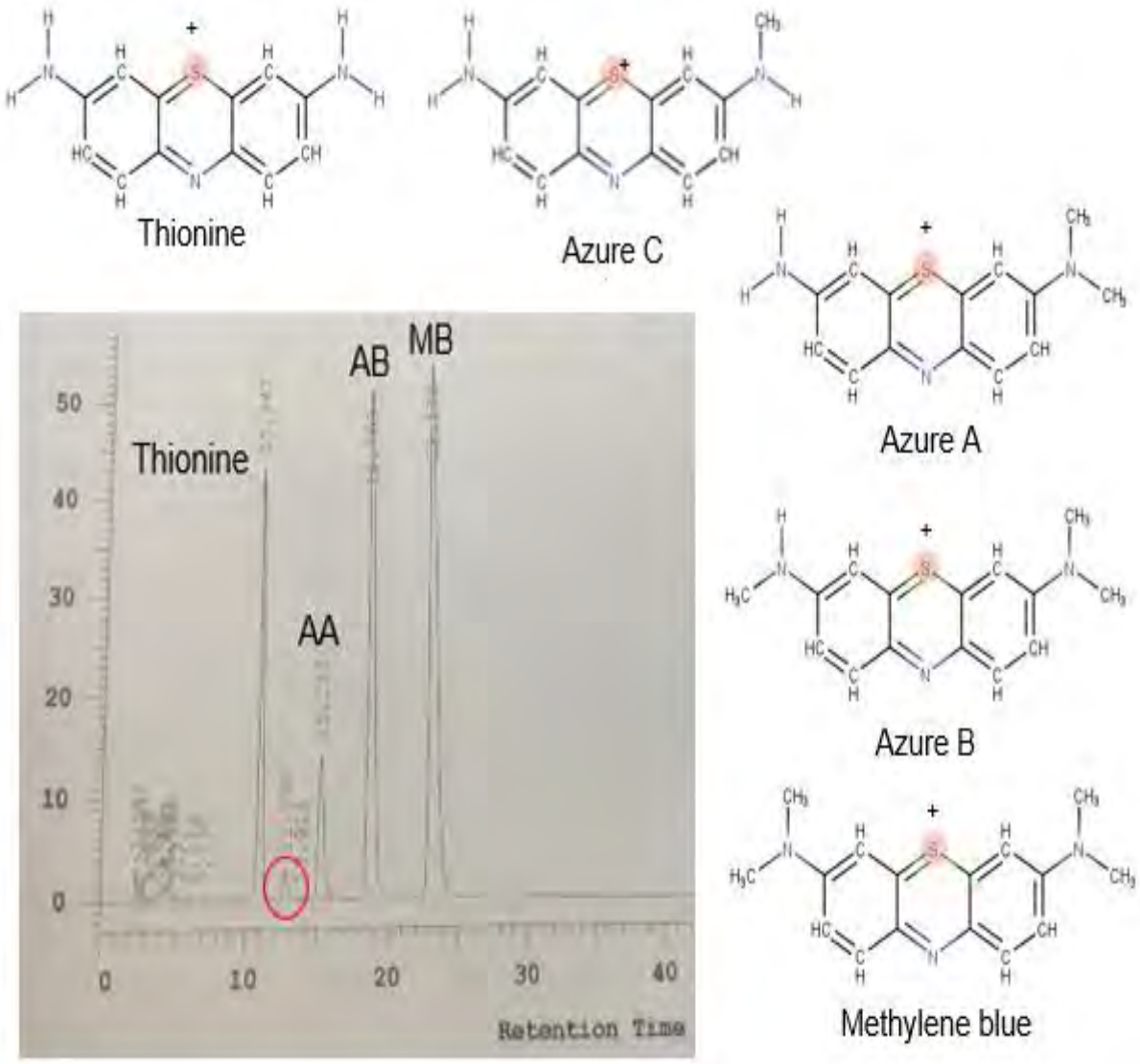


Figure 4.16: Comparison between retention times and the chemical structure of each compound and the illustration of the correlation between methylene groups and retardation.

CHAPTER 5 - METHOD VALIDATION

5.1. Introduction

The validation of a method is defined as a process in which an analytical requirement is defined and to confirm that the considered method meets the requirements for the specific intended application or purpose (Huber, 2007; Kalra, 2011; Taverniers *et al.,* 2004; UNIDO, 2009; USP, 2016, 2017a,b; Waters, 2006). During this process the competency, authenticity and predictability of the method is determined (Kalra, 2011; UNIDO, 2009). An attribute of a drug is measured by an analytical method. For any analytical method the aim would be to generate reliable, accurate and consistent data (Huber, 2010; Kalra, 2011; Ramba-Alegre *et al.,* 2012; Shabir, 2003). Results obtained during the process of validation, will enable the scientist to judge and compare the method against legal standards (Huber, 2007, 2010; Kalra, 2011). According to international validation guidelines (table 5.1), a newly developed method must be validated to be employed as the method of choice during the course of pharmaceutical drug-related studies (Huber, 2010; ISO/IEC, 17025:2005; Magnusson & Ornemark, 2014; Taverniers *et al.,* 2004; UNODC, 2009). The validation of a newly developed method is mandatory to meet the requirements of Current Good Manufacturing Practice (cGMP) and Good Laboratory Practice (GLP). One of the test method requirements stated in the cGMP regulations [21 CFR 211.194(a)] ensures that adequate accuracy and reliability standards should be met during the assessment of pharmaceutical products compliance (Es-haghi, 2011, Huber, 2010; ISO/IEC, 17025:2005; Ramba-Alegre *et al.,* 2012; UNODC, 2009; USP, 2017a). The International Organisation for Standardisation (ISO) and International Electrotechnical Commission (IEC) 17025 guideline states that for the accreditation of an analytical method, the validation of the method is mandatory (Ramba-Alegre *et al.,* 2012).

In this chapter, the importance of method validation is discussed according to the guidelines of international industrial committees and regulatory agencies. In table 5.1 an overview of these agencies is provided.

Table 5.1: An overview of basic and international guidance and regulatory agencies used as reference in this chapter.

Body	Full name
Eurachem	A Focus for Analytical Chemistry in Europe
FDA	United States Food and Drug Administration
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
AOAC	Association of Official Analytical Communities International
USP	United States Pharmacopoeia
WHO	World Health Organization
HC	Health Canada
EMEA	European medical agency
UNODC	United Nations Office on Drugs and Crime
UNIDO	United Nations Industrial Development Organisation
ISO	International Organisation for Standardisation

In practise, the focus during analytical validation of a new HPLC method lies on the following:

- Identification tests.
- Quantitative measurements of the content of related substances.
- Semiquantitative and limit tests for the control of related substances.
- Quantitative tests for the assay of major components in samples of drug substance or drug product.
- Establishing an analytical method for the required analysis.
- Evaluation of statistical data.
- Determining whether performance characteristics are met (AOAC, 2007; EMEA, 1995; Es-haghi, 2011; ICH, 1995, 1996, 2014; UNODC, 2009; USP, 2016, 2017a,b).

Definition of validation

In table 5.2, definitions of validation as defined by a few international resources are made available.

Table 5.2: Validation as defined by international resources (Es-haghi, 2011; Magnusson & Ornemark, 2014).

Definition	Reference
"Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications."	USP 30-NF 25, General Chapter <1225> Validation of Compendial Methods.
"The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose."	ICH Guideline Q2A – Text on Validation of Analytical Procedures.
"Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use."	FDA draft guidance – Analytical Procedures and Method Validation.
"Confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled."	ISO/IEC 17025: 2005. General requirements for the competence of testing and calibration laboratories, ISO Geneva.
"Confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled."	ISO 9000: 2005. Quality management systems – Fundamentals and vocabulary, ISO Geneva.
"Verification, where the specified requirements are adequate for an intended use.	International vocabulary of metrology – Basic and general concepts and associated terms (VIM), JCGM 200: 2012, www.bipm.org. A previous version is published as ISO/IEC Guide 99:2007, ISO Geneva.
[a] ISO 9000 defines 'qualification process' as "process to demonstrate the ability to fulfil specified requirements" . [b] VIM defines 'verification' as "provision of objective evidence that a given item fulfils specified requirements"	

Performance characteristics

Listed in table 5.3 below, are typical validation performance characteristics and the corresponding organisation that requires the specific parameter to be validated, however, for the validation of certain analytical methods not all performance characteristics are needed (EMEA, 1995; Es-haghi, 2011; FDA, 2015; Hansen *et al.,* 2012; Huber, 2007; ICH 1995, 1996, 2014; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; Shabir, 2013; UNODC, 2009; USP, 2016, 2017b; Waters, 2006; WHO, 2016).

Table 5.3: Performance characteristics to be validated as required by the FDA, ICH, ISO/IEC 17025, IUPAC, and USP (AOAC, 2007; Ramba-Alegre *et al.,* 2012).

Performance characteristic	**Organisation (reference)**
Specificity	ICH, USP
Selectivity	FDA, ISO/IEC 17025, IUPAC
Accuracy	FDA, ICH, ISO/IEC 17025, USP
Precision • Repeatability • Intermediate precision • Reproducibility	USP, ICH, FDA, IUPAC ICH, ISO/IEC 17025 ICH ICH, defined as ruggedness in USP, ISO/IEC 17025, FDA
Trueness	IUPAC
Linearity	ICH, ISO/IEC 17025, IUPAC, USP
Range	ICH, USP
Limit of detection	FDA, ICH, ISO/IEC 17025, IUPAC, USP
Limit of quantitation	ICH, ISO/IEC 17025, IUPAC, USP
Robustness	FDA, included in ICH as method development activity, ISO/IEC 17025, USP
Ruggedness	IUPAC, USP, defined as reproducibility in ICH
Sensitivity	FDA
Recovery	FDA, IUPAC
Applicability	IUPAC
Measurement uncertainty	IUPAC
Stability	FDA

The performance characteristics that were done during this study, and discussed in this chapter includes:

- Specificity
- Linearity ($R^2 \gg 0.998$)
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Accuracy/ trueness (98% - 102%)
- Precision (repeatability, intermediate precision, and reproducibility) ($\leq$ 2%)
- Robustness/ ruggedness
- Range (80% - 120% of nominal concentration)
- Stability

5.2. Selectivity/ specificity

Selectivity related to analytical methods is defined by various documents as: "*the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour"* (Hansen *et al.,* 2012; Magnusson & Ornemark, 2014; UNODC, 2009; Waters, 2006).

The USP and the ICH defines specificity as: *"the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components*" (EMEA, 1995; Es-haghi, 2011; Huber, 2010; USP, 2016, 2017b).

Other reputable authorities such as the International Union of Pure and Applied Chemistry (IUPAC) and Association of Official Analytical Communities International (AOAC), prefer using the term specificity (USP, 2016, 2017b).

The purpose of specificity/selectivity tests is to identify the analyte, assay and to determine the presence and level of impurities (EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; ICH, 1995, 1996, 2014; UNODC, 2009; USP, 2016, 2017b; Waters, 2006). Also, to ensure that the result obtained and measured to the proposed method, is reliable and not due to the activity of another ingredient or environmental factor, but of the analyte itself (ICH, 1995, 1996, 2014; Kalra, 2011; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; WHO, 2016). The intended objective of the analytical procedure will determine the procedures that will be used to demonstrate specificity (EMEA, 1995; Huber, 2010; ICH, 1995, 1996, 2014). In some cases, it is not possible to demonstrate the specificity of an analytical procedure for a given analyte. In this case it is recommended to use a combination of procedures in order to reach the necessary level of

discernment (Huber, 2010; EMEA, 1995; Es-haghi, 2011). For HPLC, the simplest way to demonstrate specificity is to perform the test together with a forced decomposition test. To confirm specificity, the active pharmaceutical ingredient (API) peak should be pure and individual compounds on the chromatograms should be labelled clearly (EMEA, 1995; Huber, 2010; ICH, 1995, 1996, 2014). To investigate the selectivity of a specific method, interferences is deliberately included in samples that will be analysed in order to measure the ability of the method to analyse the compound of interest (Huber, 2007; Kalra, 2011; Magnusson & Ornemark, 2014; UNODC, 2009). The ability of the method to measure the compound of interest can also be compared to that of another independent method to determine its selectivity, especially when it is not clear whether interferences are already present (Kalra, 2011; WHO, 2016). It is important to keep in mind that some compounds may exists in different forms in the same sample, for instance, bound or unbound forms of the same compound. Identification tests should be adapted accordingly to be able to discriminate between these close related compounds (EMEA, 1995; ICH, 1995, 1996, 2014; Kalra, 2011; Magnusson & Ornemark, 2014).

For HPLC, specificity of a method can be established by the choice of MP composition, the type of column, the chosen wavelength and temperature. Specificity should be done early in the validation procedure. When this criteria is not met, other performance characteristics such as accuracy, linearity and precision will be affected as well. In this case, the method should be investigated and re-evaluated for any possible faults. Further method development is recommended (Es-haghi, 2011).

5.3. Linearity

The entire analytical system must be able to generate responses which are directly proportional over the relevant concentration range for the target concentration of the analyte (EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; Huber, 2010; ICH, 1995, 1996, 2014; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; Shabir, 2003; UNODC, 2009; USP, 2016, 2017b; WHO, 2016). Another definition states: *"The method should be able to obtain results within a given range, that is directly proportional to the concentration of the analyte in the sample"* (EMEA, 1995; Hansen *et al.,* 2012; Huber, 2007, 2010; ICH, 1995, 1996, 2014; UNODC, 2009; Waters, 2006; WHO, 2016). The API should be tested for linearity separately from other related ingredients (Magnusson & Ornemark, 2014; WHO, 2016). The linearity test is done by first making stock solutions of each substance separately and a series of this solution that is diluted with each run should be injected on the HPLC (Huber, 2010; ICH, 1995, 1996, 2014; Magnusson & Ornemark, 2014). The rationale behind the separate linearity tests for each stock solution lies in the possible impurities present in them, causing the test for each component to be confounded. This is seen when the sample preparation of the active ingredient is contaminated with the

impurities that the linearity test is being performed on or when the active ingredient is present as an impurity within the impurities (Magnusson & Ornemark, 2014). Another reason for analysing the compounds separately is because it makes it easier for the relative response factor (RRF) to be determined for each compound.

According to international documents such as the ICH, a minimum of five concentrations should be used to determine linearity within the specified range, (EMEA, 1995; Hansen *et al.,* 2012; Huber, 2010; ICH, 1995, 2014; Magnusson & Ornemark, 2014; Shabir, 2013; USP, 2016, 2017b; Waters, 2006; WHO, 2016). To assess the linearity data for acceptability the correlation coefficient and the y-intercept of the linear regression line for the area response versus concentration plot and standard deviation is examined (EMEA, 1995; ICH, 1995, 1996, 2014; Kalra, 2011; Magnusson & Ornemark, 2014; Shabir, 2013; UNODC, 2009). For this data to be acceptable, correlation coefficients of >0.990 drug product (DP) of >0.998 drug substance (DS) should be evident. However, it's important to keep in mind that %RSD, y-intercept and the coefficient correlation are not true linearity measures and can sometimes present misleading data (EMEA, 1995; FDA, 2015; Magnusson & Ornemark, 2014; Shabir, 2013; UNODC, 2009). Thus, these parameters should only be considered in combination with in depth examination of the response versus concentration plot (Hansen *et al.,* 2012).

According to the USP (2016, 2017b), the linearity should be presented visually by plotting the responses generated during the study as a function of concentration (Es-haghi, 2011). Refer to figure 5.1 that graphically illustrates the definition of linearity, LOD, LOQ and range (Huber, 2010).

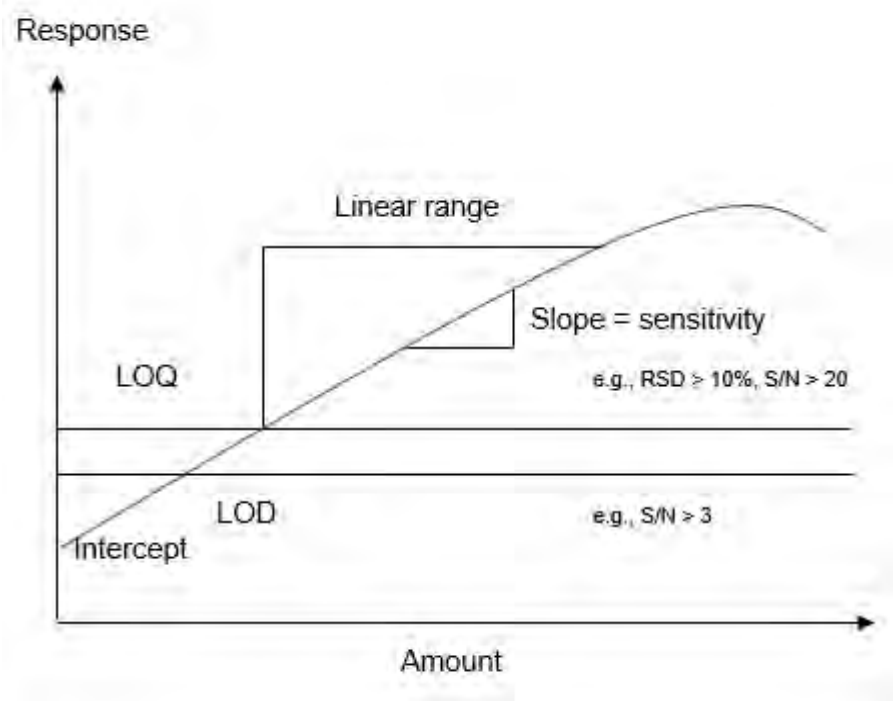


Figure 5.1: A visual illustration of range, linearity, LOD, LOQ and range (Huber, 2007, 2010; Kalra, 2011).

5.4. LOD and LOQ

The LOD and LOQ should be determined during all impurity tests. There are a few general concepts to keep in mind for measurements made at very low concentrations:

- The critical value/ decision level should be established. This indicates the result value level where an analyte differs remarkably from zero (Magnusson & Ornemark, 2014).
- At a specified level of confidence, the lowest detectable concentration of the analyte should be identified. This means that the concentration where the critical value is confidently exceeded, should be determined (Magnusson & Ornemark, 2014).
- The lowest level for a specific application at which acceptable performance is accomplished, should also be determined (Magnusson & Ornemark, 2014).

As defined by the USP the LOD is “a parameter of limit tests” (Magnusson & Ornemark, 2014; USP, 2016 2017b). The term LOD represents the lowest analyte concentration that is still detectable, however under the stated experimental conditions it is not necessarily quantitated (EMEA, 1995; Hansen *et al.,* 2012; Huber, 2007; ICH, 1995, 1996, 2014; Kalra, 2011; Ramba-Alegre *et al.,* 2012; Shabir, 2003, 2013; UNODC, 2009; Waters, 2006; WHO, 2016). It is the lowest detectable concentration that would generate a peak which is two to three times as high as the noise level on the baseline (EMEA, 1995; Huber, 2007, 2013; Kalra, 2011; Shabir, 2003; UNODC, 2009; WHO, 2016). Usually it is sufficient to only determine an approximate value for LOD at a concentration level where it starts to get difficult to detect the analyte (Magnusson & Ornemark, 2014). It is important to not confuse the instrument detection limit with the method detection limit.

The LOQ is defined by the USP as “a parameter of quantitative assays for low compound levels present in sample preparations for instance the degradation products in finished pharmaceutical preparations and impurities present in bulk drug substances” (Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; UNODC, 2009; USP, 2017b; Waters, 2006). In other words, the LOQ represents the lowest concentration present in a sample the may be measured with an acceptable level of accuracy and precision (EMEA, 1995; Hansen *et al.,* 2012; Huber, 2007, 2010; ICH, 1995, 1996, 2014; Waters, 2006; WHO, 2016). It is the lowest concentration of analyte that can be detected with acceptable performance (Magnusson & Ornemark, 2014; USP, 2017b; Waters, 2006; WHO, 2016), thus, requiring peaks of at least ten to twenty times higher than the noise level of the baseline (Huber, 2007; ICH, 1995, 1996; Kalra, 2011).

The LOD and LOQ are both normally calculated by the multiplication of a suitable factor with the standard deviation (Magnusson & Ornemark, 2014). The default value for this factor as given by IUPAC is 10 for LOQ, which correlates with a relative standard deviation (RSD) of 10% when the standard deviation stays constant at low concentrations (Hansen *et al.,* 2012; Magnusson &

Ornemark, 2014; Shabir, 2003, 2013). The number of replicates that should be used for the determination of the standard deviation should be between six and fifteen. The standard deviation should always be determined under reliability conditions (Magnusson & Ornemark, 2014). Regarding the statistics, the LOD for a HPLC separation method is defined for a peak that gives a signal-to-noise ratio of more or less 3:1 and for LOQ 10:1 (EMEA, 1995; Hansen *et al.,* 2012; Huber, 2007, 2010; ICH, 1995, 1996, 2014; Kalra, 2011; LoBrutto & Patel, 2007; Ramba-Alegre *et al.,* 2012; Shabir, 2003, 2013; UNODC, 2009), refer to figure 5.2. Another way of determining the LOD and LOQ is by injecting samples with decreasing amounts, five to six times and calculating the %RSD of each peak area (Huber, 2007, 2010; Kalra, 2011; Ramba-Alegre *et al.,* 2012), refer to figure 5.3. This approach can be used if the required precision of the method at the limit of quantitation has been specified (Huber, 2010). A value of $\leq 20\%$ regarding the LOD and $\leq 10\%$ for the LOQ is acceptable (Huber, 2007; Kalra, 2011; LoBrutto & Patel, 2007).

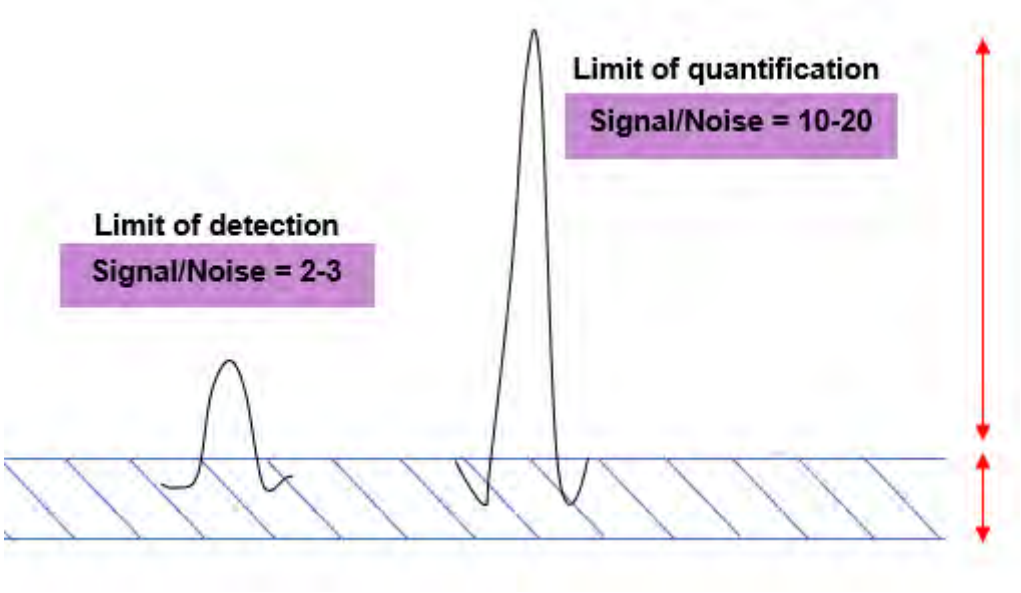


Figure 5.2: Illustration of how the limit of detection and the limit of quantification is calculated by the use of the signal-to-noise ratio (Huber, 2010; Kalra, 2011).

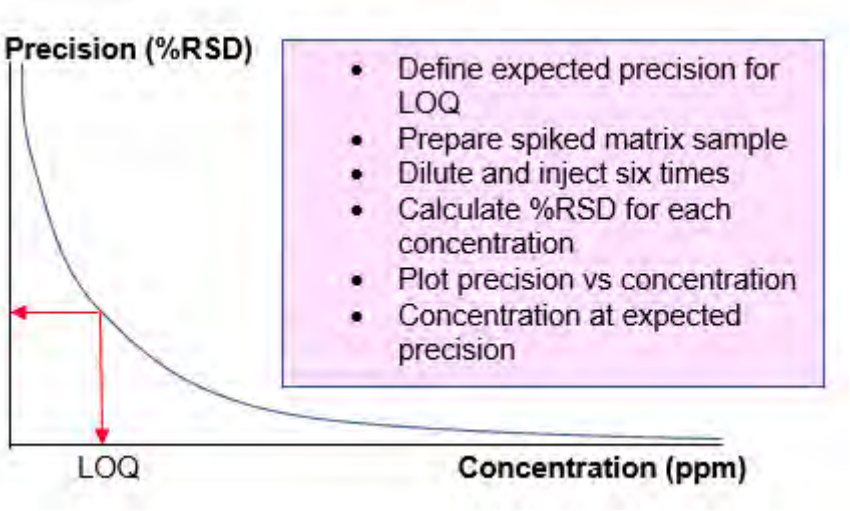


Figure 5.3: Required experimental steps for the determination of LOD and LOQ by using the EURACHEM (80) method and a typical graph showing the LOQ on selected precision (Hansen *et al.,* 2012; Huber, 2010; Kalra, 2011).

5.5. Accuracy (trueness and recovery)

According to the ICH and USP, the measure to which the value that is found corresponds to the conventional true value or an accepted reference value is determined by the test of accuracy (Huber, 2010; Kalra, 2011; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; UNODC, 2009; USP, 2016, 2017b; WHO, 2016). Accuracy can therefore be defined as the correspondence of results when the new developed method is implemented and compared to the accepted reference result, figure 5.4 (AOAC, 2007). Accuracy is also a way of determining the recovery of an active or degradation product that is present in a drug preparation (Es-haghi, 2011; Hansen *et al.,* 2012; ICH, 1995, 1996, 2014; Ramba-Alegre *et al.,* 2012; Shabir, 2013; UNODC, 2009; USP, 2016, 2017b) and of determining the difference between the mean and the accepted true value (Es-haghi, 2011; Hansen *et al.,* 2012; ICH 1995, 1996, 2014). It is a general term for measuring the quality of results gathered by a method which is described by precision, trueness and uncertainty (Magnusson & Ornemark, 2014; UNODC, 2009; USP, 2016, 2017b). During method validation, the systematic as well as random effects on a single result is studied and together determines the accuracy. The two components that are used for the determination of accuracy is precision and trueness (Magnusson & Ornemark, 2014).

The ICH and USP documents require that accuracy should be measured by using a minimum of nine determinations at a minimum if three concentrations that covers the specified range (EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; Huber, 2010; ICH, 1995, 1996; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; Shabir, 2003, 2013; USP, 2016, 2017b). Also, accuracy can be measured by means of spiking the samples at different concentrations that

covers the working range (high, medium and low) (AOAC, 2007; Es-haghi, 2011; Huber, 2010; Shabir, 2013; UNIDO, 2009; UNODC, 2009; USP, 2016, 2017b). When the uncertainty of the reference method is known, the results of the new method can be compared with results from an established reference method (Huber, 2010). Also, in the case where a reference material with a known concentration is available, the results of the new method can be compared with the true value supplied with the reference material (EMEA, 1995; Huber, 2010; ICH, 1995, 1996, 2014; Shabir, 2003).

The acceptance criteria for non-regulated products is within a range of 90% to 110% of the theoretical value. According to the United States pharmaceutical industry, the assay of an API in a DP should be 100 ± 2% when analysed over a concentration range of 80% to 120%. For dosage forms the HC requires accuracy with a bias $\leq 2\%$ and for DS $\leq 1\%$ (Shabir, 2003, 2013). According to the ICH accuracy should be reported in terms of "percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals." (Huber, 2010).

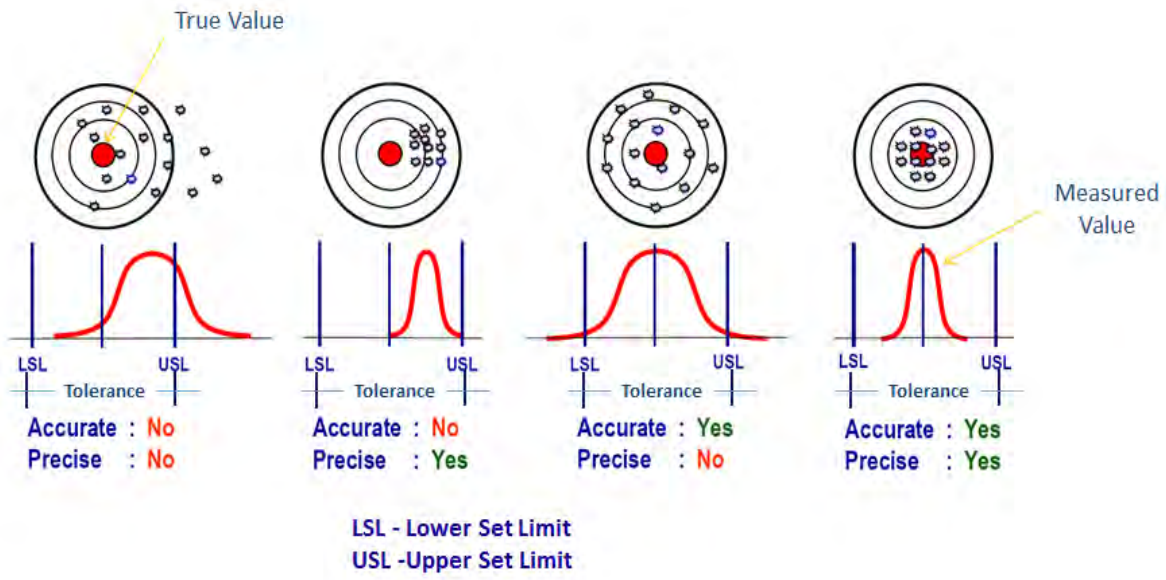


Figure 5.4: The difference between the terms accuracy and precision (Ponciano, 2017).

5.6. Precision

In order to obtain reliable approximations of method performance characteristics such as precision and bias, replication is of much importance. The aim of this test is not to determine minimum variability, but rather typical variability (Magnusson & Ornemark, 2014; UNODC, 2009; Waters, 2006). Precision is a measure of how close the results are to each other (degree of scatter) (EMEA, 1995; Huber, 2010; Hansen *et al.,* 2012; ICH, 1995, 1996, 2014; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; UNODC, 2009; WHO, 2016), refer to figure 5.4.

Precision can be divided into three sub categories according to the ICH document which are: repeatability, intermediate precision and reproducibility. It consists of measuring random errors under these three categories (ICH, 1995; Magnusson & Ornemark, 2014; Shabir, 2003; UNODC, 2009).

a) Repeatability is determined when tests are carried out by the same analyst on the same equipment, in a short period of time (AOAC, 2007; EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; Huber, 2007; ICH, 1995, 1996, 2014; Kalra, 2011; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; UNODC, 2009; Waters, 2006). Samples are analysed one injection after another by the same analyst, and the desired results is the smallest possible variation (Hansen *et al.,* 2012; Magnusson & Ornemark, 2014; UNODC, 2009). All methods that delivers numerical data should be tested for repeatability. Repeatability can also be divided into two sub categories: injection repeatability and analysis repeatability. Injection repeatability is done firstly. It is based on multiple injections done on a given day using a single sample preparation (Magnusson & Ornemark, 2014). Secondly, analysis repeatability is done by the same analyst by analysing multiple samples and multiple injections on the same day (Magnusson & Ornemark, 2014).
b) Reproducibility is the test results gathered from two different laboratories (AOAC, 2007; EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; Huber. 2007; ICH, 1995, 1996; Kalra, 2011; Magnusson & Ornemark, 2014; Ramba-Alegre *et al.,* 2012; UNODC, 2009; Waters, 2006; WHO, 2016).
c) Intermediate precision involves tests done in the same laboratory, but by different analysts, on different days and by using different apparatus, thus within-laboratory variations (AOAC, 2007; EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; Huber, 2007; ICH, 1995, 1996, 2014; Kalra, 2011; Ramba-Alegre *et al.,* 2012; Waters, 2006; WHO, 2016). Intermediate precision is carried out for the purpose of determining the variability of the analytical test. The FDA recommends that the test should be carried out on a minimum of two different days and analysts (Es-haghi, 2011). The acceptance criteria for intermediate precision is two times the RSD, however, results gathered from different laboratories should not differ from each other statistically (Es-haghi, 2011). Potential issues that may occur during method transfer can be identified by this test (Magnusson & Ornemark, 2014).

According to the ICH document, repeatability should be determined by using three concentrations over the given range, repeated three times each. Also, precision can be measured by using 6 determinations at 100% of the test concentration (EMEA, 1995; Es-haghi; 2011; Hansen *et al.,* 2012; Huber, 2010; ICH, 1995, 1996, 2014; Shabir, 2003, 2013). When intermediate precision

and repeatability is compared, it is measured under more variable conditions than repeatability, however as mentioned before, these measurements are made in the same laboratory (EMEA, 1995; Huber, 2007; ICH, 1995, 1996, 2014; Kalra, 2011; Magnusson & Ornemark, 2014). Generally, precision is dependent on the concentration of the analyte, thus it should be determined at a number of different concentrations over the range of interest. In order to assess if adequate precision has been obtained, the relative standard deviation (%RSD) is calculated (Hansen *et al.,* 2012; Kalra, 2011; Magnusson & Ornemark, 2014; USP, 2016, 2017b; WHO, 2016). Together with accuracy, precision is used to identify the error of an individual determination (Kalra, 2011; UNODC, 2009).

$$S = \sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \bar{x})^2},$$

Equation 5.1 - Equation for the calculation of the standard deviation (Kalra, 2011).

$$\% \text{ Relative Standard Deviation (RSD)} = S * 100/x$$

Equation 5.2 - Equation for the determination of the relative standard deviation (Kalra, 2011).

RSD is the standard deviation as a fraction of the mean (Kalra, 2011), figure 5.5. When multiplied with 100, the %RSD is calculated, which is a more reliable measure of precision (figure 5.6). According to the FDA and the HC, the typical RSD for DS should be ± 1% and for DP ± 2%. Reproducibility and intermediate precision are considered together with ruggedness in the USP (Es-haghi, 2011; Shabir, 2003, 2013).

5.7. Robustness/ruggedness

Robustness is defined as the producibility of tests done on the same samples, but in different laboratories, different instruments/apparatus and on different days (Es-haghi, 2011; Hansen *et al.,* 2012; Kalra, 2011; Magnusson & Ornemark, 2014; Shabir, 2003, 2013; Waters, 2006; WHO, 2016). It describes to what extent the method is reliable during the course of normal circumstances and usage (EMEA, 1995; Es-haghi, 2011; ICH, 1995, 1996, 2014; Ramba-Alegre *et al.,* 2012). This measures to what degree environmental and operational variables of the method have no influence on the test results (EMEA, 1995; Huber, 2007, 2010; ICH, 1995, 1996, 2014; Kalra, 2011; Waters, 2006; WHO, 2016). This test involves the investigation on the

performance of the method when changes are deliberately made to some aspects of the method (EMEA, 1995; Es-haghi, 2011; Hansen *et al.,* 2012; Magnusson & Ornemark, 2014; Shabir, 2003; Waters, 2006). The purpose of this test is to identify the most influential variables and to ensure in the future that they are closely monitored when the method is in use, thus it should be done early in the method development stage (FDA, 2015; Magnusson & Ornemark, 2014). From a practical perspective, another name for robustness is intermediate precision where an identical sample is tested by different chemists, in different laboratories on different days, using different instruments, columns, solvents, chemicals and reagents (Magnusson & Ornemark, 2014).

Possible factors that could influence robustness:

- stability of test and standard samples and solutions
- reagents (e.g. different suppliers)
- different columns (e.g. different lots and/or suppliers)
- extraction time
- variations of pH of a mobile phase
- variations in mobile phase composition
- temperature
- flow rate (EMEA, 1995; Huber, 2010; ICH, 1995, 1996, 2014; WHO, 2016).

Robustness can be determined by using one of the following approaches:

- Uni-variable
    - Varying each parameter sequentially within a range of 5-10% above and below the set value.
- Multi-variable
    - Implementation of a statistical design such as fractional factorials, Hendrix, central composites and/or the two-factor Plackett-Burman statistical approach.

5.8. Working range

The working range is defined as the range in which the method provides results with acceptable uncertainty (EMEA, 1995; Huber, 2007, 2010; ICH, 1995, 1996, 2014; Kalra, 2011; Ramba-Alegre *et al.,* 2012; Shabir, 2003; USP, 2017b). The LOQ determines the lower end of the range (Magnusson & Ornemark, 2014). Concentrations at which significant anomalies in the analytical sensitivity is observed determines the upper end of this range (Magnusson & Ornemark, 2014) (figure 5.1). During the process of validation both the method working range and the instrument working range should be determined. The response of the instrument follows a certain relationship between the LOQ and the upper end of the range that is not yet known to the analyst (Magnusson & Ornemark, 2014). When validation is done, it is important to confirm this relationship, prove that

the working range of the instrument is compatible to that of one stated in the method validation scope and verify that the instrument calibration procedure is adequate (Magnusson & Ornemark, 2014).

To determine the working range of the method, samples with known concentrations and blanks should be prepared, and the samples that is used should be used through the whole course of the procedure (Magnusson & Ornemark, 2014). Also, the sample concentrations should cover the whole working range (WHO, 2016). The instrument that is used should've been calibrated before starting with this test. Calculate the measures by the equations given in table and plot these values on the y-axis against the concentration of the samples on the x-axis (Magnusson & Ornemark, 2014).

By visual inspection of the plot and comparing it with a residuals plot from a linear regression, the working range and linearity is assessed (Magnusson & Ornemark, 2014; USP, 2016, 2017b). Results gathered from precision and bias tests (proving that these studies cover concentrations over the whole method working range) will support the results gathered during the test for working range.

According to the ICH and USP (2016, 2017b), minimum specified ranges that should be considered are the following:

- The assay of a DP or DS should be done over a test concentration range of 80% to 120%.
- A minimum test concentration range of 70% to 130% should be covered to prove content uniformity.
- More or less 20% of the specified range should be covered for dissolution testing.
- Impurity determination should be done from the level at which the impurity is visible to 120% of the specified range.
- In the case where the pharmaceutical/ toxic effects of impurities are unknown, the LOQ should correlate with the level at which impurities must be controlled (EMEA, 1995; Huber, 2010; ICH, 1995, 1996, 2014; Shabir, 2003; USP, 2016, 2017b).

5.9. Stability

The reason for this test is to determine and make sure whether the sample preparations that is prepared and analysed using a given method, are stable for the duration of a normal analytical sequence (Huber, 2007; Ramba-Alegre *et al.,* 2012; UNODC, 2009). Within a preselected time frame, the bias of assay results is measured by using a single preparation solution (Huber, 2010; Ramba-Alegre *et al.,* 2012). It is of much importance to determine whether the method has the ability to analyse and measure the DP's in the presence of their degradation products (Huber, 2010; Ramba-Alegre *et al.,* 2012). The ICH recommends stress tests in which 5%-20% of the

drug is degraded (Ramba-Alegre *et al.,* 2012). The recommendation is to carry out this test at 24, 48 and 72 hours respectively. Directly after the samples have been prepared, it should be analysed, where after the same samples are then stored at 5ºC and at normal ambient laboratory conditions (Huber, 2010; Ramba-Alegre *et al.,* 2012). After a period of time, the solutions are then reanalysed and compared against freshly made and analysed preparations. It is acceptable to compare the peak response at the initial time and at the defined time (24, 48 and 72 hours) for drug substances on condition that the HPLC system is running, the same MP is used and suitability is run daily. The change in the responses are compared to the initial. The basis of this test is to determine for how long a sample is stable in the respective diluents. These tests allow scientists to determine the allowed time span between the preparations of samples and the analyses there of (Huber, 2007; Ramba-Alegre *et al.,* 2012).

5.10. Results and discussion

During the method validation procedure, two different HPCL systems were utilised:

- Hitachi Chromaster chromatographic system. The system consists of a 5410 UV detector, an auto-sampler (5260) with a sample temperature controller and a solvent delivery module (5160) – system 1.
- Shimadzu (Kyoto, Japan) UFLC (LC-20AD) high performance chromatographic system. The apparatus consisted of a SPD-M20A UV/VIS Photodiode Array detector, and autosampler (SIL-20AC) with a sample temperature controller and a solvent delivery module (LC-20A) – system 2.

The validation studies were performed on system 1 and system 2 was used for the purposes of some of the robustness studies and shall be indicated as such.

5.10.1. Specificity

Peak identification was done by first analysing the samples separately. Each sample was injected individually. Thereafter, the standard 20 µg/ml samples were spiked with 100 µg/ml of MB and AB respectively, to be able to identify the individual peaks. Chromatograms are given in figures 5.5 – 5.10.

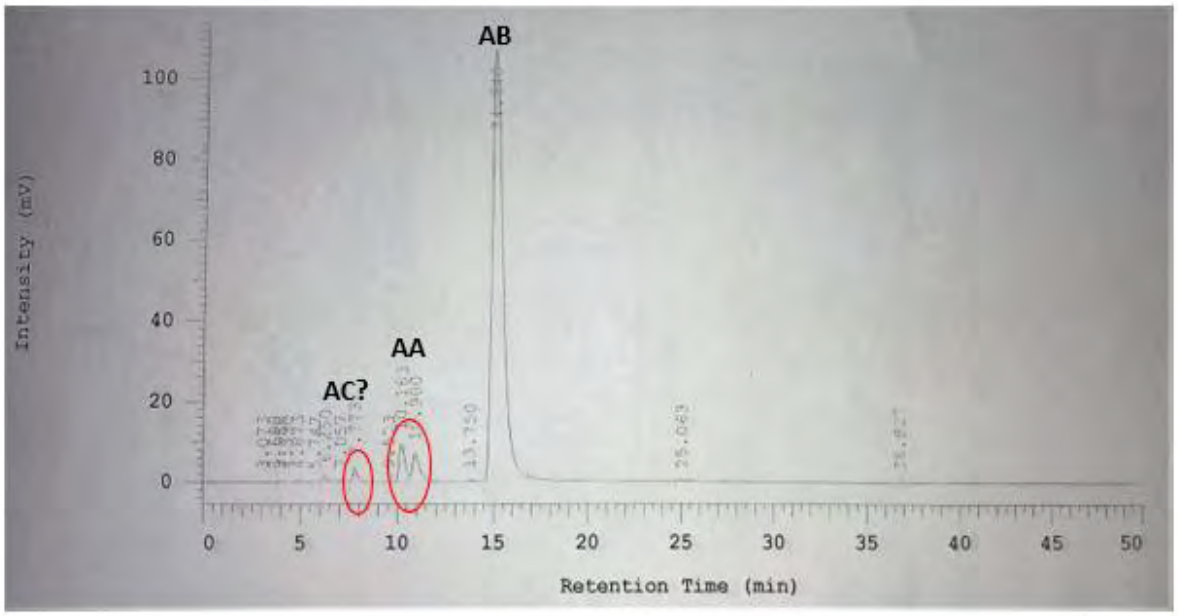


Figure 5.5: Chromatogram of 20ug/ml azure B.

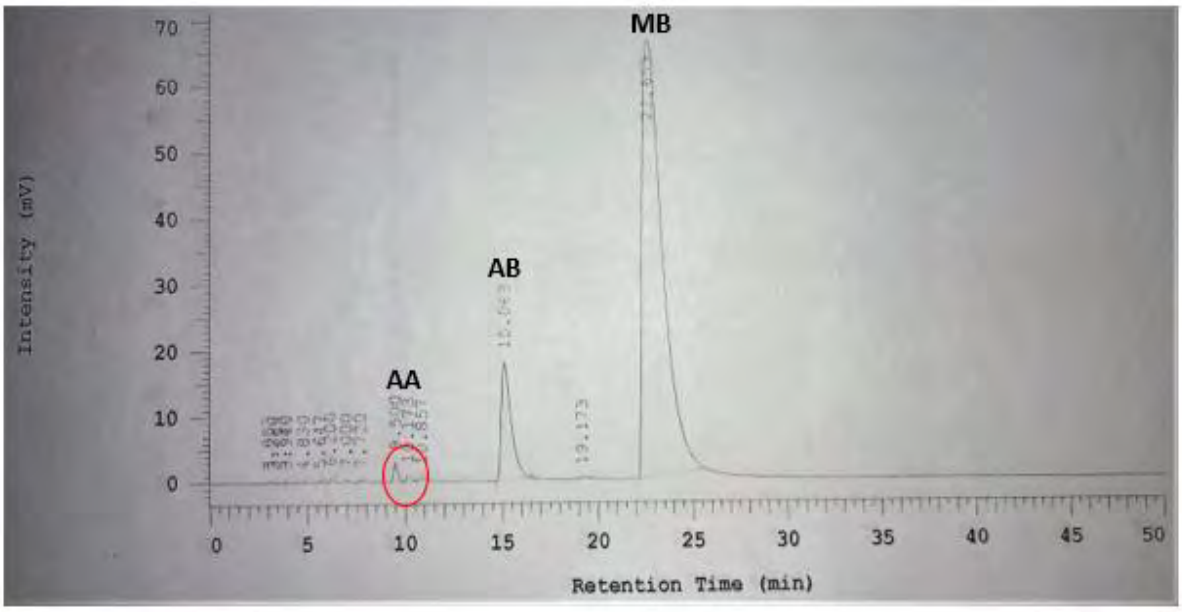


Figure 5.6: Chromatogram of 20ug/ml methylene blue.

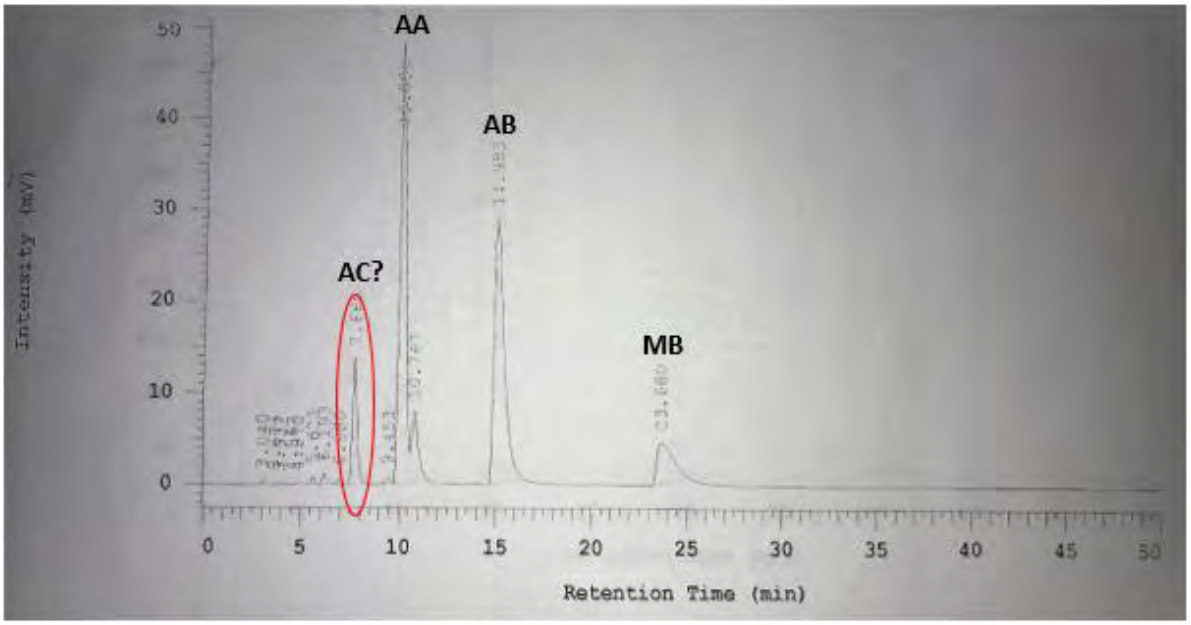


Figure 5.7: Chromatogram of 20ug/ml azure A.

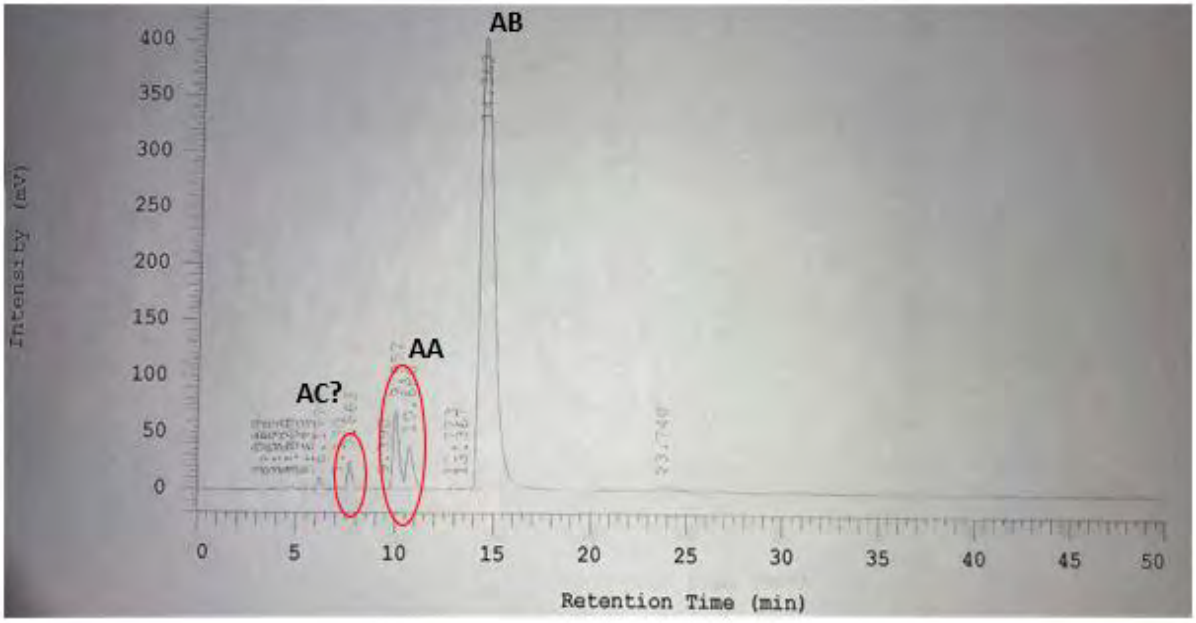


Figure 5.8: Chromatogram of 20ug/ml azure A + 100ug/ml azure B.

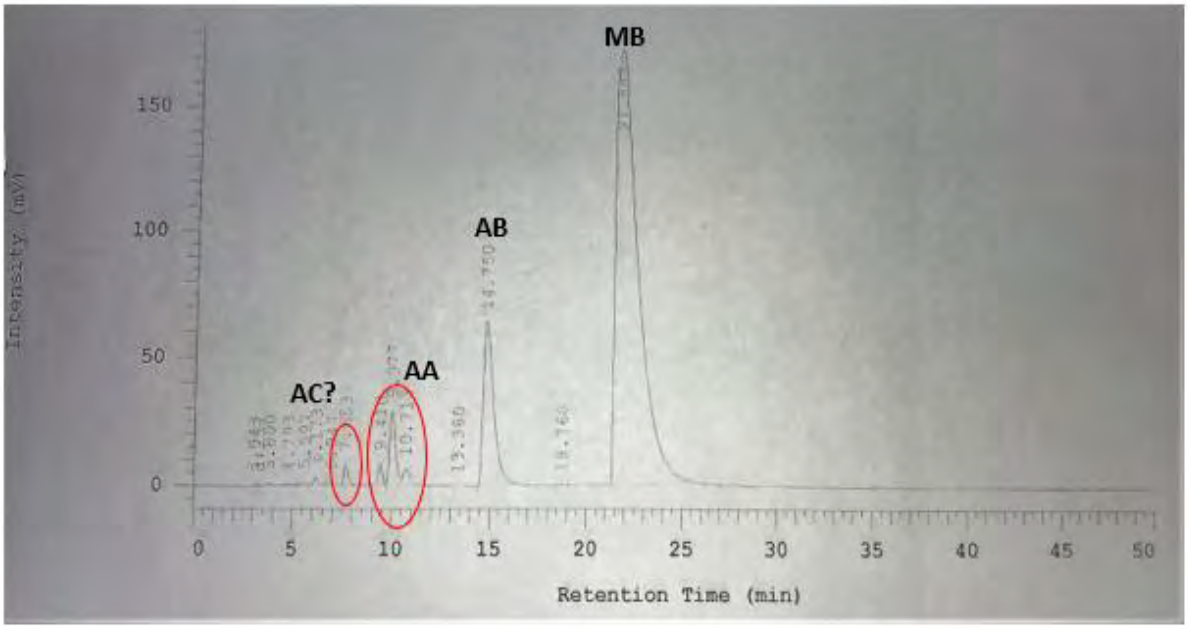


Figure 5.9: Chromatogram of 20ug/ml azure A + 100ug/ml methylene blue.

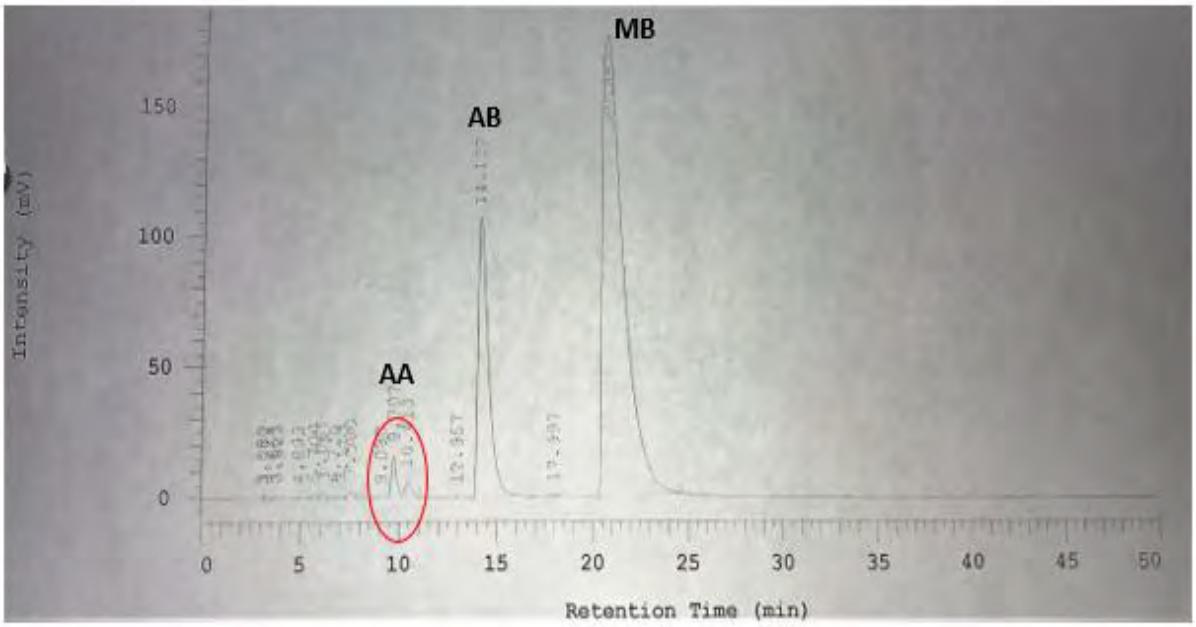


Figure 5.10: Chromatogram of 20ug/ml azure B + 100ug/ml methylene blue.

From this study, it has been proved that all of these compounds are impure andare contaminated with one or more of the other metabolites. According to the retention times as seen in figure 5.5 azure B contains a small amount of azure A which is indicated in red (and possibly a trace of azure C – refer to chapter 4, figure 4.16). In figure 5.6, it is clear that methylene blue contains azure B and a slight amount of azure A indicated in red (contains no azure C). According to figure 5.7, almost half of azure A is contaminated with different concentrations of azure B, methylene

blue and azure C (refer to chapter 4, figure 4.16). By spiking each sample, the individual peaks of azure A, azure B and methylene blue could easily be identified from the chromatograms in figures 5.8 to 5.10. Azure A elutes first at around 10 minutes. Azure B elutes at 15 minutes and methylene blue elutes last at about 23 minutes.

5.10.2. LOD and LOQ

A stock solution containing MB, Azure A and Azure B was prepared. The stock solution was diluted progressively 13 times, each time with a factor of 0.5. Each diluted sample was injected six times. The average peak area and the %RSD for each concentration was calculated and given in tables 5.4 - 5.6.

Table 5.4: Progressive dilutions of azure A and the corresponding average peak area and %RSD.

Concentration (µg/ml)	Average peak area of six injections	RSD (%)
0,10	2662	12,57
0,24	21857	27,61
0,49	52115	10,67
0,98	123630	4,28
1,95	191480	3,35
3,91	417088	1,34
7,81	743231	0,94
15,63	1554804	0,77
31,25	3042619	0,31
62,50	5978569	0,38
125,00	12032889	0,48
250,00	23690139	0,20
500,00	45420427	0,21
1000,00	86873270	0,93

From the results presented in table 5.4, it could be seen that azure A could still be detected at a concentration of 0.1 µg/ml and this value can safely be indicated as the LOD. At a concentration level of 3,9 µg/ml a %RSD lower than 2% was achieved and at a concentration level of 1,95 µg/ml a %RSD of 3,35 was achieved. A concentration level of 5-6 µg/ml could thus be a good indication of the LOQ value.

Table 5.5: Progressive dilutions of azure B and the corresponding average peak area and %RSD.

Concentration (µg/ml)	Average peak area of six injections	RSD (%)
0,10	15636	7,86
0,24	47399	18,73
0,49	107971	4,65
0,98	250112	2,65
1,95	422609	2,65
3,91	845455	1,58
7,81	1577559	0,46
15,63	3222811	1,90
31,25	6489584	0,56
62,50	12762798	0,25
125,00	25141926	0,26
250,00	47879409	0,29
500,00	95072355	0,23
1000,00	Concentration too high	-

From the results presented in table 5.5 it could be seen that azure B could still be detected at a concentration of 0.1 µg/ml and this value can safely be indicated as the LOD. At a concentration level of 3,9 µg/ml a %RSD lower than 2% was achieved and at a concentration level of 1,95 µg/ml a %RSD of 2,65 was achieved. A concentration level of 2 µg/ml could thus be a good indication of the LOQ value.

Table 5.6: Progressive dilutions of methylene blue and the corresponding average peak area and %RSD.

Concentration (µg/ml)	Average peak area of six injections	RSD (%)
0,10	8135	34,09
0,24	34205	4,79
0,49	59322	24,86
0,98	139489	9,87
1,95	194333	3,39
3,91	646438	2,45
7,81	1281478	1,74
15,63	2608357	1,02
31,25	5320736	0,35
62,50	11146714	0,31
125,00	25023763	1,95
250,00	48380176	0,12
500,00	95257460	0,17
1000,00	185399592	0,17

From the results presented in table 5.6, it could be seen that methylene blue could still be detected at a concentration of 0.1 µg/ml and this value can safely be indicated as the LOD. At a concentration level of 7.8 µg/ml a %RSD lower than 2% was achieved and at a concentration level of 3.9 µg/ml a %RSD of 2.45 was achieved. A concentration level of 5 µg/ml could thus be a good indication of the LOQ value.

5.10.3. Linearity and range

From the LOD/LOQ study it was evident that a concentration range of 10 – 500 µg/ml can be considered as an appropriate working range for methylene blue, azure A, azure B and thionin. Five concentrations that covers the whole working range were used to determine the linearity of each of these compounds (figures 5.11-5.14). First a mixture of azure A, azure B and methylene blue were analysed by injecting the mixture six times at five different concentrations that covers the whole range. However, after processing the data the observation was made that the data of methylene blue from the mixture is incomparable to that of the other two methylene blue standards. This finding can be explained by the additive effect that the compounds have on each

other due to its impurities. For example, due to the fact that azure A contains great amounts of azure B and methylene blue, greater peak areas than expected for both azure B and methylene blue were observed. In order to have data that is comparable to that of the other two methylene blue standards, methylene blue used in the mixture were analysed separately from the other compounds (table 5.7). A mixture of azure A, azure B and thionin was also analysed (tables 5.9-5.14). Refer to Addendum B for detailed data processing of each compound.

Table 5.7: Concentrations, corresponding average peak area and % RSD for methylene blue for the determination of linearity and range.

Concentration (µg/ml)	Average peak area	RSD (%)
0,10	8135	34,09
4,94	704276	1,96
9,69	2029912	0,90
48,45	9980864	0,54
96,90	20921115	0,23
242,25	51513535	0,10
484,50	104016667	0,29

Table 5.8: Linearity data obtained for methylene blue.

Regression statistics	
Multiple R	0,999978898
R Square	0,999957796
Adjusted R square	0,999943728
Standard Error	311806,77
Observations	5

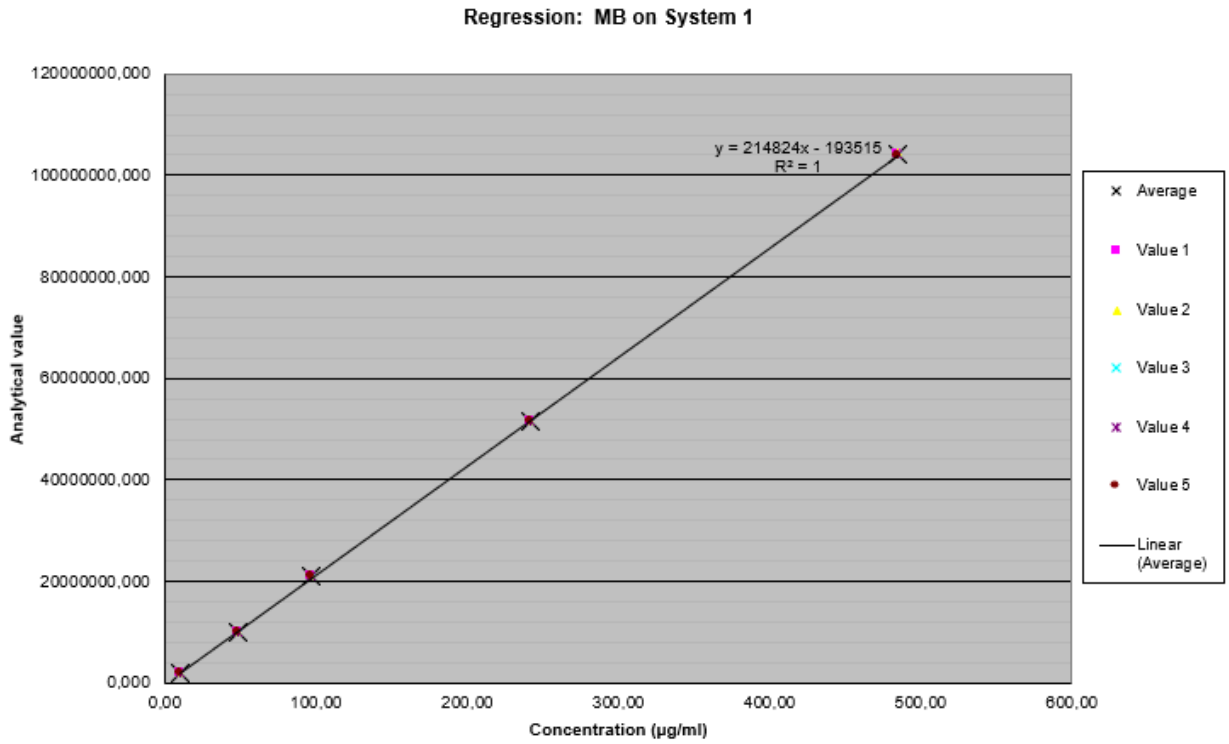


Figure 5.11: Regression line of methylene blue analysed on System 1.

For methylene blue (table 5.7, figure 5.11), the range was postulated to be between 10-500 µg/ml. However, according to the data, a %RSD of 1,96 is still obtained at a concentration of 4,98 µg/ml. Also, the %RSD obtained at a concentration of 484,50 µg/ml, was 0,29. Thus, it is safe to say that the optimal working range of MB is between 5-500+ µg/ml.

As for linearity, excellent data that proves that this method is able to obtain results within a given range, which is directly proportional to the concentration of the analyte in the sample, was obtained and is presented in table 5.8. (Please refer to Addendum B).

Table 5.9: Concentrations, corresponding average peak area and % RSD for azure A

Concentration(µg/ml)	Average peak area for AA	RSD (%)
9,69	1319397	1,07
48,45	2621476	0,65
96,90	8091090	1,04
242,25	7009728	0,36
484,50	13341595	1,31

Table 5.10: Linearity data obtained for azure A.

Regression statistics	
Multiple R	0,999579301
R Square	0,999158779
Adjusted R square	0,998878372
Standard Error	179755,33
Observations	5

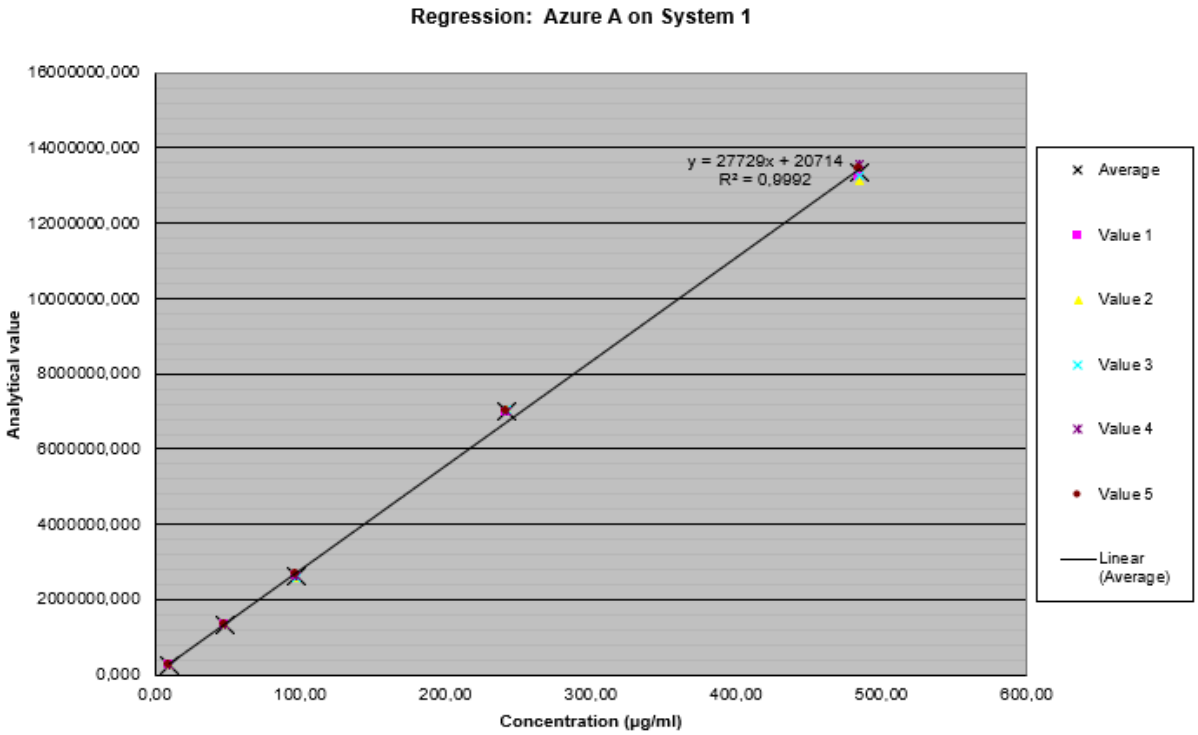


Figure 5.12: Regression line of azure A analysed on System 1.

For azure A (table 5.9, figure 5.12), the range was postulated to be between 10-500 µg/ml. According to the data, a %RSD of 1,07 is obtained at a concentration of 9,69 µg/ml. Also, the %RSD obtained at a concentration of 484,50 µg/ml, was 1,31. Thus, it is safe to say that the optimal working range of AA is between 10-500 µg/ml.

As for linearity, despite of the additive effect the metabolites have on each other in the sample mixture, acceptable data that proves that this method is able to obtain results within a given range, which is directly proportional to the concentration of the analyte in the sample, was still obtained and is presented in table 5.10. (Please refer to Addendum B).

Table 5.11: Concentrations, corresponding average peak area and % RSD for azure B.

Concentration(µg/ml)	Average peak area for AB	RSD (%)
9,69	804291	1,00
48,45	4052709	0,26
96,90	8091090	0,18
242,25	21332280	0,41
484,50	40764326	1,56

Table 5.12: Linearity data obtained for azure B.

Regression statistics	
Multiple R	0,999661403
R Square	0,99932292
Adjusted R square	0,999097227
Standard Error	492104,8
Observations	5

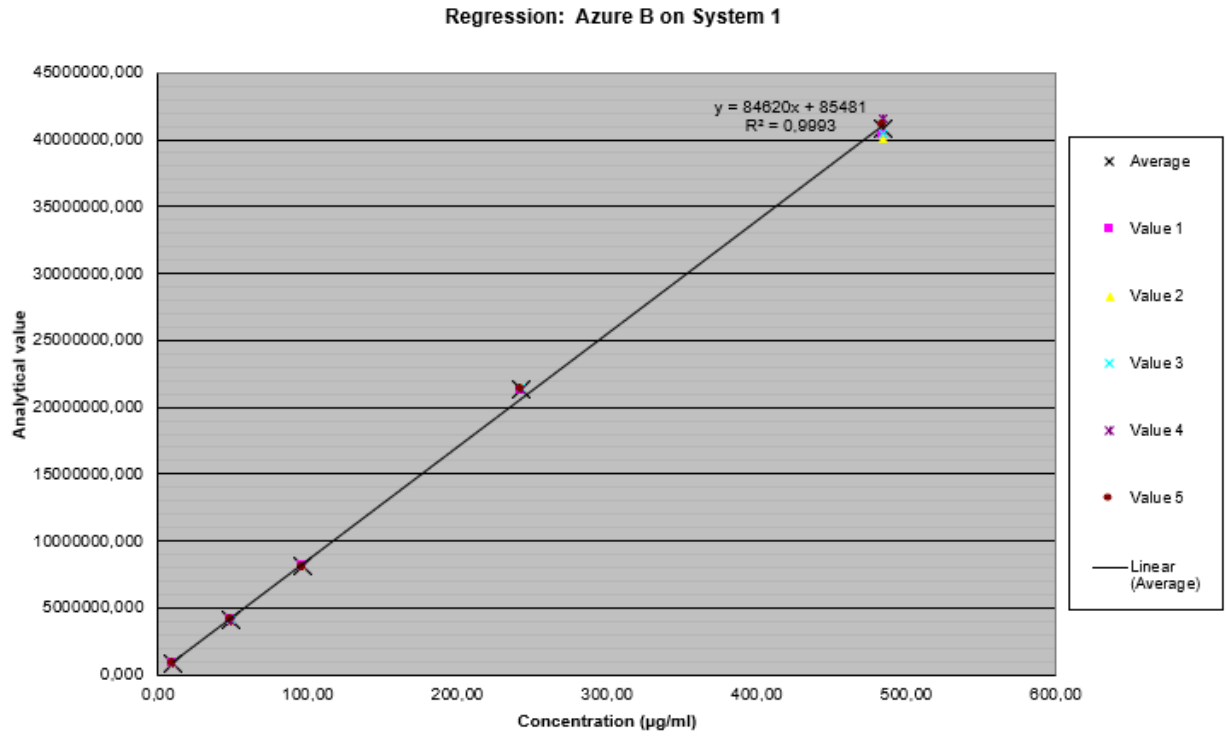


Figure 5.13: Regression line of azure B analysed on System 1.

For azure B (table 5.11, figure 5.13), the range was postulated to be between 10-500 µg/ml. According to the data, a %RSD of 1,00 is obtained at a concentration of 9,69 µg/ml. Also, the %RSD obtained at a concentration of 484,50 µg/ml, was 1,56. Thus, it is safe to say that the optimal working range of AB is between 10-500 µg/ml.

As for linearity, despite of the additive effect the metabolites have on each other in the sample mixture, acceptable data that proves that this method is able to obtain results within a given range, which is directly proportional to the concentration of the analyte in the sample, was still obtained and is presented in table 5.12. (Please refer to Addendum B).

Table 5.13: Concentrations, corresponding average peak area and % RSD for thionin.

Concentration(µg/ml)	Average peak area for thionin	RSD (%)
9,69	347351	0,67
48,45	1724390	0,45
96,90	3417788	0,46
242,25	8720693	0,26
484,50	16061107	0,99

Table 5.14: Linearity data obtained for thionin.

Regression statistics	
Multiple R	0,99904424
R Square	0,998089394
Adjusted R square	0,997452525
Standard Error	324670,54
Observations	5

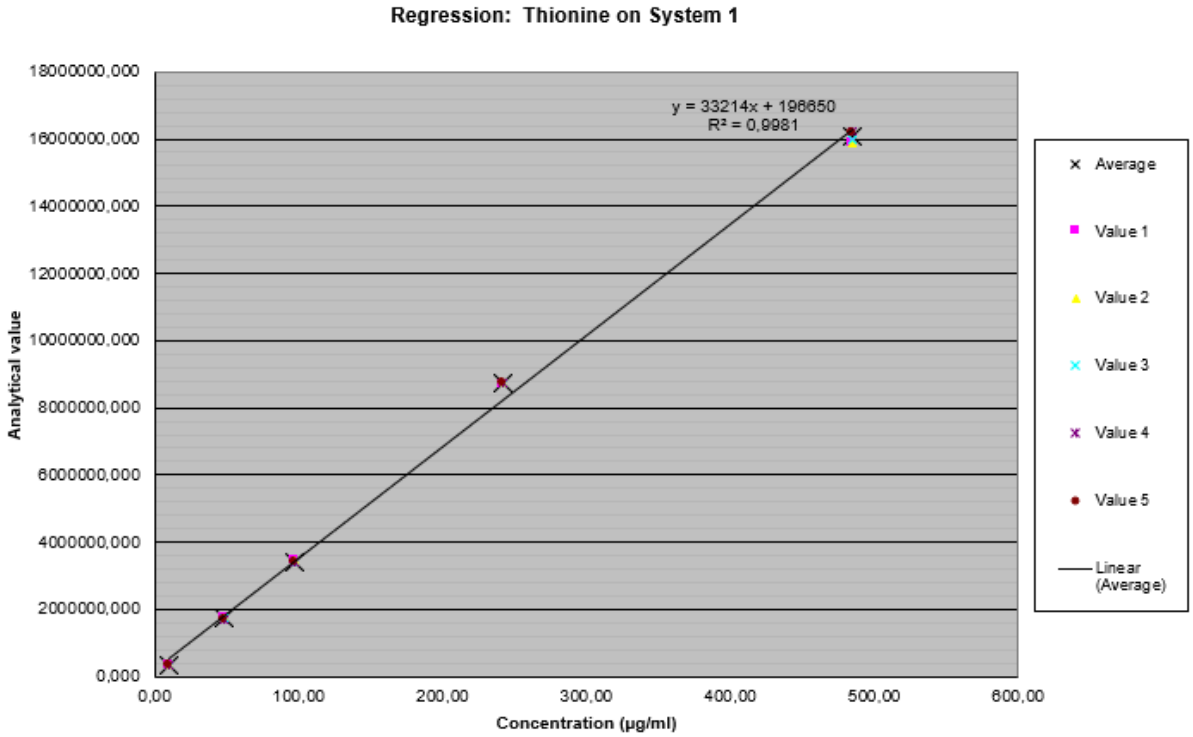


Figure 5.14: Regression line of thionin analysed on System 1.

For thionin (table 5.13, figure 5.14), the range was postulated to be between 10-500 µg/ml. According to the data, a %RSD of 0,67 is obtained at a concentration of 9,69 µg/ml. Also, the %RSD obtained at a concentration of 484,50 µg/ml, was 0,99. Thus, it is safe to say that the optimal working range of AB is between 10-500 µg/ml.

As for linearity, despite of the additive effect the metabolites have on each other in the sample mixture, acceptable data that proves that this method is able to obtain results within a given range, which is directly proportional to the concentration of the analyte in the sample, was still obtained and is presented in table 5.14. (Please refer to Addendum B).

5.10.4. Precision

In tables 5.7, 5.9, 5.11 and 5.13 the five concentrations within the chosen range with their corresponding average peak area and % RSD are given for each metabolite, respectively. The % RSD of all the concentrations for each metabolite were less than 2%, thus acceptable repeatability and precision was accomplished.

5.10.5. Accuracy and recovery

Two MB standards from different suppliers were also analysed. Methylene blue 1 (purity given as 98,5%) was purchased from Associated Chemical enterprises (ace), and methylene blue 2 (no purity indicated), was purchased from Merck. Four concentrations that covers the working range (10 – 500 µg/ml) were used for each analyte. These concentrations were prepared in triplicate for each analyte. Each of the prepared samples were then injected and analysed six times. The recovery of each was calculated with the regression line generated from the data for the working range. The results are presented in tables 5.15 and 5.16.

Table 5.15: Expected concentrations with the corresponding % recovery for methylene blue 1.

Expected concentration (µg/ml)	Average recovery (%)
10,05	149,9*
50,45	96,7
100,47	93,9
502,35	100,2

*The high value that were obtained are due to human error and/or too low sample concentration.

Table 5.16: Expected concentrations with the corresponding % recovery for methylene blue 2.

Expected concentration (µg/ml)	Average recovery (%)
10,33	108,6
51,70	124,2*
103,33	98,9
516,67	102,9

*The high value that were obtained are due to human error.

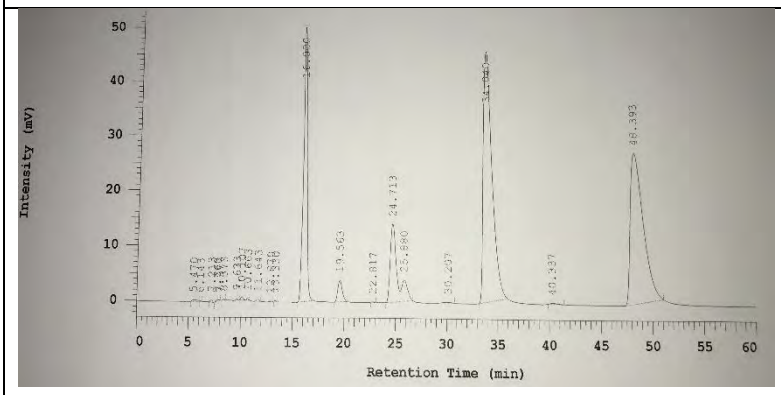
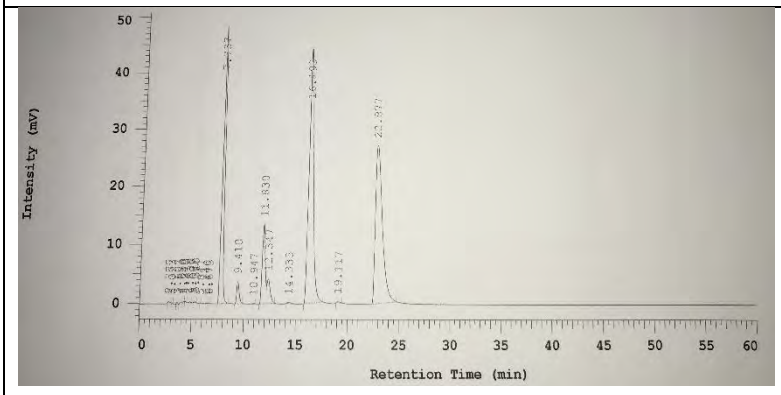
5.10.6. Robustness

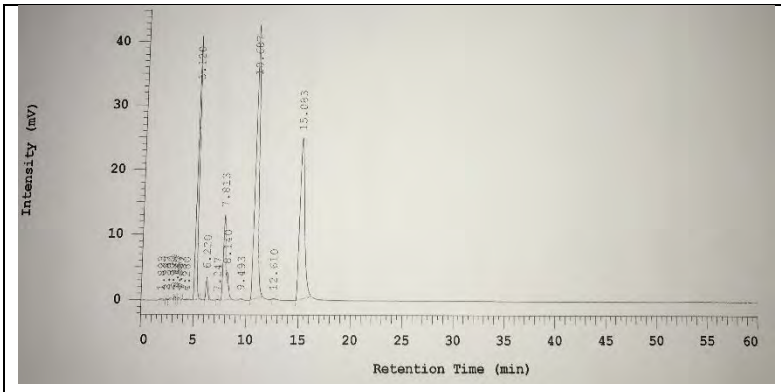
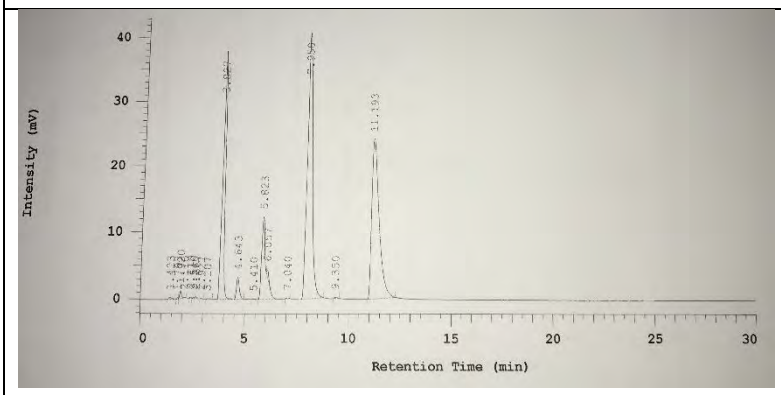
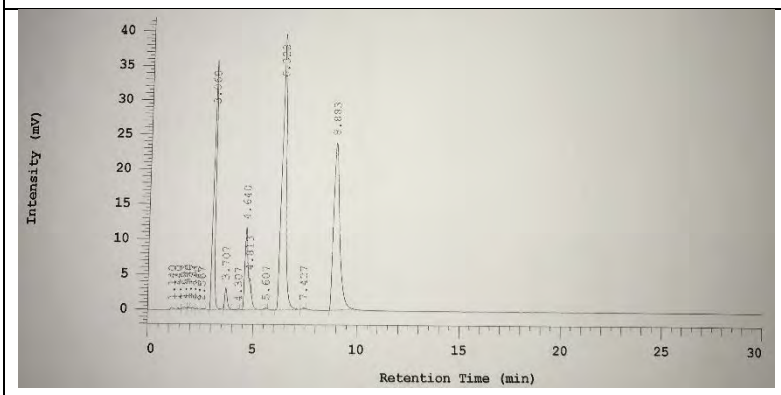
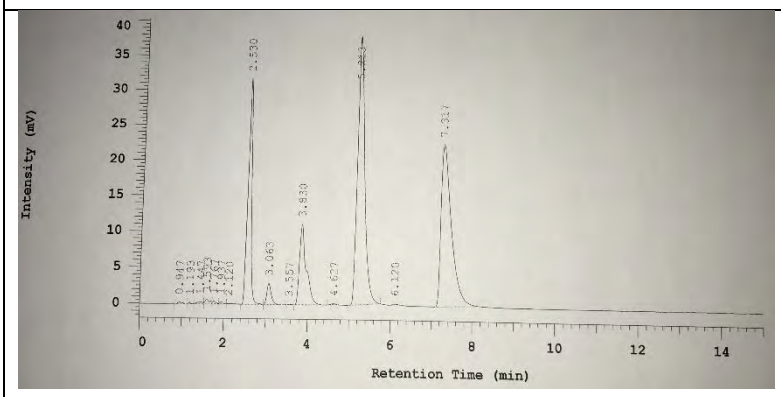
In this study, different parameters were deliberately changed in order to determine to what degree environmental and operational variables of the method will influence the test results.

- *Flow rate*

During the test for the determination of the influence that the flow rate might have, the flow rate was deliberately adjusted with 0.25 ml/min for each run, while all the other parameters were kept unchanged.

Table 5.17: The deliberate change of flow rate and the resulting chromatograms.

Chromatogram	Description
	Flow rate: 0.25 ml/min
	Flow rate: 0.5 ml/min

	Flow rate: 0.75 ml/min
	Flow rate: 1.0 ml/min
	Flow rate: 1.25 ml/min
	Flow rate: 1.5 ml/min

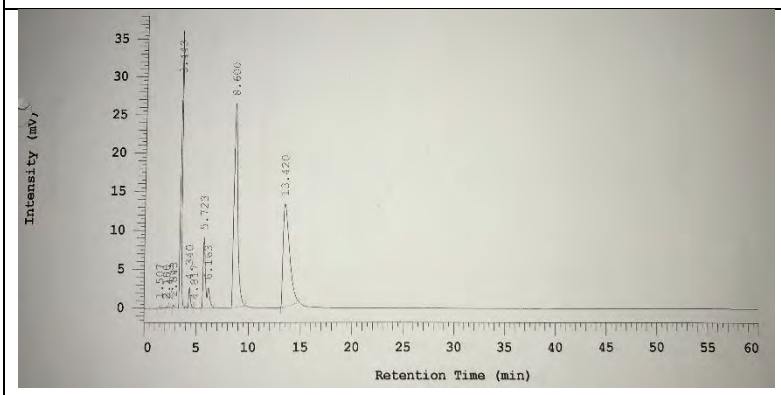
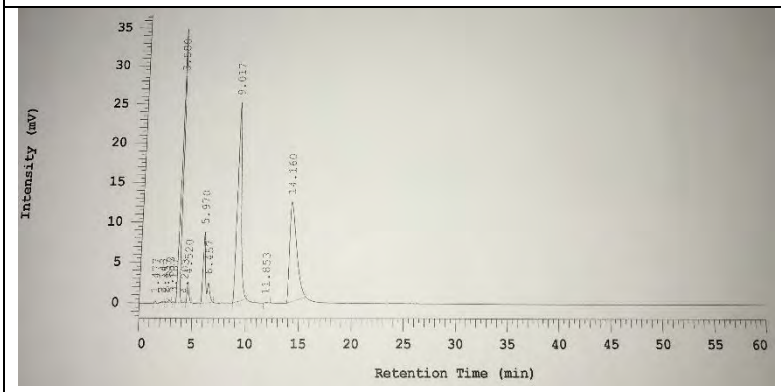
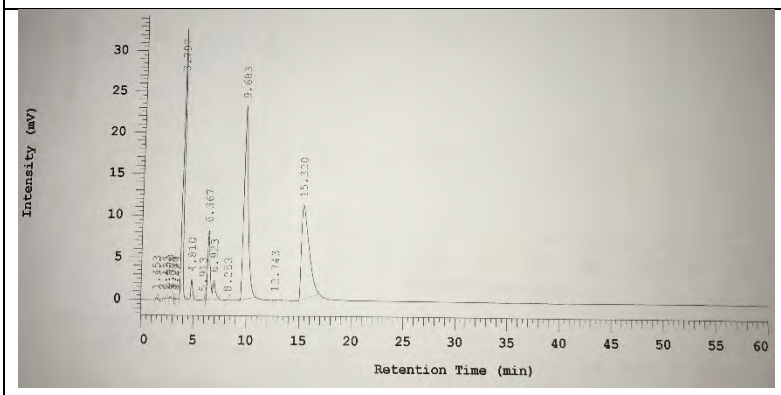
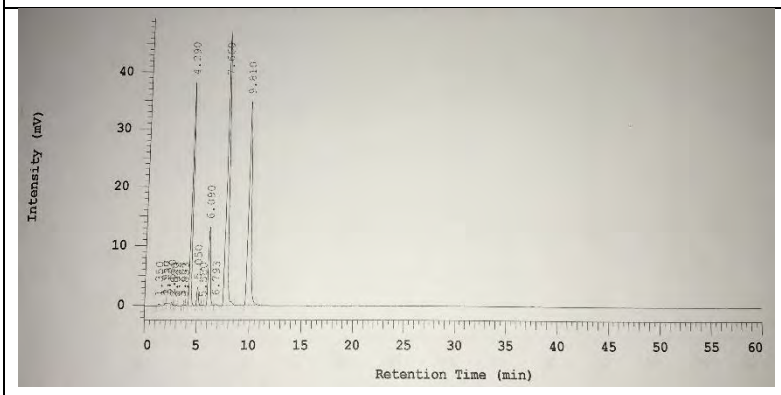
A great disadvantage of significant back pressure that is related to higher flow rates was noticed during this test, thus for the optimisation of this analytical method the focus was rather on flow rates lower than 1ml/min. In table 5.17, the implementation of a flow rate of 0.75 ml/min led to

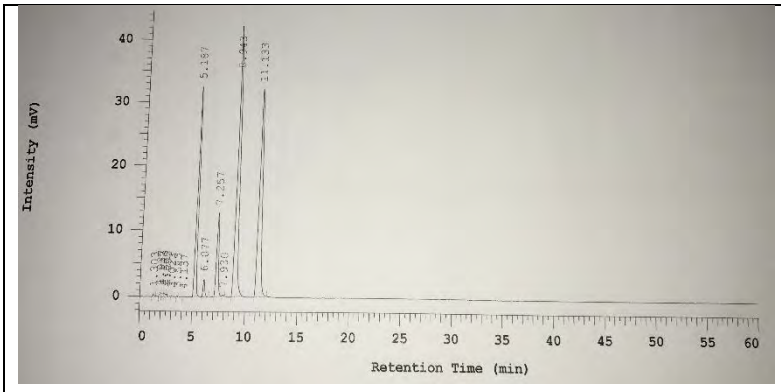
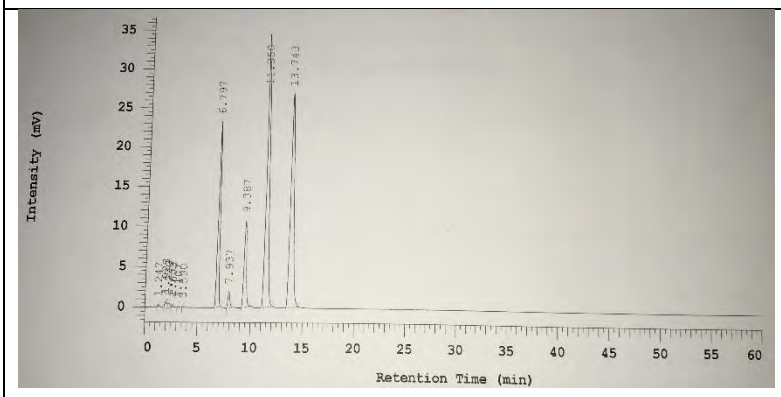
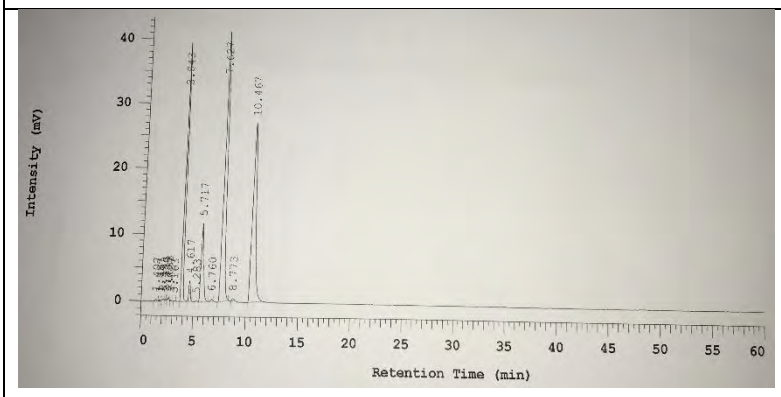
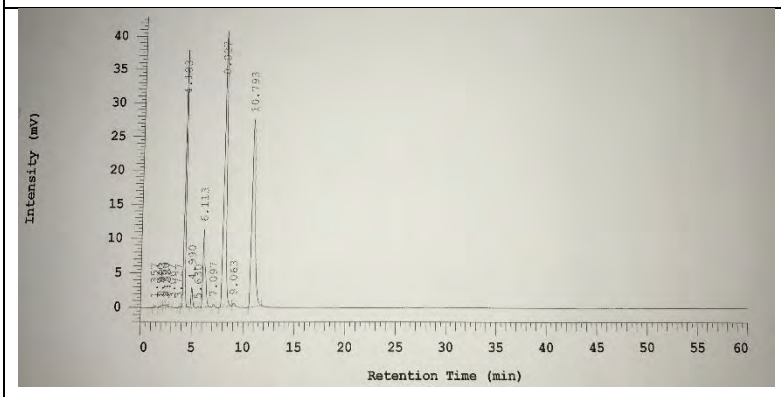
very small retention times and the peaks eluted significantly closer to each other. With the implementation of a flow rate of 0.5 ml/min the retention times were longer and the peaks eluted further each other, which is considered as an advantage of the lower flow rate. However, it was argued that to combine the advantage of optimal retention times and optimum peak separation as achieved with 0.75 and 0.5 ml/min respectively, the decision was made to use a flow rate between those two values. The implementation of 0.65 ml/min as the preferred flow rate, resulted in optimal retention times (all peaks still eluted within 25 minutes), and optimal peak separation (not to near to each other).

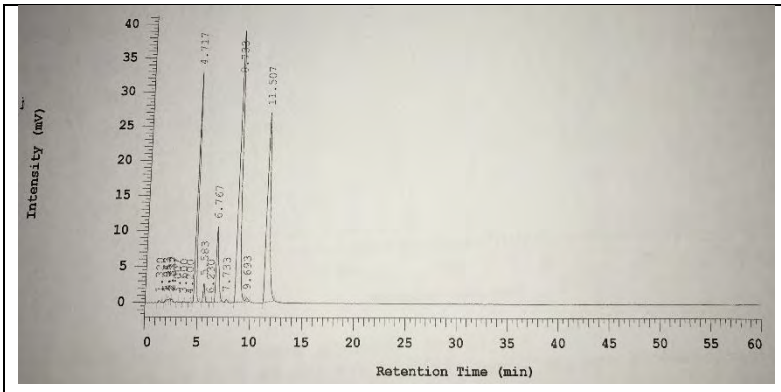
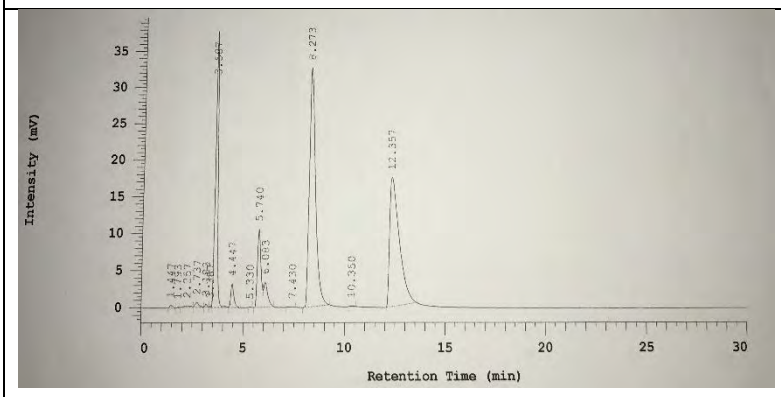
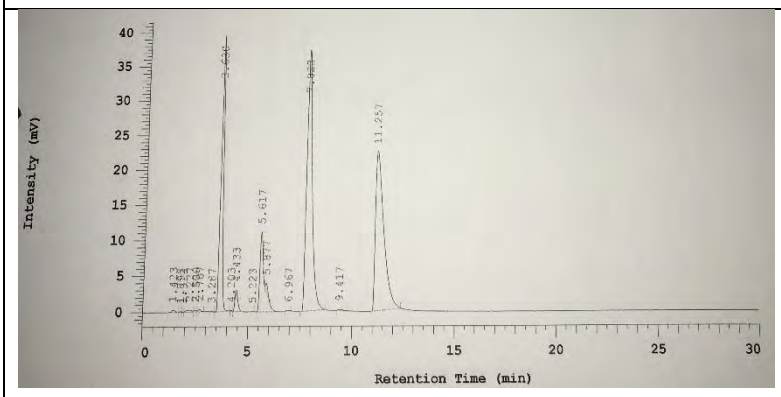
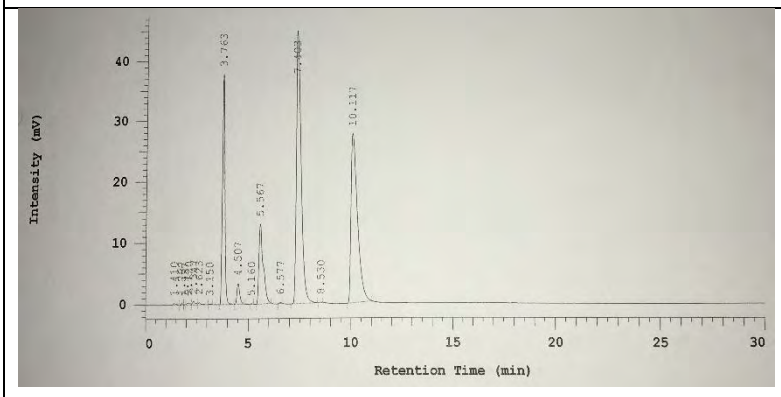
- *Mobile phase composition*

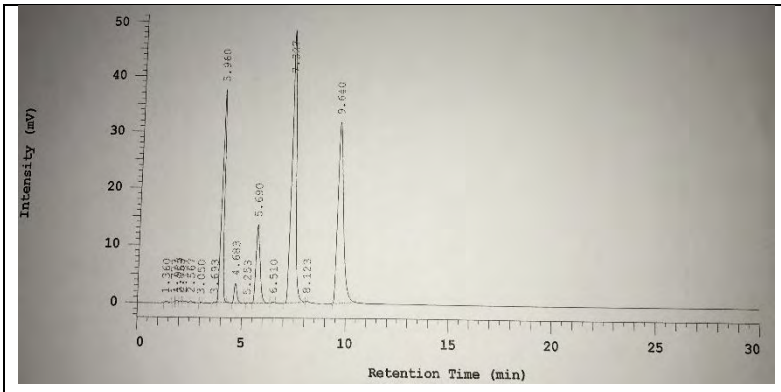
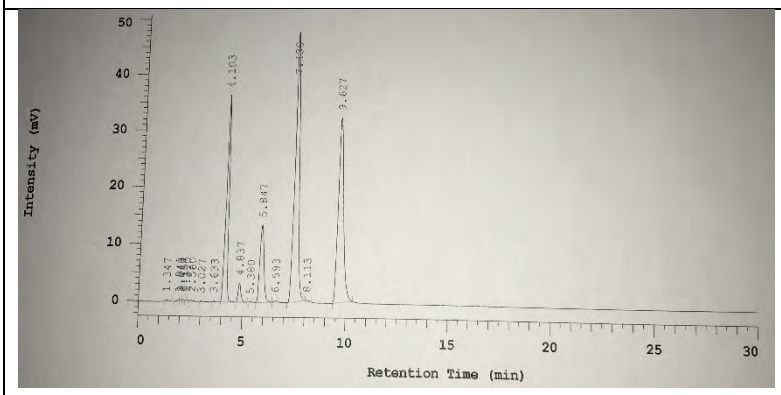
In this test, the possible effect of the MP composition was determined by comparing chromatograms from the series injected during the method development stage (refer to chapter 4, table 4.3) and choosing a possible range where acceptable separation was observed. The chosen MP composition range was then injected again to determine the optimum ratio interval of part A (5 mM ammonium acetate dissolved in 10:90 water-methanol) to part B (acetonitrile of methanol) (table 5.18). After the ratio of part A to part B was established, the composition of part B (mixture of methanol and acetonitrile) was also adjusted to optimise the separations.

Table 5.18: The deliberate change of the mobile phase composition and the resulting chromatograms.

Chromatogram	Description
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol 40% Part B: methanol Sample: AA + AB + MB + Thionin
	50% Part A: 5 mM ammonium acetate in 10:90 water to methanol 50% Part B: methanol Sample: AA + AB + MB + Thionin
	40% Part A: 5 mM ammonium acetate in 10:90 water to methanol 60% Part B: methanol Sample: AA + AB + MB + Thionin
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol 40% Part B: acetonitrile Sample: AA + AB + MB + Thionin

	50% Part A: 5 mM ammonium acetate in 10:90 water to methanol 50% Part B: acetonitrile Sample: AA + AB + MB + Thionin
	40% Part A: 5 mM ammonium acetate in 10:90 water to methanol 60% Part B: acetonitrile Sample: AA + AB + MB + Thionin
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 20% methanol + 20% acetonitrile Sample: AA + AB + MB + Thionin
	50% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 25% methanol + 25% acetonitrile Sample: AA + AB + MB + Thionin

	40% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 30% methanol + 30% acetonitrile Sample: AA + AB + MB + Thionin
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 35% methanol + 5% acetonitrile Sample: AA + AB + MB + Thionin
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 30% methanol + 10% acetonitrile Sample: AA + AB + MB + Thionin
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 20% methanol + 20% acetonitrile Sample: AA + AB + MB + Thionin

	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 10% methanol + 30% acetonitrile Sample: AA + AB + MB + Thionin
	60% Part A: 5 mM ammonium acetate in 10:90 water to methanol Part B: 5% methanol + 35% acetonitrile Sample: AA + AB + MB + Thionin

Both methanol and acetonitrile have advantages when used for the separation of the analytes. Acetonitrile shows less band broadening, however, the peaks are separated better when methanol is used. Thus, the decision was made to incorporate both methanol and acetonitrile as a mixture in part B of the MP. As seen in table 5.18, he best results were seen with a MP composition of 60% part A and 40% part B, where part B consisted of 25% methanol and 15% acetonitrile.

- *HPLC system*

  In the study to determine the influence of different HPLC systems and different laboratories, a linear regression study was done on system 2 in a concentration range of 0.1 – 500µg/ml. the results are presented in tables 5.19-5.21 and figures 5.17-5.19.

Table 5.19: Concentrations, corresponding average peak area and % RSD for azure A as analysed on system 2.

Concentration (µg/ml)	Average peak area for AA	RSD (%)
0,10	No peak detected	No peak detected
4,94	225029	1,47
49,40	1078395	0,18
247,00	5436688	0,08
494,00	10835914	0,45

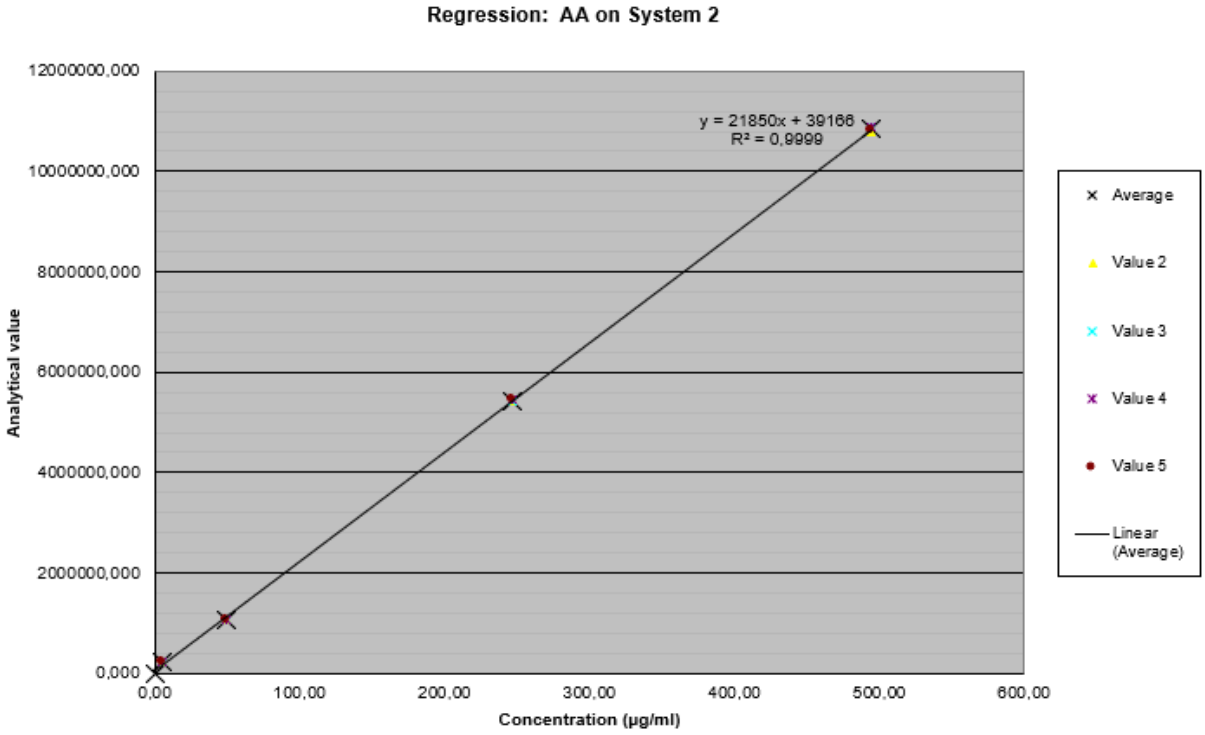


Figure 5.15: Regression line of azure A analysed on System 2.

Table 5.20: Concentrations, corresponding average peak area and % RSD for azure B as analysed on system 2.

Concentration (µg/ml)	Average peak area for AB	RSD (%)
0,10	No peak detected	No peak detected
4,94	820575	0,17
49,40	3947880	0,08
247,00	19869046	0,05
494,00	39539475	0,30

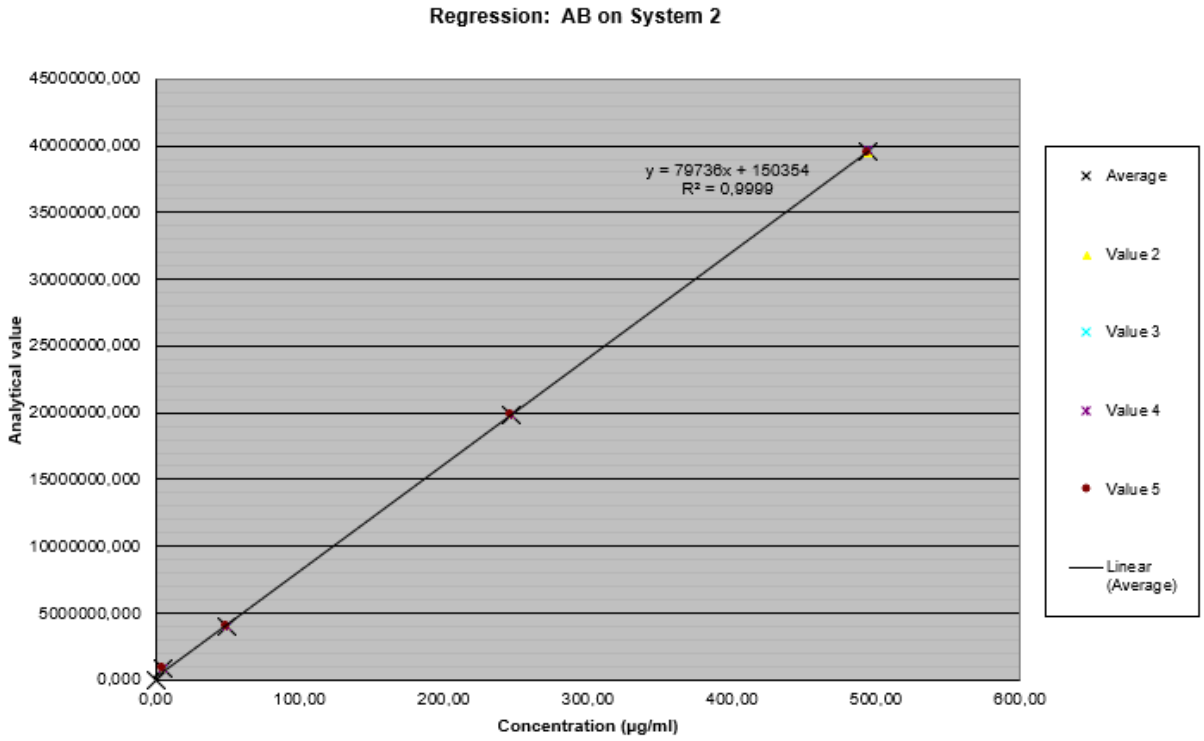


Figure 5.16: Regression line of azure B analysed on System 2.

Table 5.21: Concentrations, corresponding average peak area and % RSD for methylene blue.

Concentration (µg/ml)	Average peak area for AB	RSD (%)
0,10	No peak detected	No peak detected
4,94	715724	0,14
49,40	3537278	0,07
247,00	17919788	0,08
494,00	35838876	0,07

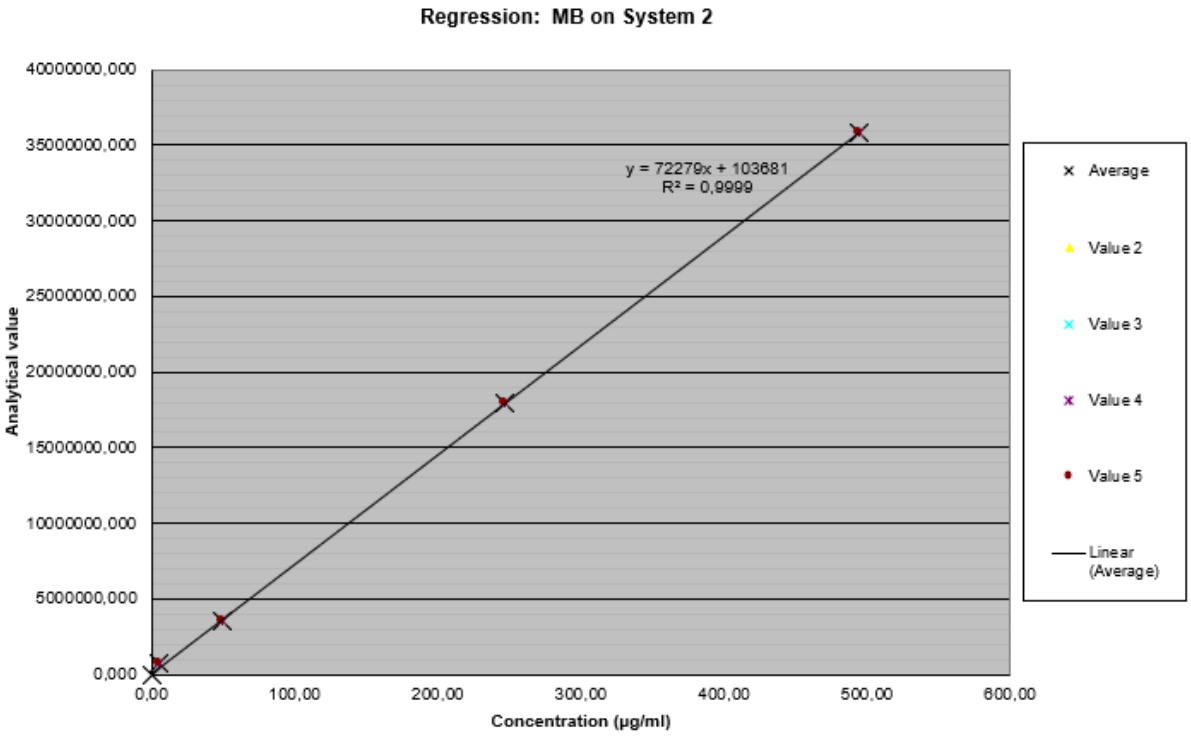


Figure 5.17: Regression line of methylene blue analysed on System 2.

System 2 was not able to identify any peaks when a sample concentration of 0.1 µg/ml was analysed (tables 5.19-5.21). However, the results indicate that linearity was achived for MB, azure A and azure B in the concentration range of 4-500 µg/ml with R^2 values larger than 0.99 for all three actives (figures 5.15-5.17).

- *Product analyses*

Two MB standards from different suppliers were also analysed from which the data was processed and compared to that of the primary standard MB. These standards were analysed by using the regression information of the primary standard and the resulting recoveries are presented in tables 5.15 and 5.16.

- *Column*

Different columns were used for analysis. All chromatograms are available, however, only the results of the two columns on which successful separation were observed, are included in this dissertation. Refer to chapter 4, tables 4.2 and 4.3.

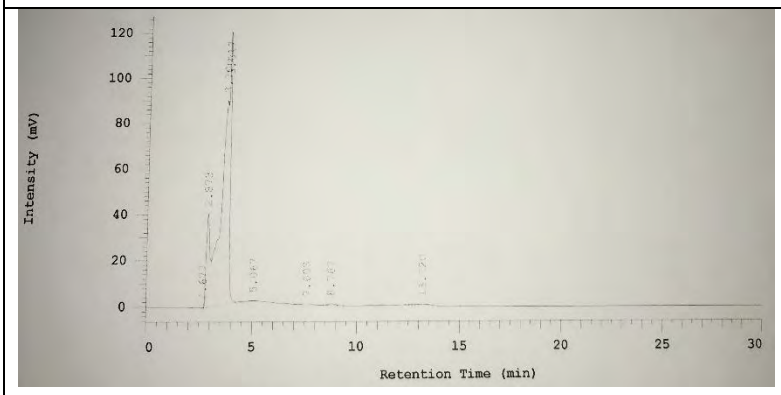
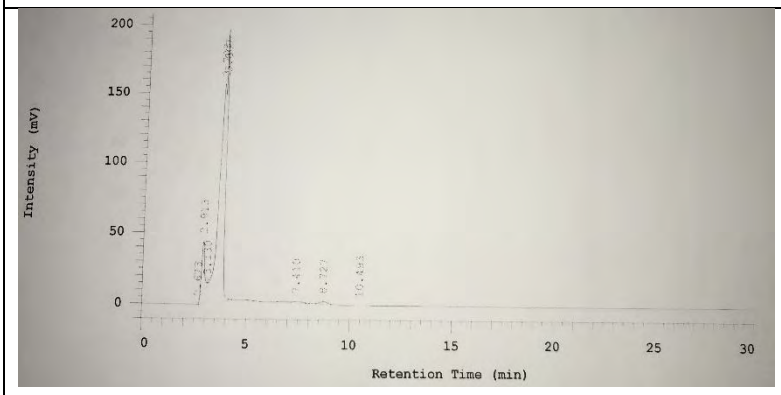
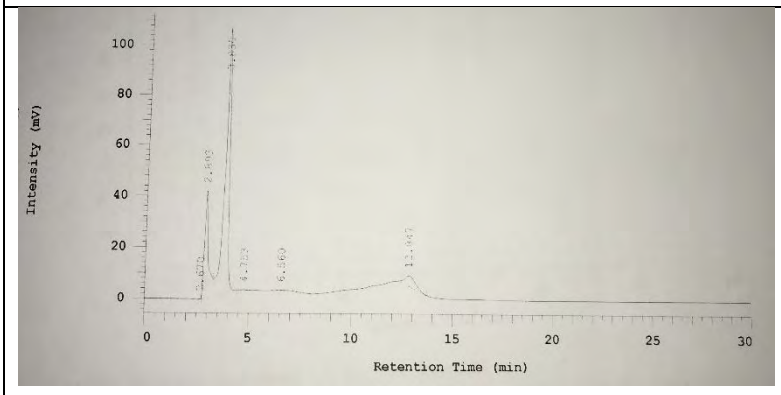
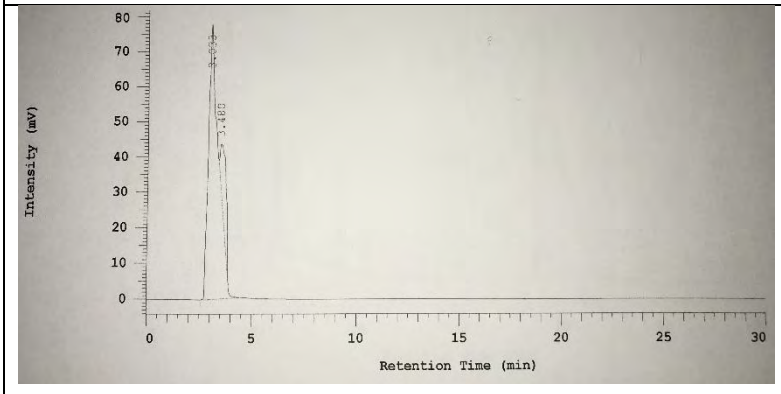
5.10.7. Stability

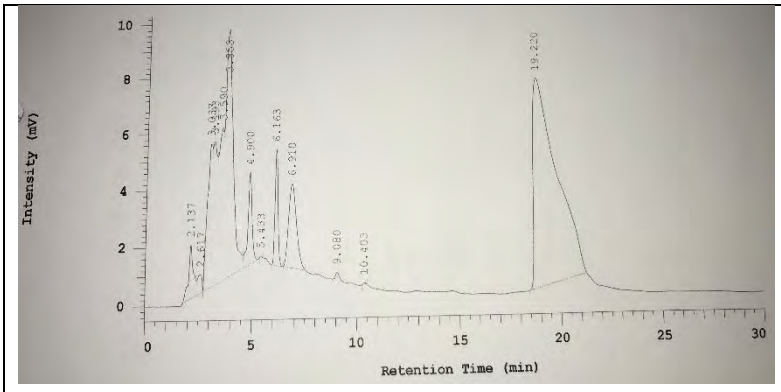
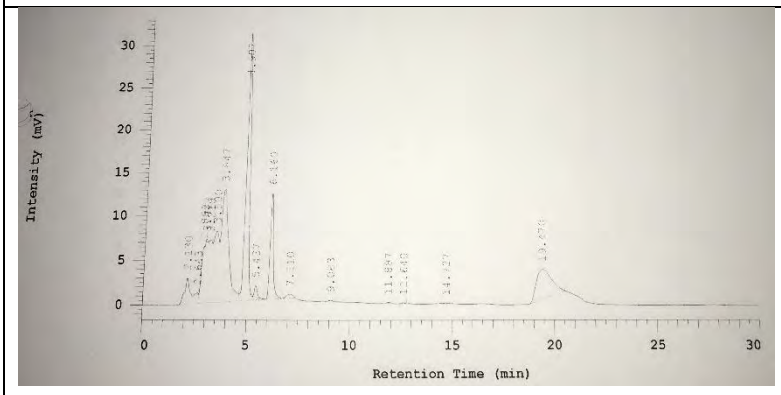
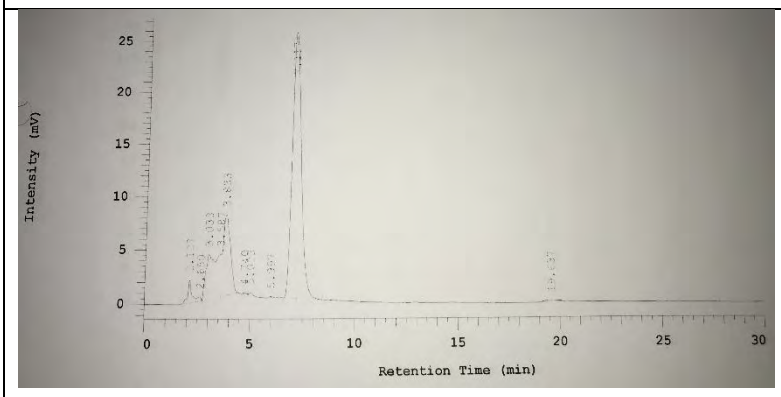
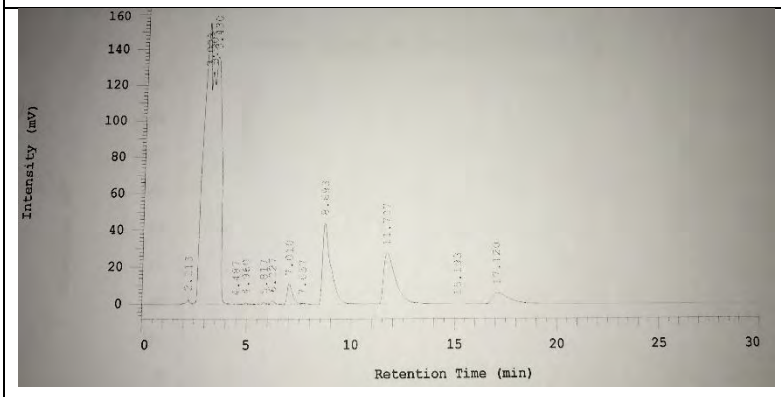
In this study, five 20 µg/ml samples of each analyte were prepared by using each of the following solvents:

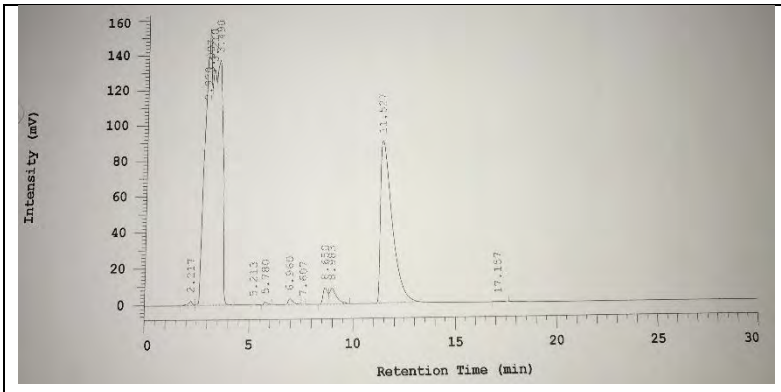
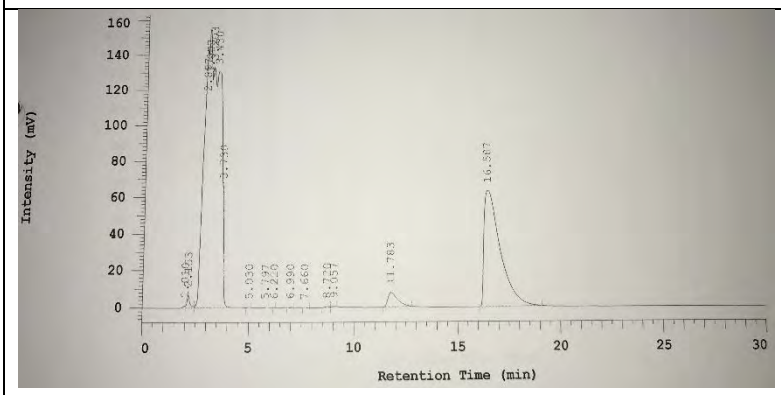
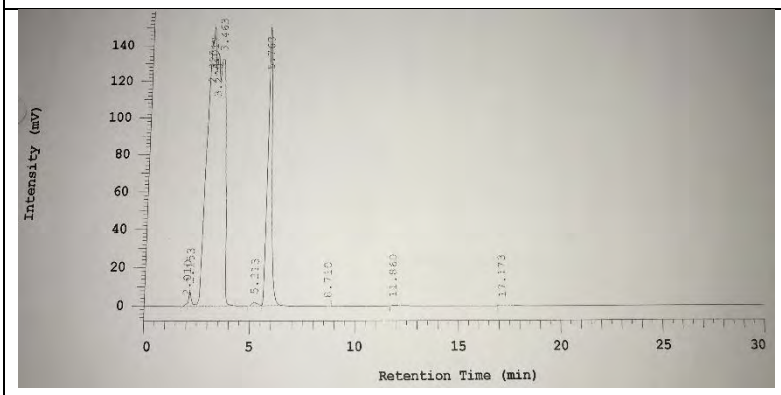
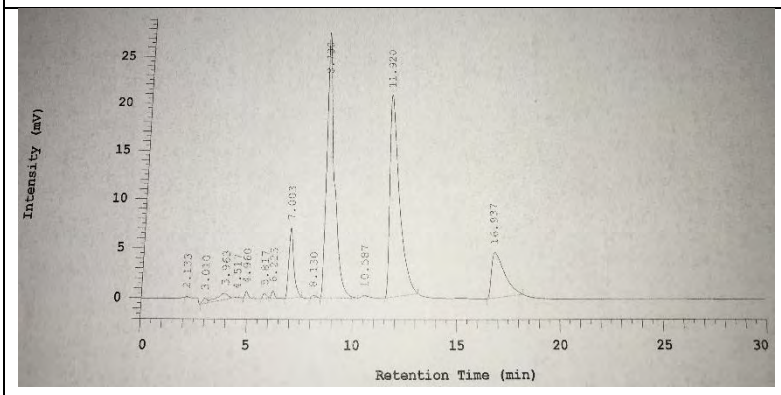
- 1 N Hydrogen chloride (HCl)
- 1 N Sodium chloride (NaOH)
- 10% Hydrogen peroxide (H_2O_2)
- Water (H_2O)
- 60:40 part A: 5 Mm ammonium acetate dissolved in 10:90 water-methanol, part B: 15:25 acetonitrile-methanol. (MP)

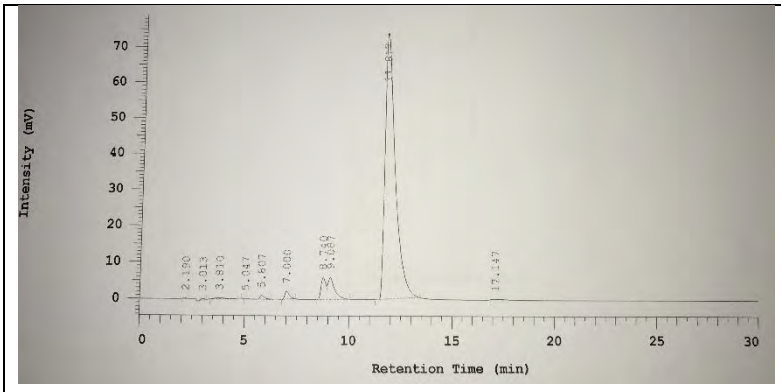
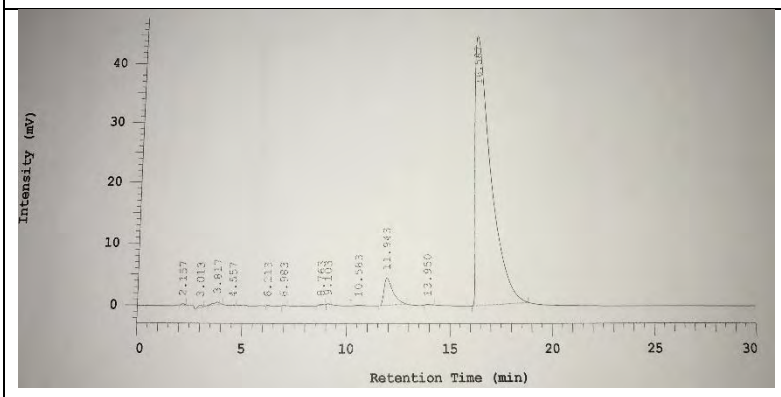
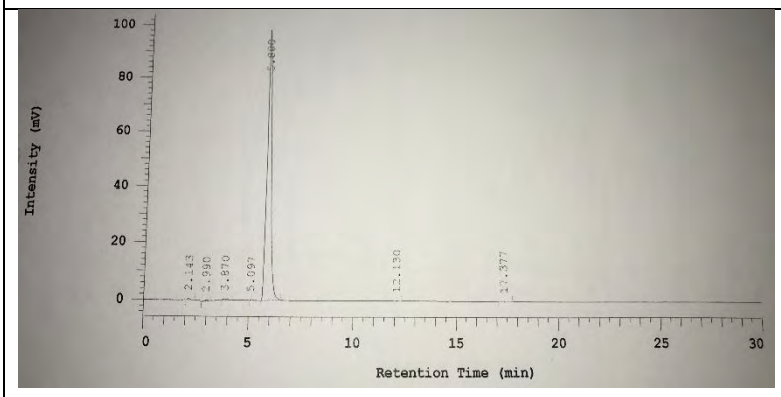
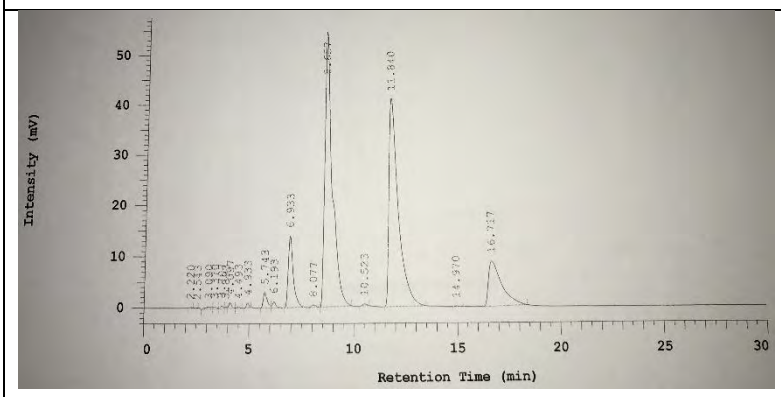
Each sample were analysed on days one, two, three and on day seven. Also, samples from the same batches were put in a refrigerator (0 ºC) and left on the laboratory bench (sun exposure) for seven days, respectively. The resulting chromatograms of the compounds analysed in the different solvents on day one are presented in table 5.22.

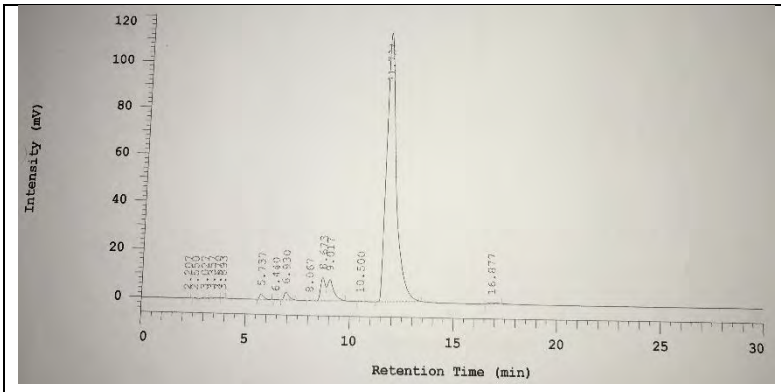
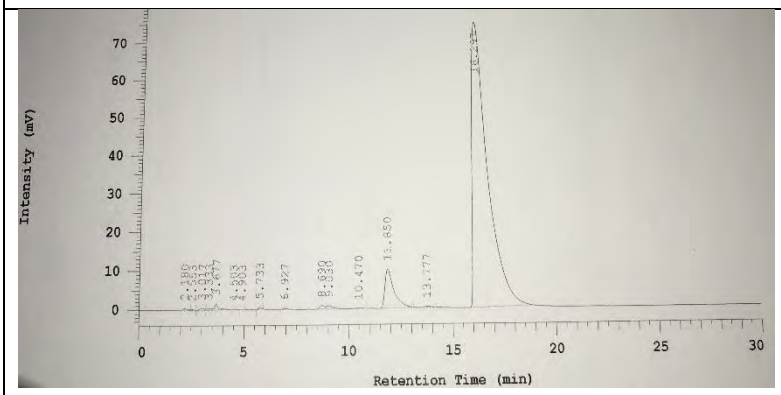
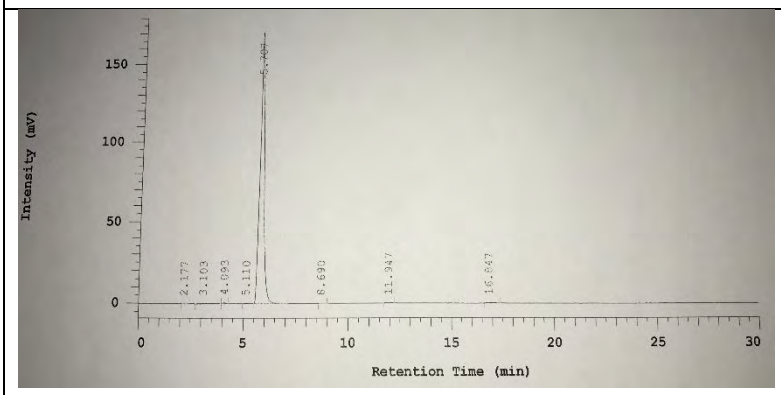
Table 5.22: Stability tests done on each sample on day one.

Chromatograph	Description
	• Sample: AA • Solvent: HCl
	• Sample: AB • Solvent: HCl
	• Sample: MB • Solvent: HCl
	• Sample: Thionin • Solvent: HCl

	• Sample: AB • Solvent: NaOH
	• Sample: MB • Solvent: NaOH
	• Sample: Thionin • Solvent: NaOH
	• Sample: AA • Solvent: H_2O_2

	• Sample: AB • Solvent: H_2O_2
	• Sample: MB • Solvent: H_2O_2
	• Sample: Thionin • Solvent: H_2O_2
	• Sample: AA • Solvent: H_2O

	• Sample: AB • Solvent: H_2O
	• Sample: MB • Solvent: H_2O
	• Sample: Thionin • Solvent: H_2O
	• Sample: AA • Solvent: MP

	• Sample: AB • Solvent: MP
	• Sample: MB • Solvent: MP
	• Sample: Thionin • Solvent: MP

From the chromatograms presented in table 5.22, it is evident that HCl, NaOH and H_2O_2 are not suitable solvents for either of the actives as no peaks could be identified due to significant degradation within the respective solvents. However, for the samples that were dissolved in MP and water, peaks could be identified one day one, two, three and seven as well as after exposure to more certain temperatures. It has been concluded that azure A, azure B and methylene blue undergo extreme degradation in high acidic and/or alkaline solvents. Slight degradation was observed to when the analytes were dissolved in water. The least degradation was observed when the analytes were dissolved in MP. Generally, when dissolved in MP or water, the analytes were able to withstand the influence of moderate temperature changes. In tables 5.23-5.28 are the average peak areas and % RSD given for each compound, after being analysed in water and MP respectively.

Table 5.23: Average peak area and %RSD of azure A samples dissolved in water.

	Average peak area of 2 injections	RSD (%)
Day 1	729324	5,98
Day 2	742778	1,52
Day 3	702839	2,04
Day 7	568741	6,57
0 ºC (after 7 days)	705292	2,48
Sun exposure (after 7 days)	651461	6,25

Table 5.24: Average peak area and %RSD of azure A samples dissolved in mobile phase.

	Average peak area of 2 injections	RSD (%)
Day 1	1321027	1,09
Day 2	1319610	0,32
Day 3	1323622	1,18
Day 7	1327371	1,98
0 ºC (after 7 days)	1261336	0,16
Sun exposure (after 7 days)	1117114	0,87

Table 5.25: Average peak area and %RSD of azure B samples dissolved in water.

	Average peak area of 2 injections	RSD (%)
Day 1	2519081	1,09
Day 2	2663306	2,03
Day 3	2491956	1,03
Day 7	2110183	2,83
0 ºC (after 7 days)	2593160	0,34
Sun exposure (after 7 days)	2406830	2,06

Table 5.26: Average peak area and %RSD of azure B samples dissolved in mobile phase.

	Average peak area of 2 injections	RSD (%)
Day 1	3707858	0,81
Day 2	3679663	0,92
Day 3	3677264	0,66
Day 7	3650351	1,04
0 ºC (after 7 days)	3753901	0,84
Sun exposure (after 7 days)	3534683	0,85

Table 5.27: Average peak area and %RSD of methylene blue samples dissolved in water.

	Average peak area of 2 injections	RSD (%)
Day 1	217474	9,88
Day 2	2124820	3,42
Day 3	2313052	2,19
Day 7	2237813	2,49
0 ºC (after 7 days)	2282540	1,22
Sun exposure (after 7 days)	2005755	1,18

Table 5.28: Average peak area and %RSD of methylene blue samples dissolved in mobile phase.

	Average peak area of 2 injections	RSD (%)
Day 1	3770308	1,19
Day 2	3731458	1,01
Day 3	3703112	1,10
Day 7	3702861	1,33
0 ºC (after 7 days)	3717700	0,98
Sun exposure (after 7 days)	3170165	0,31

From the data it is evident that methylene blue and azure B remained stable when dissolved in water or MP. For azure A it was noted that smaller peak areas were after 7 days when dissolved in water and not kept in a refrigerator. When dissolved in MP smaller peak areas were observed only where the samples were left exposed on the bench.

5.10.8. Conclusion

The analytical method is able to separate MB and its metabolites. The method was successfully validated and acceptable results were obtained. A summary of these results are presented in tables 5.29 and 5.30.

Table 5.29: Summary of results obtained during the validation of methylene blue.

Parameter	**Results obtained**
Specificity	• No solvent interference was detected. • Different metabolites contaminating MB had some interference, but MB could easily be identified by spiking the samples.
Linearity	R square > 0,998 achieved for all
Range	10-500 µg/ml
Repeatability	%RSD < 2% achieved across the working range
Robustness	
Flow rate	0.650 ml/min
Mobile phase	60:40 part A (5 mM ammonium acetate dissolved in 10:90 water-methanol) to part B (15:25 acetonitrile-methanol)
HPLC system	System 1 was able to detect peaks at 0,1 µg/ml, that system 2 was not able to detect.
Product analyses	Acceptable results of two other standards from different suppliers.
Column	The Synergi polar-RP column is the only column that separated the metabolites with acceptable precision and accuracy.
Recovery	Recovery was variable over the concentration ranges and may be attributed to the impurity profiles.

Table 5.30: Summary of results obtained during the validation of related substances.

	Results obtained				
Parameter	**Methylene blue 1**	**Methylene blue 2**	**Azure A**	**Azure B**	**Thionin**
Linearity (r^2)	0,999	0,999	0,999	0,999	0,998
Range (µg/ml)	10-500	10-500	10-500	10-500	10-500
Repeatability (%RSD)	< 2%	< 2%	< 2%	< 2%	< 2%
Limit of detection (µg/ml)	0,1	0,1	0,1	0,1	0,1
Limit of quantification (µg/ml)	5	5	5-6	2	N/A
Recovery (%)	90-125% across the 50-500 µg/ml range		Not determined		

CHAPTER 6 - CONCLUSION

6.1. Introduction

As mentioned in chapter 1, the growing interest of methylene blue (MB) as a potential treatment and prevention of Alzheimer's disease have led to a large number of new studies developed for the determination of the exact effects of MB (Gura, 2008; Sullivan, 2008; Wischik *et al*., 2008). A lot of research has been done on the advantages of using MB not only for AD, but also for a variety of other illnesses and disorders. However, recently it has been proven that azure B have superior effects in comparison to MB (Buchholz *et al*., 2008; Petzer *et al*., 2012; Petzer *et al*., 2014; Taniguchi *et al*., 2005; Wischik *et al*., 1996). During earlier studies, scientists did not know about the existence of azure B and the other metabolites (Shirmer *et al.,* 2011; Warth *et al.,* 2009). Therefore, all pharmacological effects observed in their studies were regarded as the effects of MB (Bruchey & Gonzales-Lima, 2008; Shirmer *et al*., 2011). After the discovery of azure B, many attempts have been made to develop analytical methods in order to analyse and separate these compounds. However, the structural similarity between these compounds complicated the development process and as a result only a few methods are made available and published (Kim *et al*., 2014; Schirmer *et al*., 2011). These methods all suffer from great disadvantages such as low sensitivity, uneconomically high costs, the need of professionally trained personnel and very expensive, high technology apparatus (Kim *et al*., 2014; Warth *et al*., 2009). The lack of sensitive, reliable, and easy to implement analytical methods, have delayed and limited further research on key aspects regarding these compounds. The impurity of commercially available MB and azure B batches have caused misleading and inaccurate conclusions that were made during previous studies on the pharmacological effects of these compounds (Bruchey & Gonzales-Lima, 2008; Shirmer *et al*., 2011). The fact that these batches were contaminated with the metabolites of MB, were just recently discovered and the amount of contamination remains unknown (Shirmer *et al*., 2011). Azure A has recently been removed from the market due to the inaccurate stated purity given with each batch. Also, azure C products are not available for similar reasons. This led to the need to develop and validate accurate and cost effective chromatographic methods that can be used for the analysis and separation of MB and its metabolites.

6.2. Findings and conclusions.

During this study, reverse phase and normal phase thin layer chromatography (TLC) methods have successfully been developed by which separation of MB and its metabolites have been accomplished. Also, successful separation was observed with high performance liquid

chromatography (HPLC) methods using two different types of HPLC columns, namely a Synergi polar-RP and a Luna phenyl-hexyl colum. In both analytical methods (TLC and HPLC), each analyte could be identified within samples that contained a mixture of these metabolites. The chosen HPLC method was fully validated and acceptable results regarding its accuracy, precision, stability and repeatability were obtained. In table 6.1 a summary of the findings regarding the metabolites that were present in each sample, is presented.

Table 6.1: Summary of the findings in the current study regarding the purity of the samples and with which metabolites they are contaminated.

Sample	Contamination	Manufacturer	Given purity (%)
Methylene blue (primary standard)	• Moderate amount of azure B • Small amount of azure A	Sigma-Aldrich	95%
Methylene blue 1	• Moderate amount of azure B • Small amount of azure A	Merck	Not indicated
Methylene blue 2	• Moderate amount of azure B • Small amount of azure A	Accociated Chemical Enterprises (ace)	98,5%
Azure B	• Small amount of azure A • Very small amount of azure C (possibly)	Sigma-Aldrich	Not indicated
Azure A	• Significant amounts of azure B and methylene blue • Moderate amount of azure C (possibly)	Sigma-Aldrich	Not indicated
Thionin	• Pure	Sigma-Aldrich	Not indicated

6.3. Recommendations for future studies

1. The HPLC method together with fraction collection may be utilised to purify commercially available MB, Azure A and Azure B.
2. The identification of Azure C as another related substance/metabolite should be investigated.
3. Clarification on the activity/ies of the individual metabolites should be investigated.
4. Further method development should be investigated to separate the double peak that occurs with azure A, and to be able to identify the peak.

CHAPTER 7 - REFERENCE LIST

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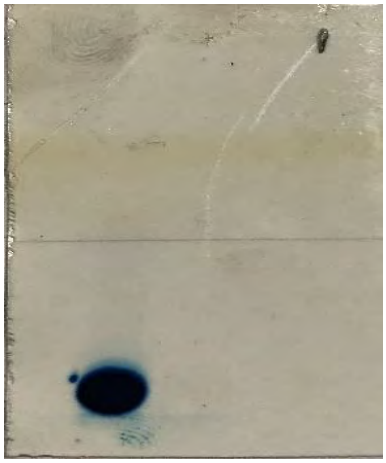
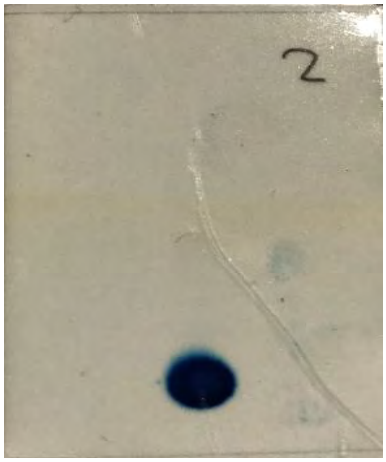
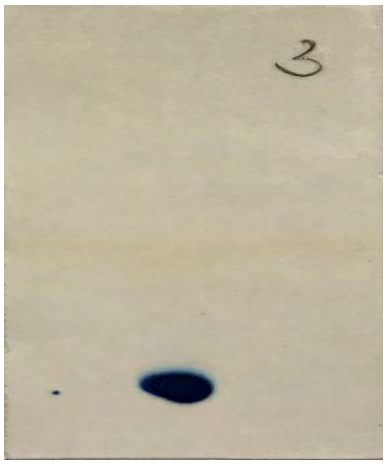
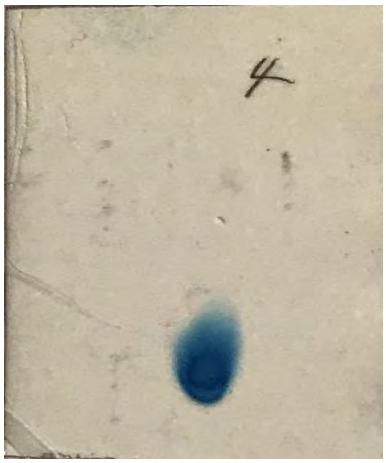
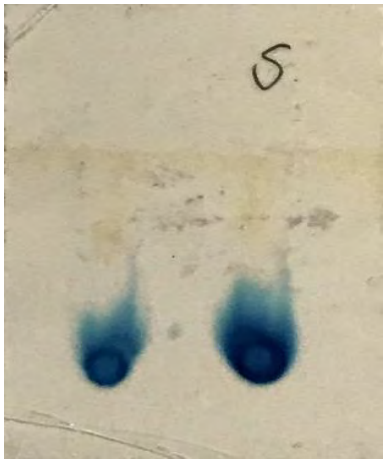
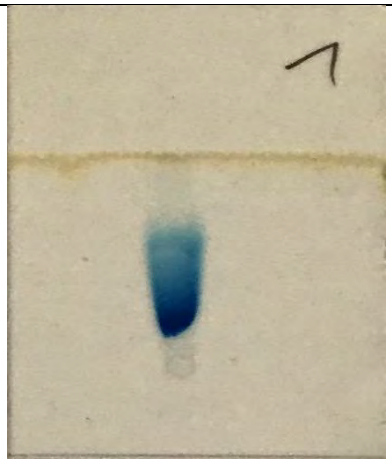
S.D.G

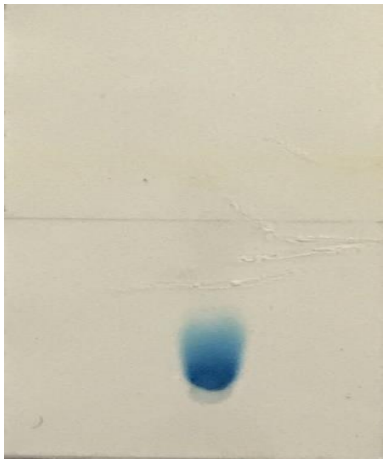
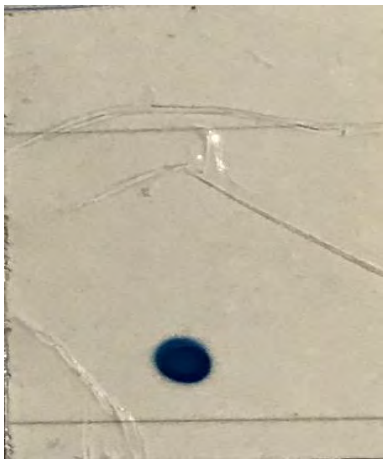
ADDENDUM A

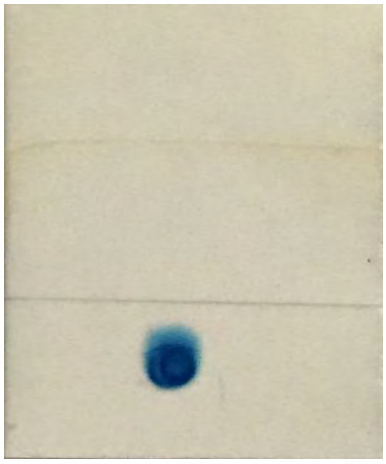
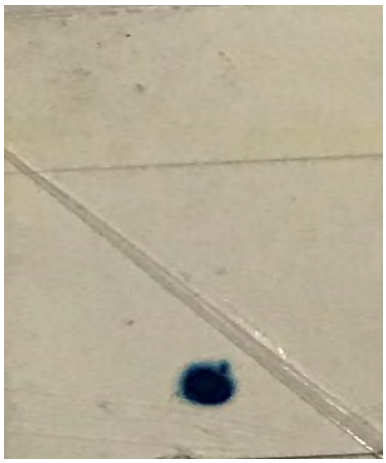
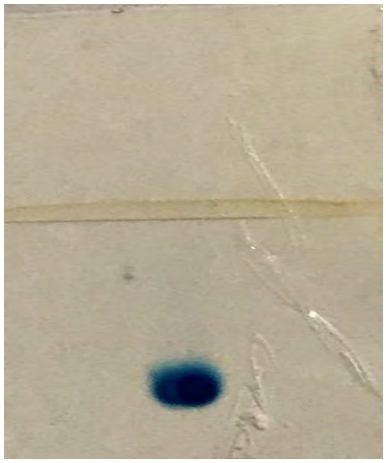
Table 7.1: Normal phase TLC (unknown sample concentration).

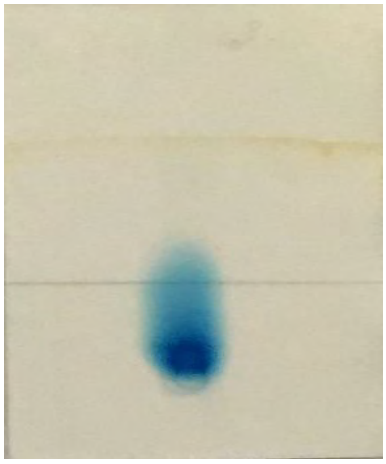
TLC plate	Description
	• Sample: MB • Solvent: ethanol • MP: 60:40 ethyl-acetate to ethanol
	• Sample: MB • Solvent: methanol • MP: 60:40 dichloromethane to ethyl-acetate

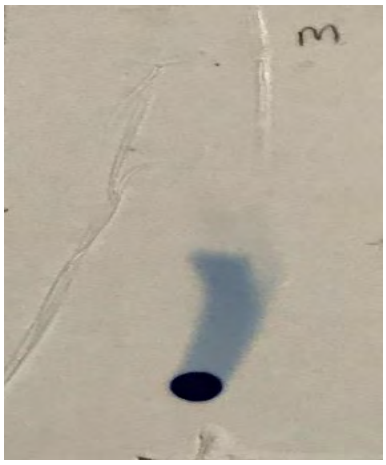
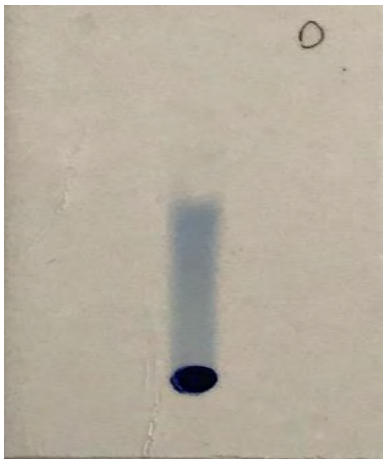
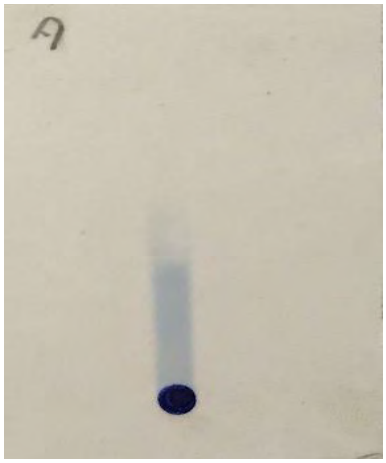
	• Sample: MB • Solvent: ethanol • MP: 60:40 dichloromethane to ethyl-acetate
	• Sample: MB • Solvent: methanol • MP: 50:50 dichloromethane to acetonitrile
	• Sample: MB • Solvent: methanol • MP: 3:1 acetonitrile to dichloromethane

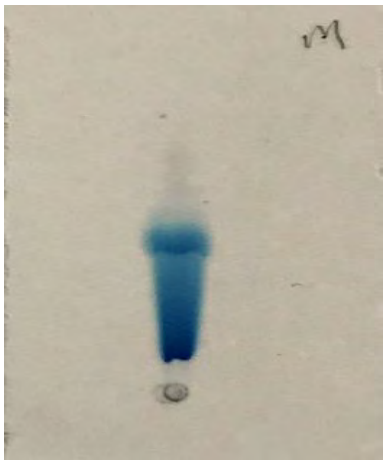
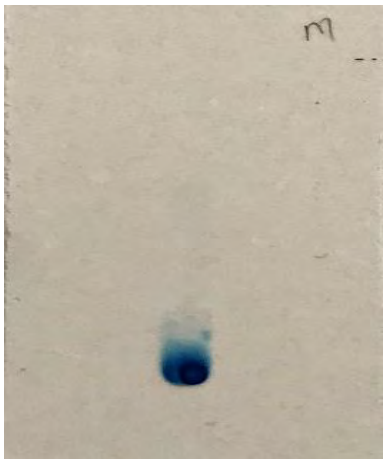
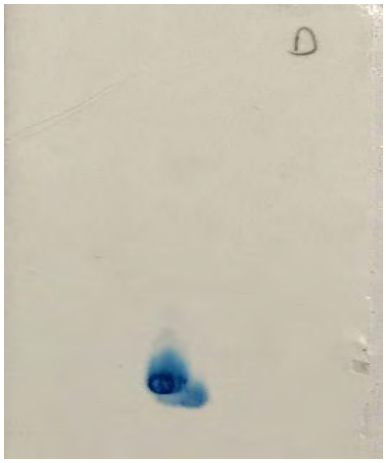
	• Sample: MB • Solvent: methanol • MP: 3:1:2 acetonitrile to dichloromethane to propanol
	• Sample: MB • Solvent: methanol • MP: 2:1 dichloromethane to methanol
	• Sample: MB • Solvent: methanol • MP: 2:1:1 dichloromethane to methanol to acetonitrile

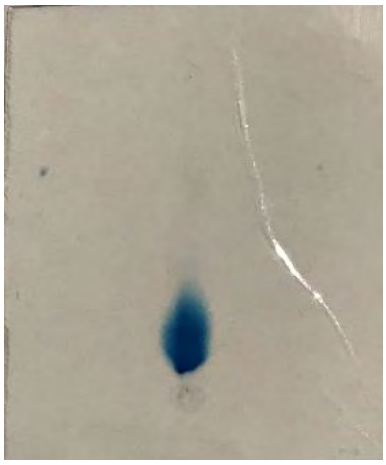
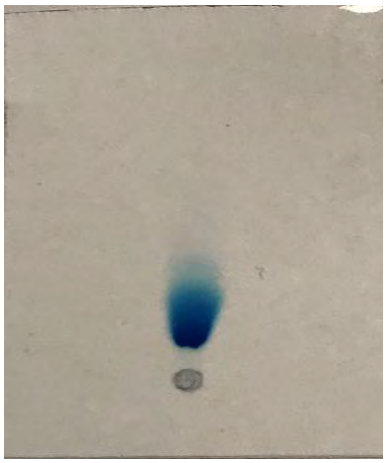
	• Sample: MB • Solvent: methanol • MP: 2:1:1:1 dichloromethane to methanol to acetonitrile to ethyl-acetate
	• Sample: MB • Solvent: methanol • MP: 1:1 petroleum-ether to dichloromethane
	• Sample: MB • Solvent: methanol • MP: 1:1:1 petroleum-ether to dichloromethane to acetonitrile

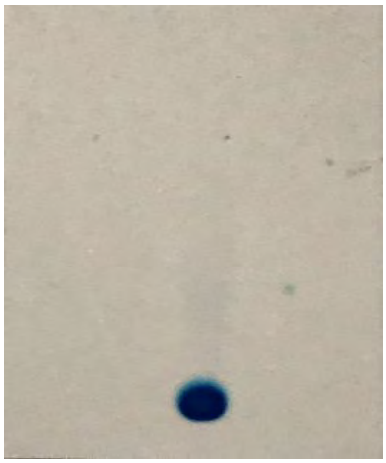
	• Sample: MB • Solvent: methanol • MP: 2:1 acetonitrile to acetone
	• Sample: MB • Solvent: methanol • MP: 1:1 acetonitrile to ethyl-acetate
	• Sample: MB • Solvent: methanol • MP: 2:1 acetonitrile to ethanol

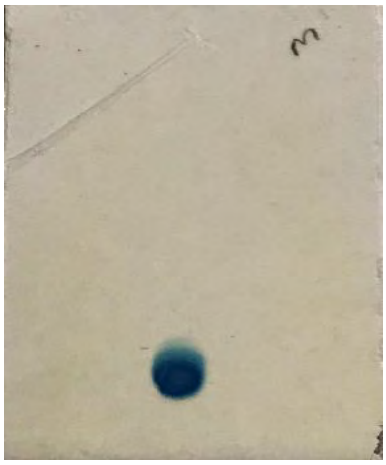
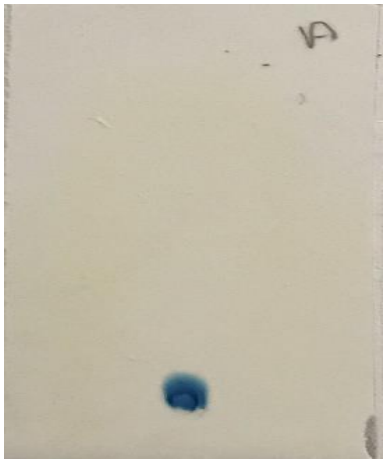
	• Sample: MB • Solvent: methanol • MP: 2:1:1 acetonitrile to ethanol to dichloromethane
	• Sample: MB • Solvent: methanol • MP: 1:1 dichloromethane to ethanol
	• Sample: MB • Solvent: methanol • MP: 1:1 dichloromethane to ethanol

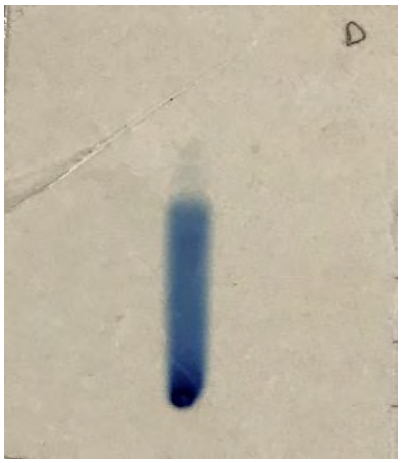
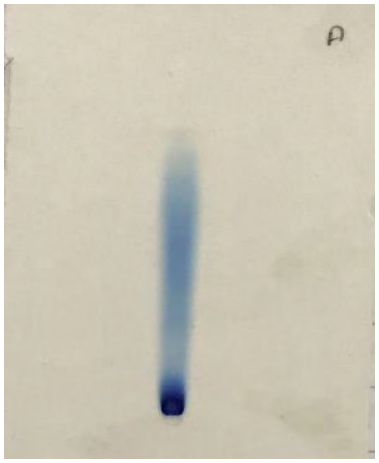
	• Sample: MB • Solvent: methanol • MP: 5:3 dichloromethane to trimethylamine
	• Sample: MB • Solvent: dichloromethane • MP: 5:3 dichloromethane to trimethylamine
	• Sample: MB • Solvent: acetonitrile • MP: 5:3 dichloromethane to trimethylamine

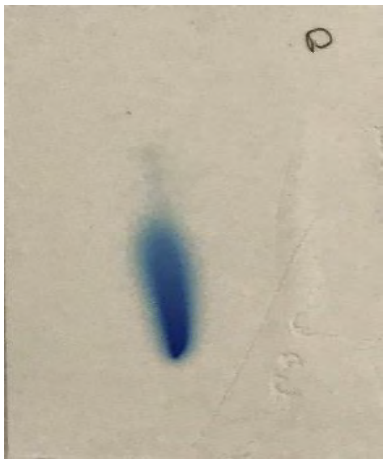
	• Sample: MB • Solvent: methanol • MP: 1:1 dichloromethane to methanol
	• Sample: MB • Solvent: methanol • MP: 1:1:1 dichloromethane to methanol to ethyl-acetate
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 dichloromethane to methanol to ethyl-acetate

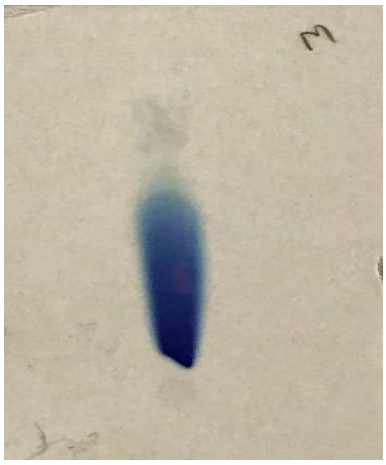
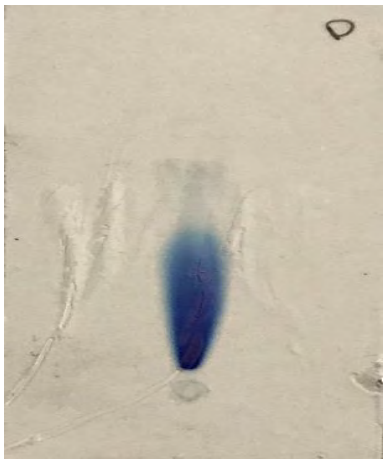
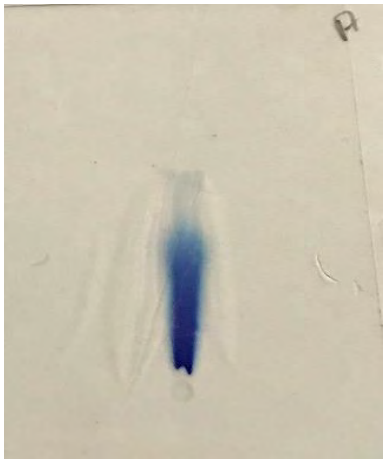
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 dichloromethane to methanol to petroleum-ether
	• Sample: MB • Solvent: methanol • MP: 1:1:1 dichloromethane to methanol to petroleum-ether
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 dichloromethane to methanol

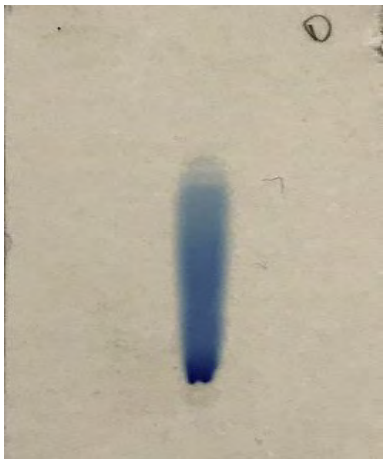
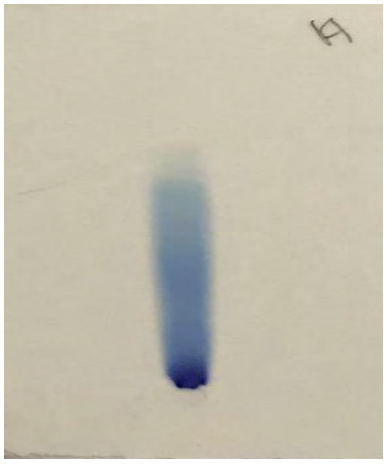
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 acetonitrile to methanol
	• Sample: MB • Solvent: methanol • MP: 1:1 acetonitrile to methanol
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 petroleum-ether to methanol

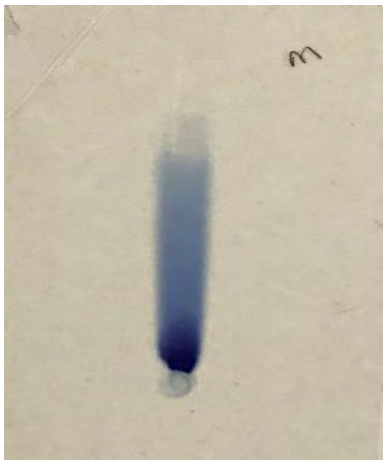
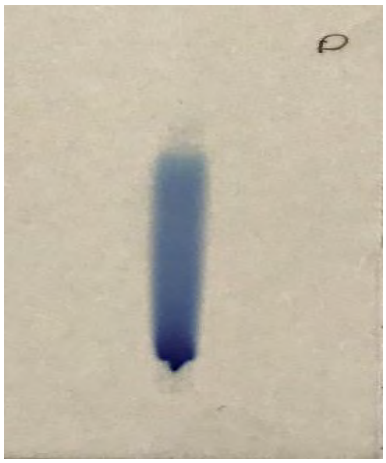
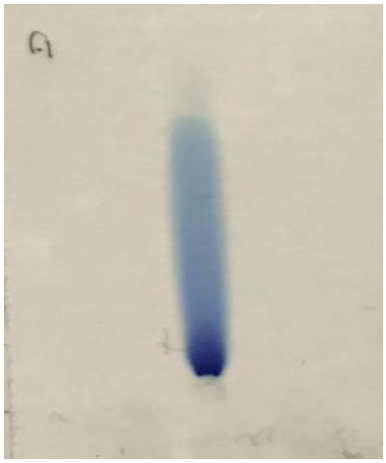
	• Sample: MB • Solvent: methanol • MP: 5:8 dichloromethane to tetrahydrofuran
	• Sample: MB • Solvent: dichloromethane • MP: 5:8 dichloromethane to tetrahydrofuran
	• Sample: MB • Solvent: acetonitrile • MP: 5:8 dichloromethane to tetrahydrofuran

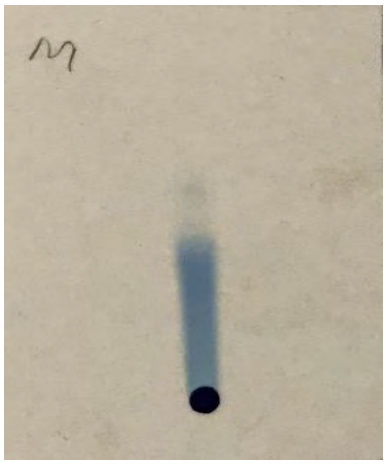
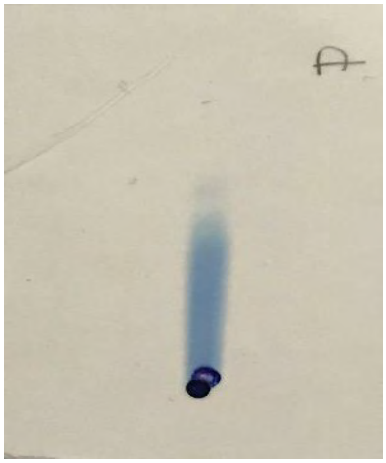
	• Sample: MB • Solvent: methanol • MP: 1:1 methanol to tri-ethylamine
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 methanol to tri-ethylamine
	• Sample: MB • Solvent: acetonitrile • MP: 1:1 methanol to tri-ethylamine

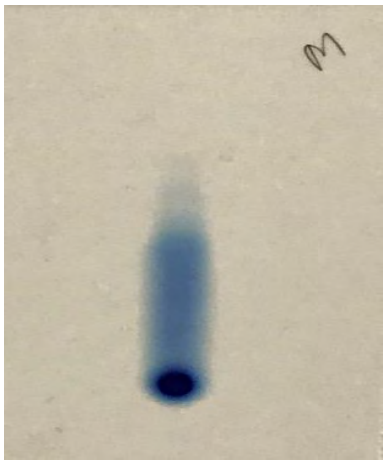
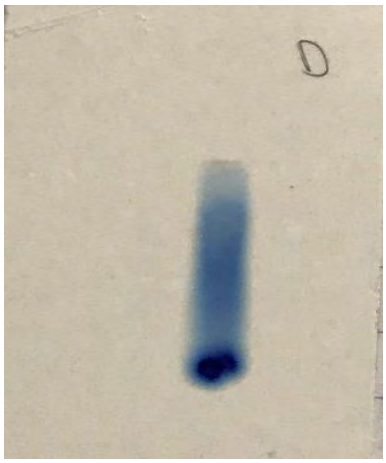
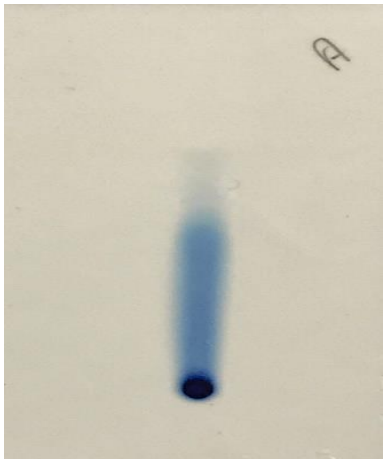
	• Sample: MB • Solvent: methanol • MP: 1:1:1 methanol to trimethylamine to tri-sodium citrate hydrate
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 methanol to trimethylamine to tri-sodium citrate hydrate
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 methanol to trimethylamine to tri-sodium citrate hydrate

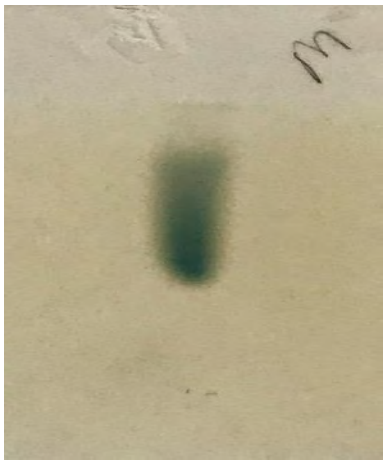
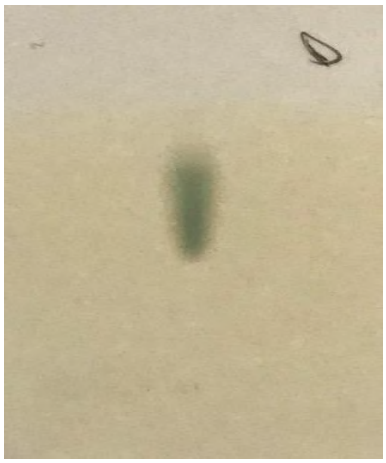
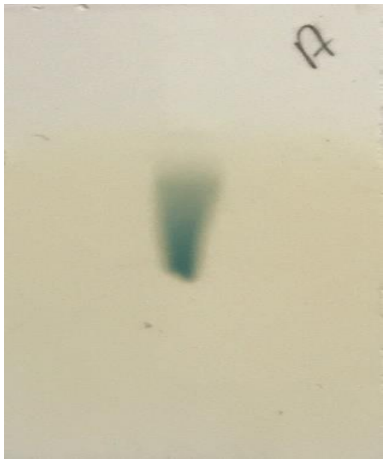
	• Sample: MB • Solvent: methanol • MP: 1:1:1:1 methanol to trimethylamine to tri-sodium citrate hydrate to acetonitrile
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1:1 methanol to trimethylamine to tri-sodium citrate hydrate to acetonitrile
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1:1 methanol to trimethylamine to tri-sodium citrate hydrate to acetonitrile

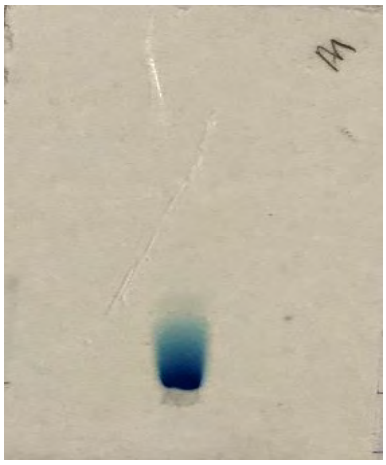
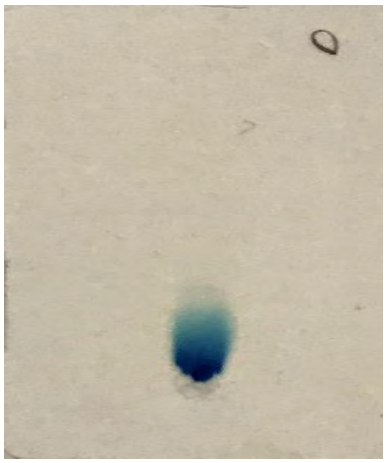
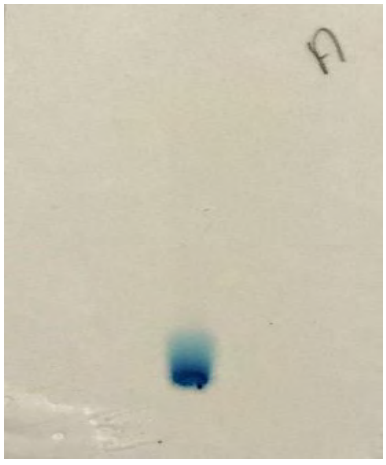
	• Sample: MB • Solvent: methanol • MP: 1:1:1 methanol to tri-methylamine to dichloromethane
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 methanol to tri-methylamine to dichloromethane
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 methanol to tri-methylamine to dichloromethane

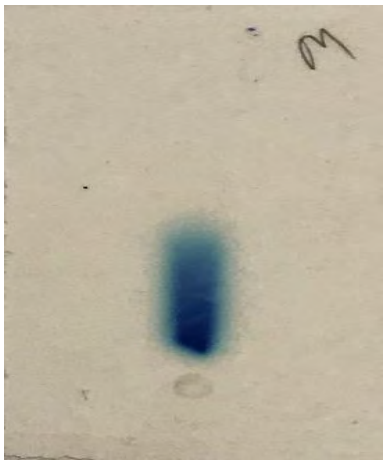
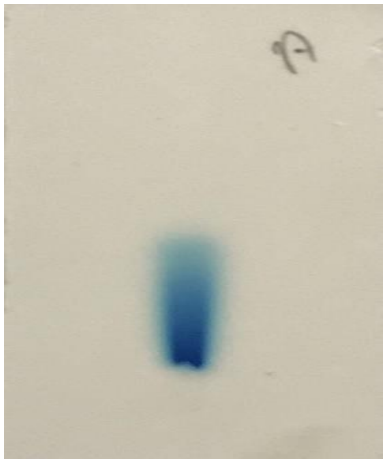
	• Sample: MB • Solvent: methanol • MP: 1:1:1:1 methanol to tri-methylamine to dichloromethane to acetonitrile
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1:1 methanol to tri-methylamine to dichloromethane to acetonitrile
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1:1 methanol to tri-methylamine to dichloromethane to acetonitrile

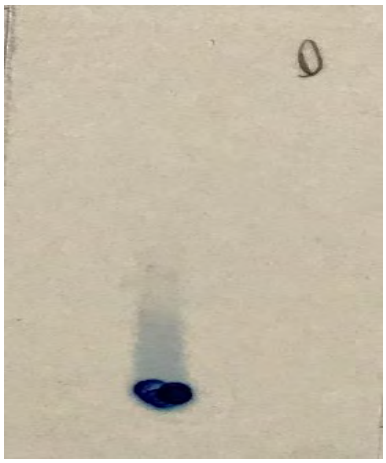
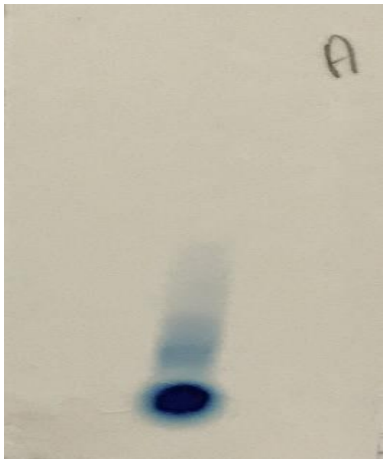
	• Sample: MB • Solvent: methanol • MP: 1:1 trimethylamine to propanol
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 trimethylamine to propanol
	• Sample: MB • Solvent: acetonitrile • MP: 1:1 trimethylamine to propanol

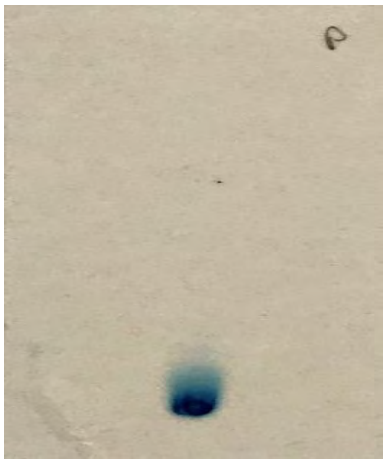
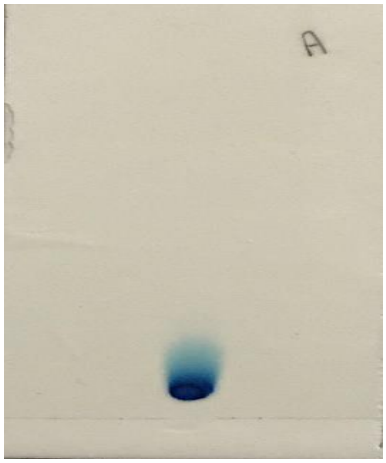
	• Sample: MB • Solvent: methanol • MP: 1:1:1 trimethylamine to propanol to methanol
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 trimethylamine to propanol to methanol
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 trimethylamine to propanol to methanol

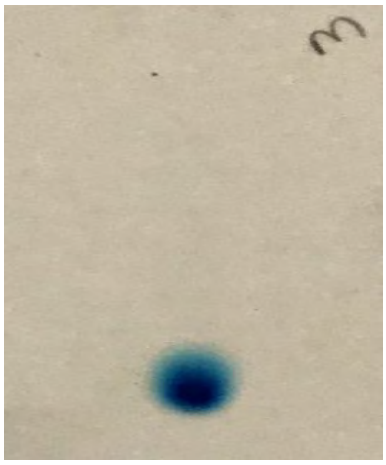
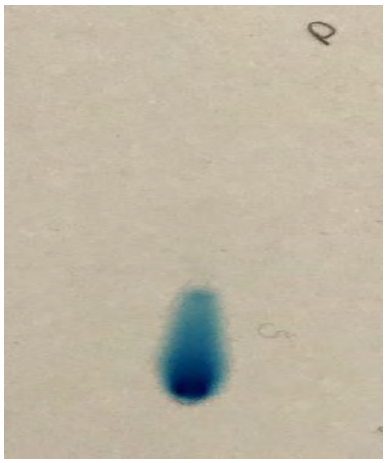
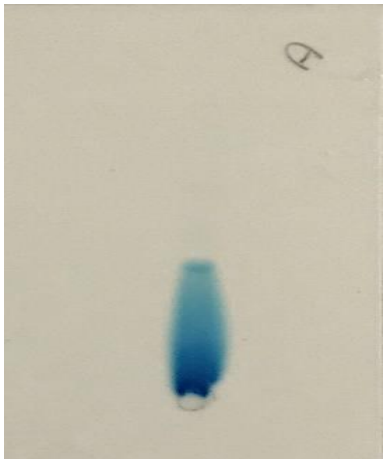
	• Sample: MB • Solvent: methanol • MP: 1:1:1 trimethylamine to methanol to tetrahydrofuran
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 trimethylamine to methanol to tetrahydrofuran
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 trimethylamine to methanol to tetrahydrofuran

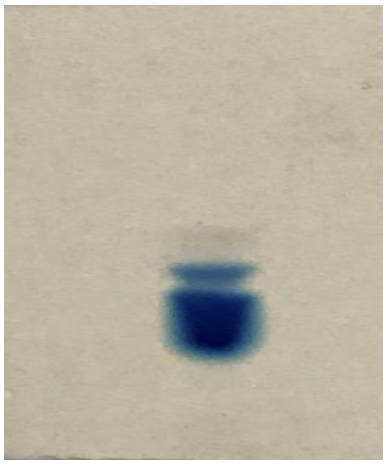
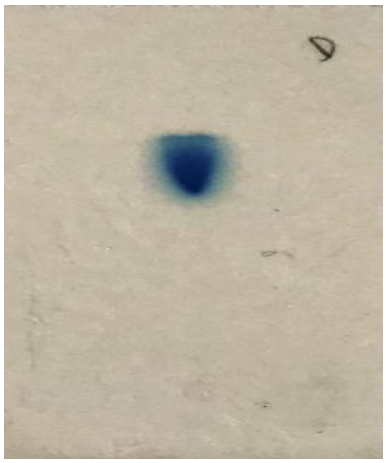
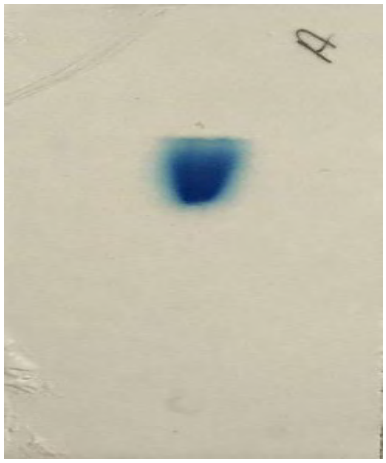
	• Sample: MB • Solvent: methanol • MP: 1:1 ammonium acetate to methanol
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 ammonium acetate to methanol
	• Sample: MB • Solvent: acetonitrile • MP: 1:1 ammonium acetate to methanol

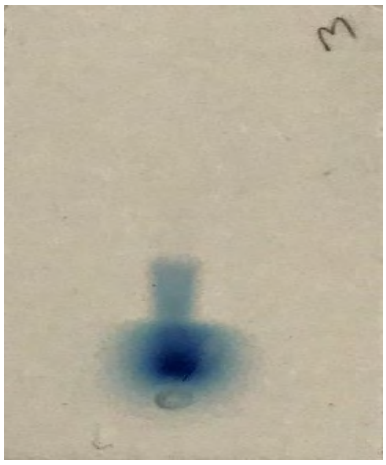
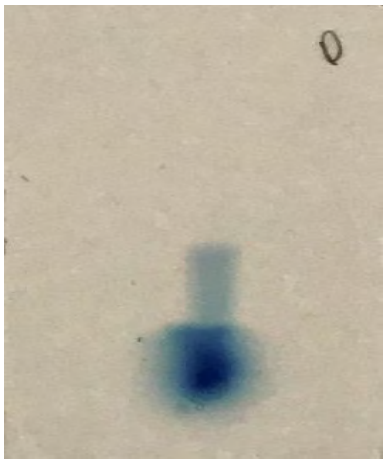
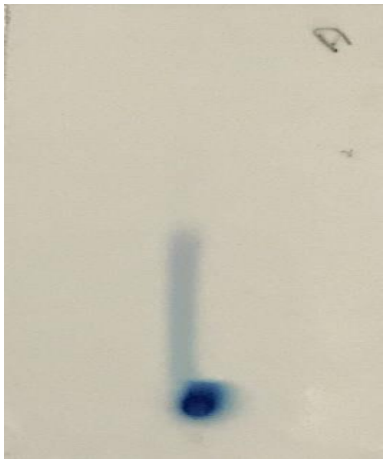
	• Sample: MB • Solvent: methanol • MP: 1:1:1 ammonium acetate to methanol to dichloromethane
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 ammonium acetate to methanol to dichloromethane
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 ammonium acetate to methanol to dichloromethane

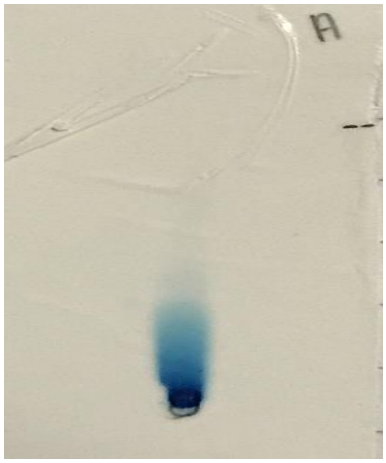
	• Sample: MB • Solvent: methanol • MP: 1:1 ammonium acetate to tri-ethylamine
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 ammonium acetate to trimethylamine
	• Sample: MB • Solvent: acetonitrile • MP: 1:1 ammonium acetate to tri-ethylamine

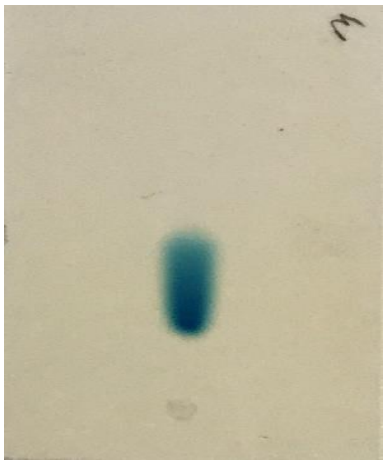
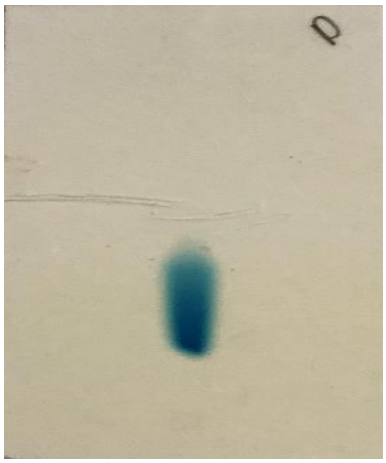
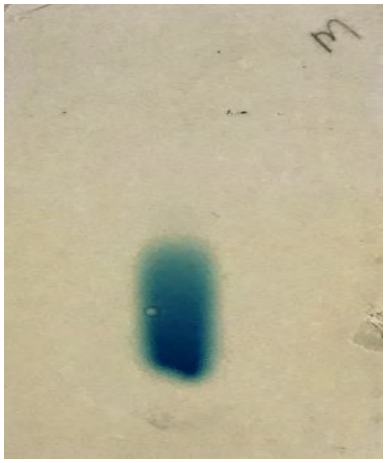
	• Sample: MB • Solvent: methanol • MP: 1:2:4 ammonium acetate to water to methanol
	• Sample: MB • Solvent: dichloromethane • MP: 1:2:4 ammonium acetate to water to methanol
	• Sample: MB • Solvent: acetonitrile • MP: 1:2:4 ammonium acetate to water to methanol

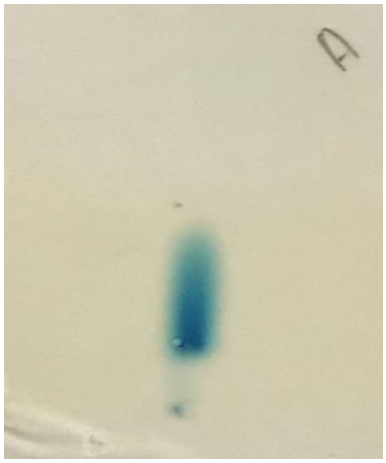
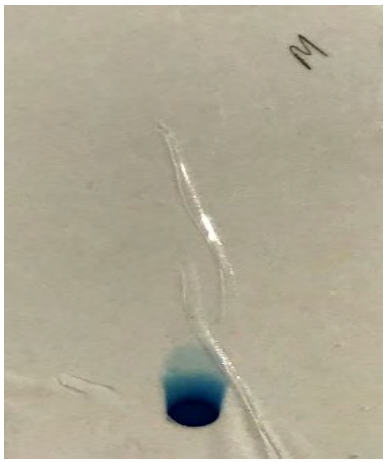
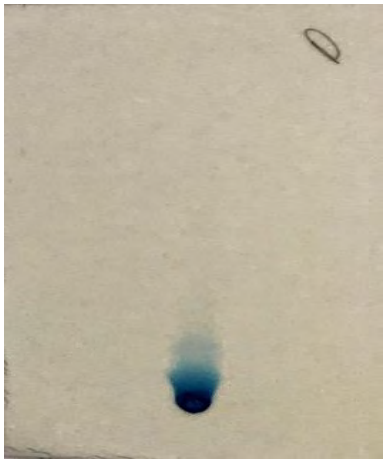
	• Sample: MB • Solvent: methanol • MP: 1:1 ammonium acetate to acetonitrile
	• Sample: MB • Solvent: dichloromethane • MP: 1:1 ammonium acetate to acetonitrile
	• Sample: MB • Solvent: acetonitrile • MP: 1:1 ammonium acetate to acetonitrile

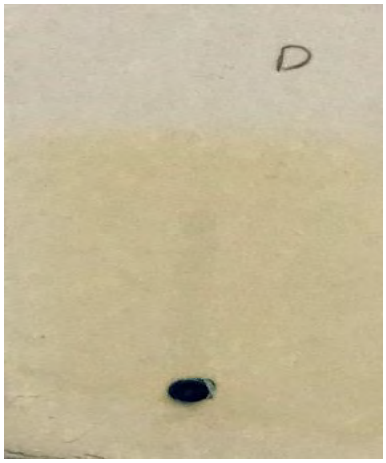
	• Sample: MB • Solvent: methanol • MP: 1:1:1 ammonium acetate to acetonitrile to tri-ethylamine
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 ammonium acetate to acetonitrile to tri-ethylamine
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 ammonium acetate to acetonitrile to tri-ethylamine

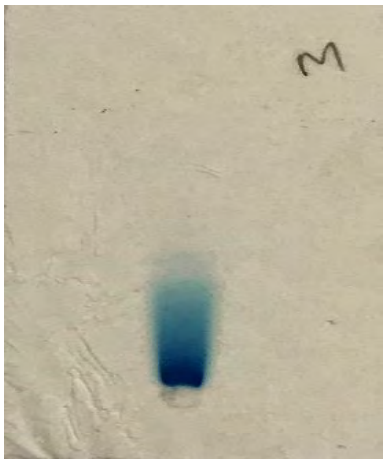
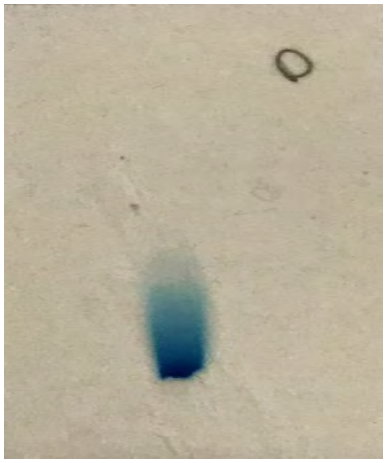
	• Sample: MB • Solvent: methanol • MP: 1:1:1 ammonium acetate to dichloromethane to tri-ethylamine
	• Sample: MB • Solvent: dichloromethane • MP: 1:1:1 ammonium acetate to dichloromethane to tri-ethylamine
	• Sample: MB • Solvent: acetonitrile • MP: 1:1:1 ammonium acetate to dichloromethane to tri-ethylamine

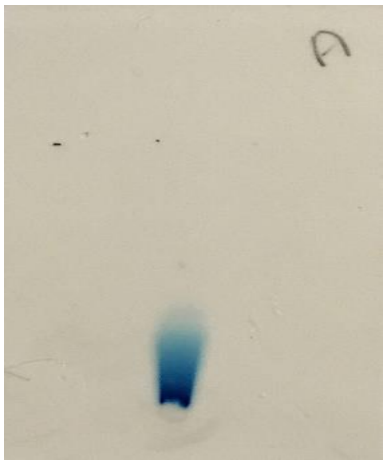
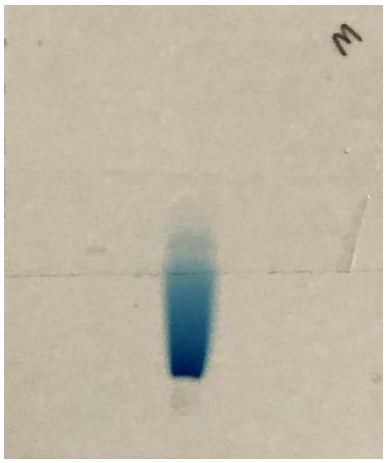
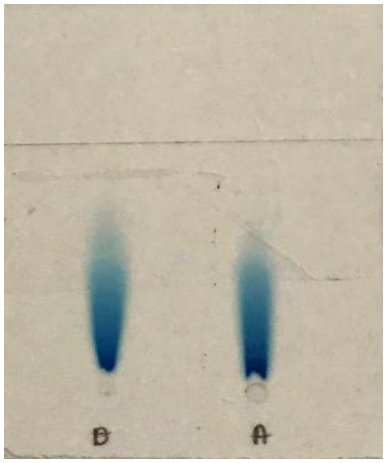
	• Sample: MB • Solvent: methanol • MP: 1:2 ammonium acetate to methanol
	• Sample: MB • Solvent: dichloromethane • MP: 1:2 ammonium acetate to methanol
	• Sample: MB • Solvent: acetonitrile • MP: 1:2 ammonium acetate to methanol

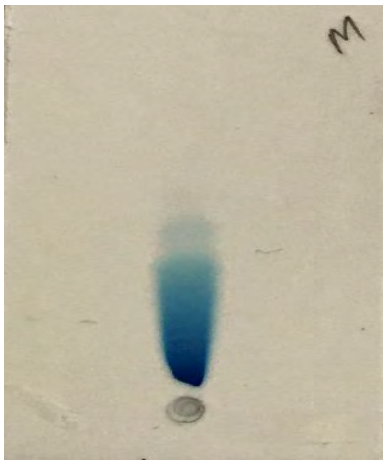
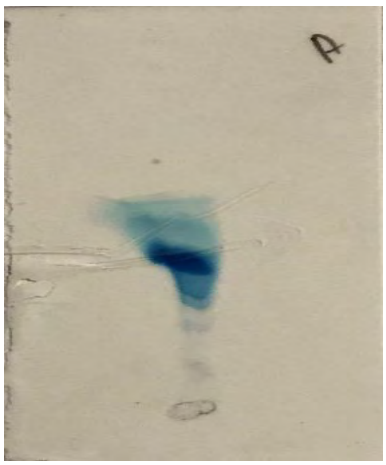
	• Sample: MB • Solvent: methanol • MP: ammonium acetate, methanol, acetonitrile, propanol and tetrahydrofuran in equal quantities
	• Sample: MB • Solvent: dichloromethane • MP: ammonium acetate, methanol, acetonitrile, propanol and tetrahydrofuran in equal quantities
	• Sample: MB • Solvent: methanol • MP: ammonium acetate, acetonitrile, propanol, tetrahydrofuran, ethyl-acetate and sodium chloride in equal quantities

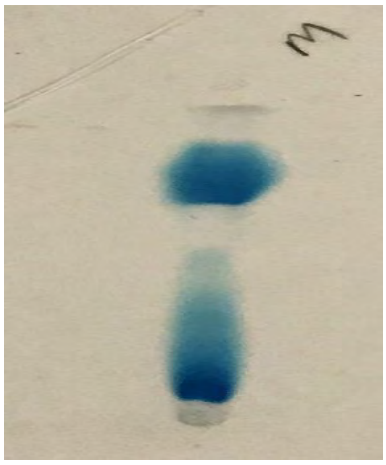
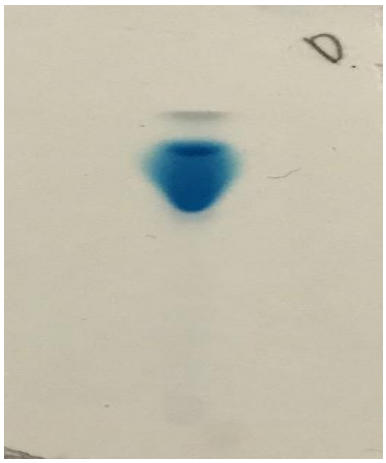
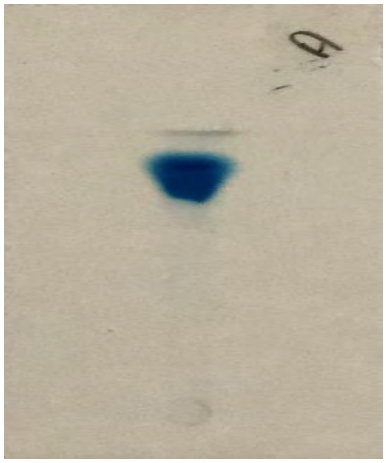
	• Sample: MB • Solvent: acetonitrile • MP: ammonium acetate, acetonitrile, propanol, tetrahydrofuran, ethyl-acetate and sodium chloride in equal quantities
	• Sample: MB • Solvent: methanol • MP: 3:3:4 ethyl-acetate to propanol to ammonium acetate
	• Sample: MB • Solvent: dichloromethane • MP: 3:3:4 ethyl-acetate to propanol to ammonium acetate

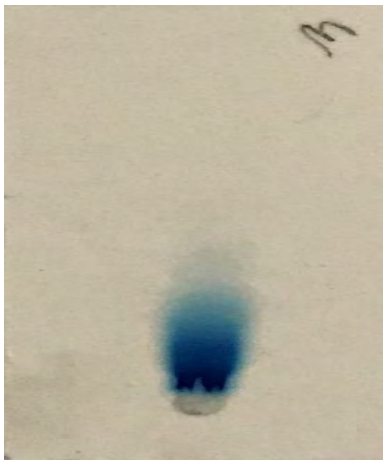
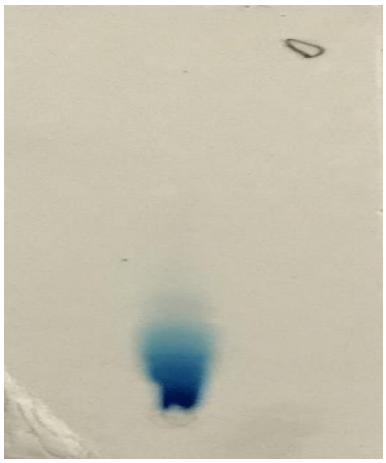
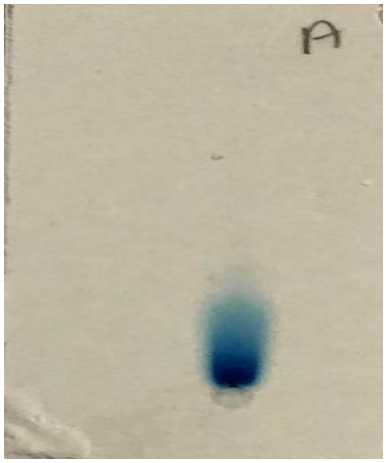
	• Sample: MB • Solvent: acetonitrile • MP: 3:3:4 ethyl-acetate to propanol to ammonium acetate
	• Sample: MB • Solvent: methanol • MP: trimethylamine, tetrahydrofuran and ammonium acetate in equal quantities
	• Sample: MB • Solvent: dichloromethane • MP: trimethylamine, tetrahydrofuran and ammonium acetate in equal quantities

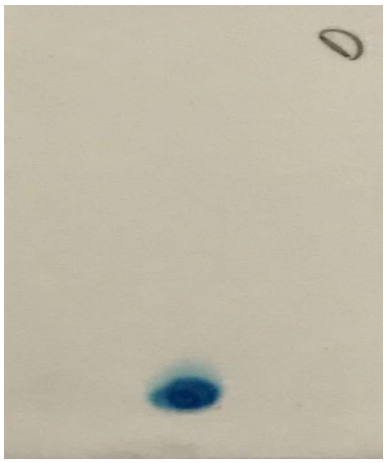
	• Sample: MB • Solvent: acetonitrile • MP: trimethylamine, tetrahydrofuran and ammonium acetate in equal quantities
	• Sample: MB • Solvent: methanol • MP: 1:3:3 propanol to acetonitrile to ammonium acetate
	• Sample: MB • Solvent: dichloromethane • MP: 1:3:3 propanol to acetonitrile to ammonium acetate

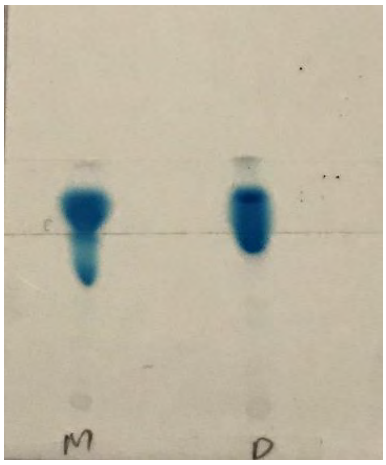
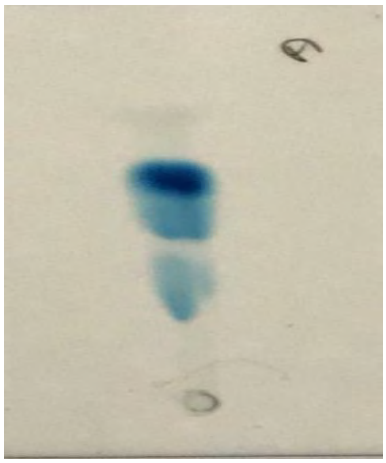
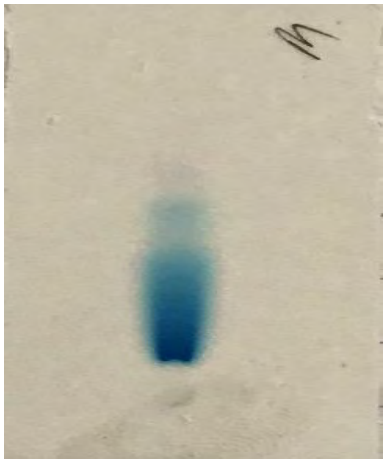
	• Sample: MB • Solvent: acetonitrile • MP: 1:3:3 propanol to acetonitrile to ammonium acetate
	• Sample: MB • Solvent: methanol • MP: ammonium acetate, sodium chloride, acetonitrile and methanol in equal quantities
	• Sample: MB • Solvent: dichloromethane (left) acetonitrile (right) • MP: ammonium acetate, sodium chloride, acetonitrile and methanol in equal quantities

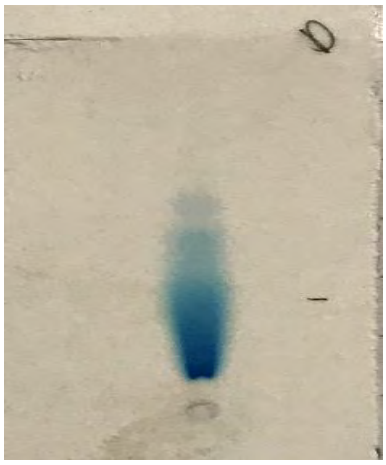
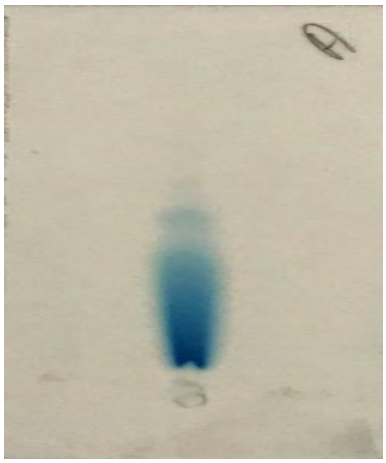
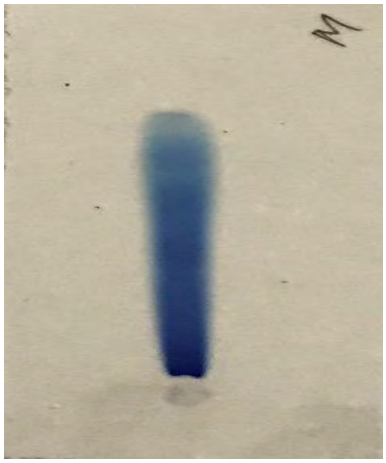
	• Sample: MB • Solvent: methanol • MP: 2:1:1 methanol to dichloromethane to ammonium acetate
	• Sample: MB • Solvent: dichloromethane • MP: 2:1:1 methanol to dichloromethane to ammonium acetate
	• Sample: MB • Solvent: acetonitrile • MP: 2:1:1 methanol to dichloromethane to ammonium acetate

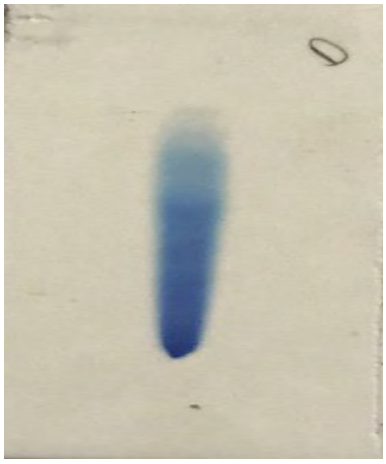
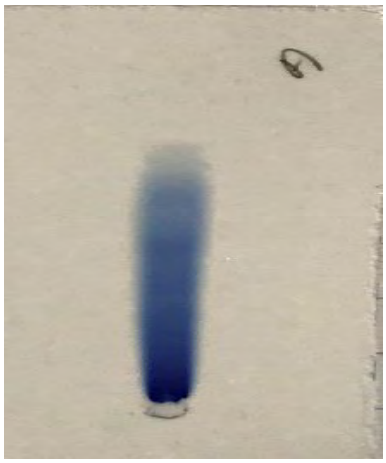
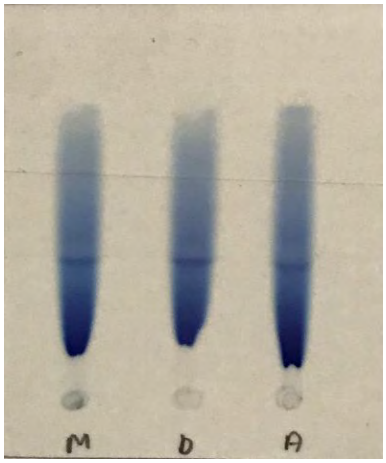
	• Sample: MB • Solvent: methanol • MP: 30:70 methanol to chloroform
	• Sample: MB • Solvent: dichloromethane • MP: 30:70 methanol to chloroform
	• Sample: MB • Solvent: acetonitrile • MP: 30:70 methanol to chloroform

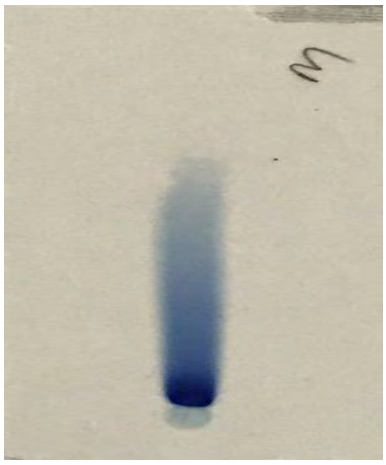
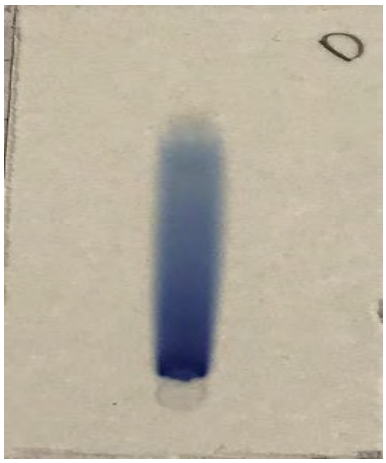
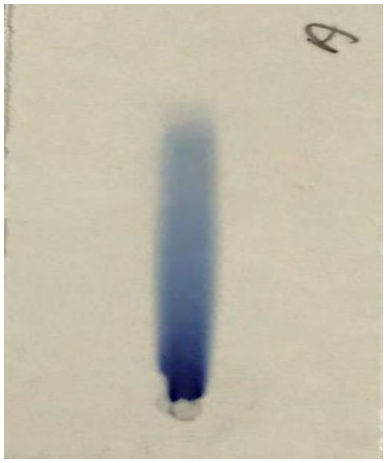
	• Sample: MB • Solvent: methanol • MP: 3:2 tetrabuthyl-ammonium-hydrogensulfate to methanol
	• Sample: MB • Solvent: dichloromethane • MP: 3:2 tetrabuthyl-ammonium-hydrogensulfate to methanol
	• Sample: MB • Solvent: acetonitrile • MP: 3:2 tetrabuthyl-ammonium-hydrogensulfate to methanol

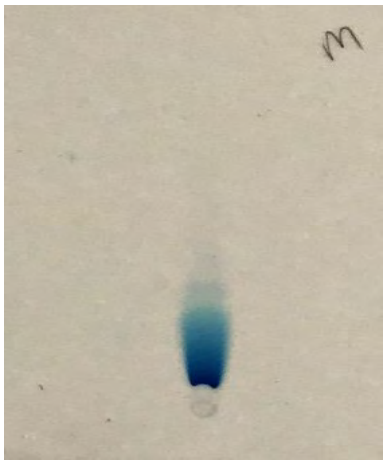
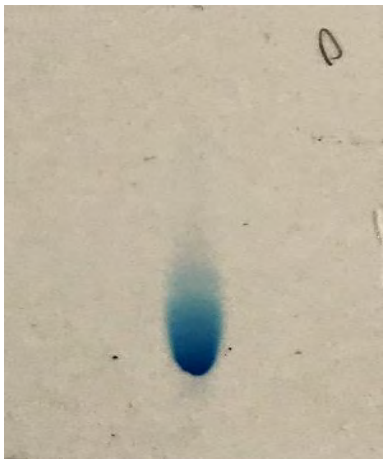
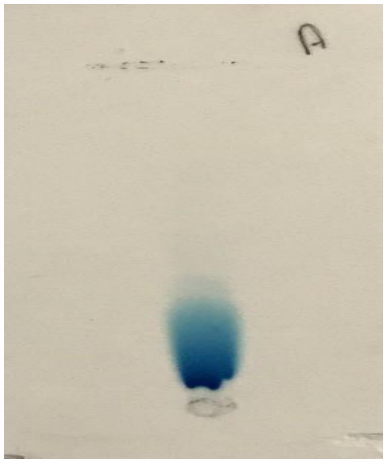
	• Sample: MB • Solvent: methanol • MP: 3:2:5 tetrabuthyl-ammonium-hydrogensulfate to methanol to chloroform
	• Sample: MB • Solvent: dichloromethane • MP: 3:2:5 tetrabuthyl-ammonium-hydrogensulfate to methanol to chloroform
	• Sample: MB • Solvent: acetonitrile • MP: 3:2:5 tetrabuthyl-ammonium-hydrogensulfate to methanol to chloroform

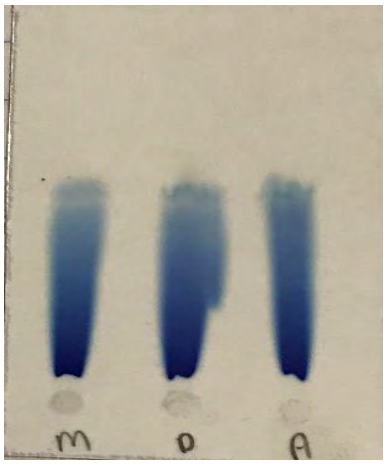
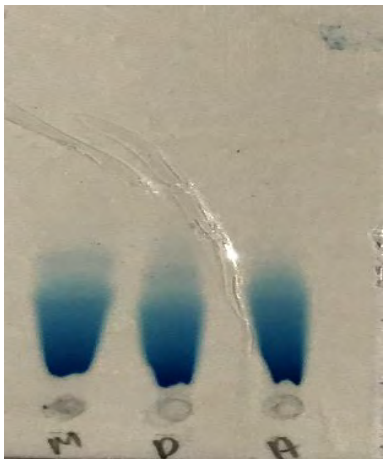
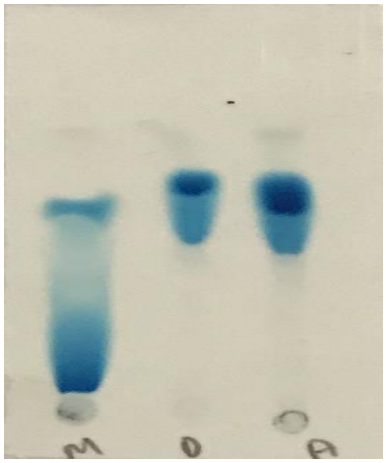
	• Sample: MB • Solvent: methanol (left) dichloromethane (right) • MP: 5:4:1 chloroform to methanol to dichloromethane
	• Sample: MB • Solvent: acetonitrile • MP: 5:4:1 chloroform to methanol to dichloromethane
	• Sample: MB • Solvent: methanol • MP: chloroform, methanol and propanol in equal quantities

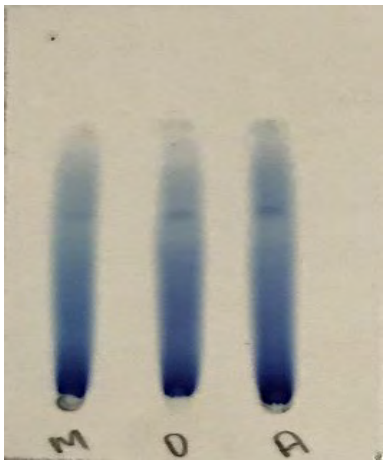
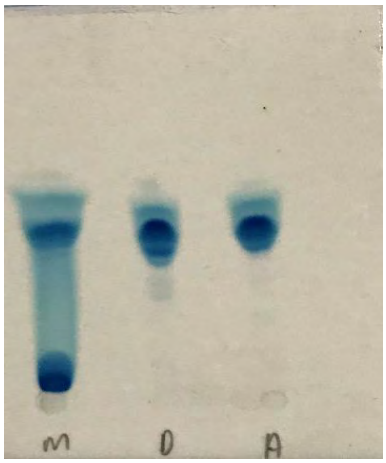
	• Sample: MB • Solvent: dichloromethane • MP: chloroform, methanol and propanol in equal quantities
	• Sample: MB • Solvent: acetonitrile • MP: chloroform, methanol and propanol in equal quantities
	• Sample: MB • Solvent: methanol • MP: 5:4:2 chloroform, methanol and tertabuthyl-ammonium-hydrogensufate

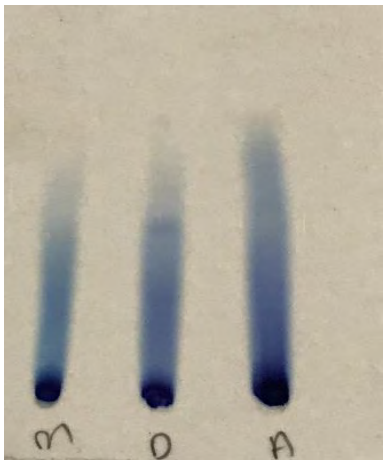
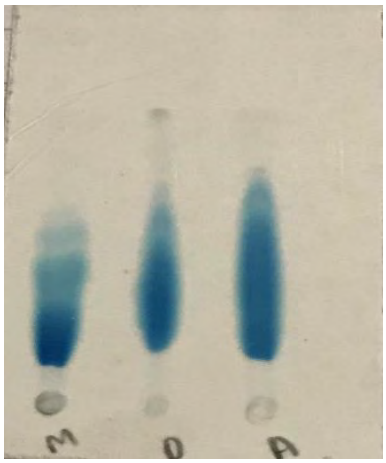
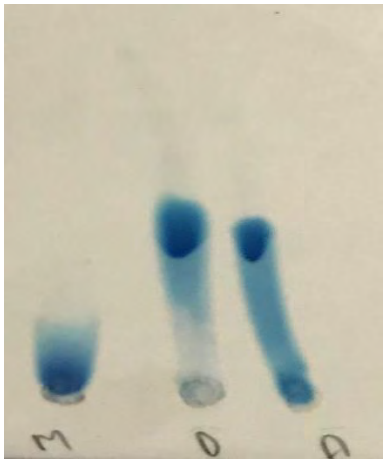
	• Sample: MB • Solvent: dichloromethane • MP: 5:4:2 chloroform, methanol and tertabuthyl-ammonium-hydrogensufate
	• Sample: MB • Solvent: acetonitrile • MP: 5:4:2 chloroform, methanol and tertabuthyl-ammonium-hydrogensufate
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 5:4:2 chloroform, methanol and tertabuthyl-ammonium-hydrogensufate

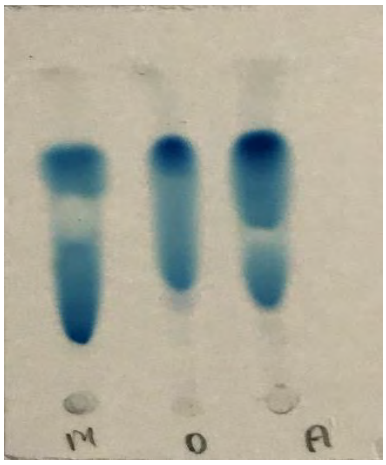
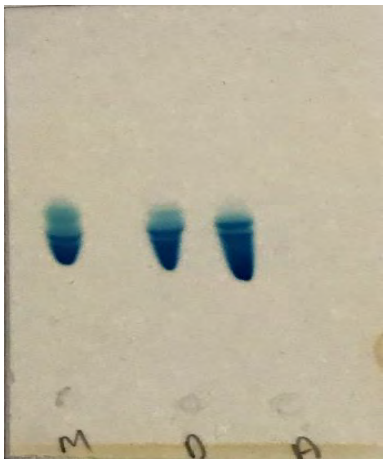
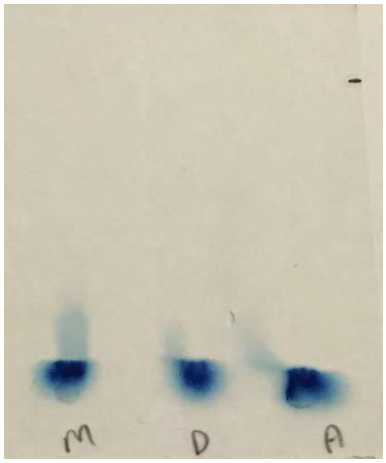
	• Sample: MB • Solvent: methanol • MP: 5:5:2:2 chloroform, methanol, trimethylamine and dichloromethane
	• Sample: MB • Solvent: dichloromethane • MP: 5:5:2:2 chloroform, methanol, trimethylamine and dichloromethane
	• Sample: MB • Solvent: acetonitrile • MP: 5:5:2:2 chloroform, methanol, trimethylamine and dichloromethane

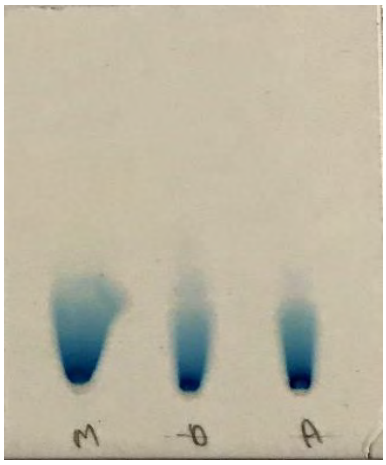
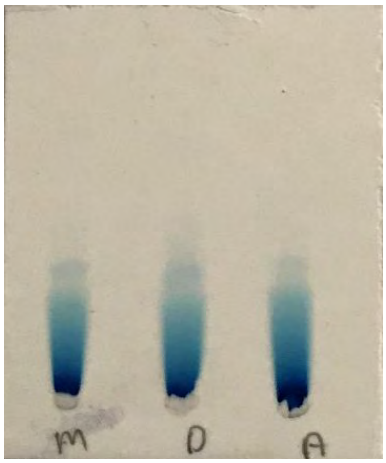
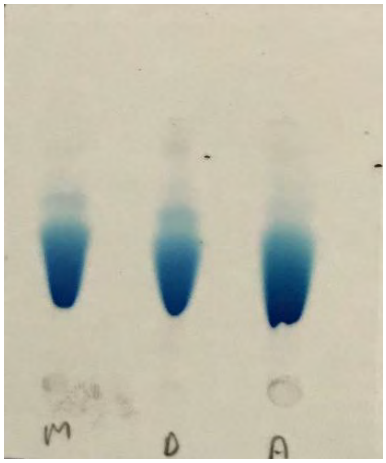
	• Sample: MB • Solvent: methanol • MP: 5:4:2 methanol to ethylene-di-amine-tetra-acetic-acid to acetonitrile
	• Sample: MB • Solvent: dichloromethane • MP: 5:4:2 methanol to ethylene-di-amine-tetra-acetic-acid to acetonitrile
	• Sample: MB • Solvent: acetonitrile • MP: 5:4:2 methanol to ethylene-di-amine-tetra-acetic-acid to acetonitrile

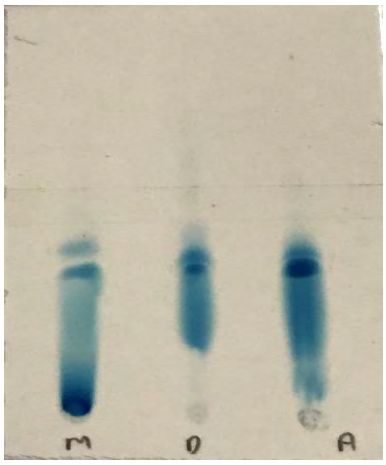
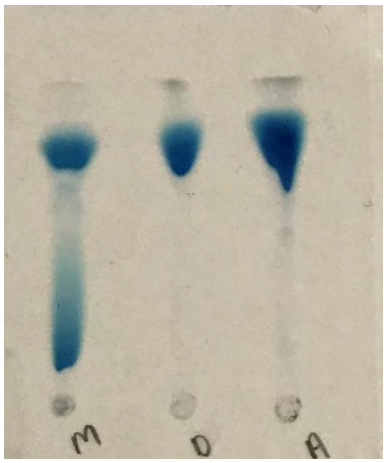
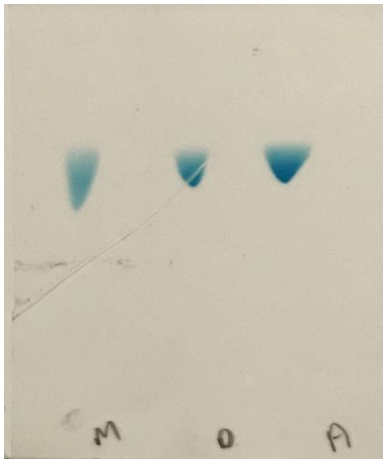
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 4:1:1:1 methanol to ethylene-di-amine-tetra-acetic-acid to chloroform to tri-ethylamine
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 3:2:5 methanol to ethylene-di-amine-tetra-acetic-acid to dichloromethane
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 5:2:2:4 chloroform to dichloromethane to acetonitrile to methanol

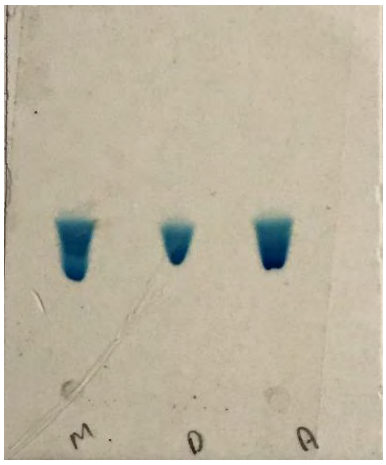
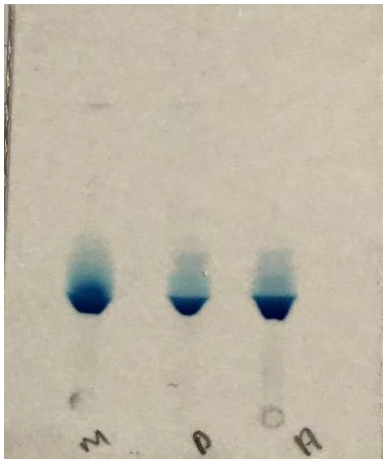
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 5:3:2 chloroform to methanol to trimethylamine
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 5:2:3 chloroform to dichloromethane to methanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 8:4:1 chloroform to propanol to ethyl-acetate

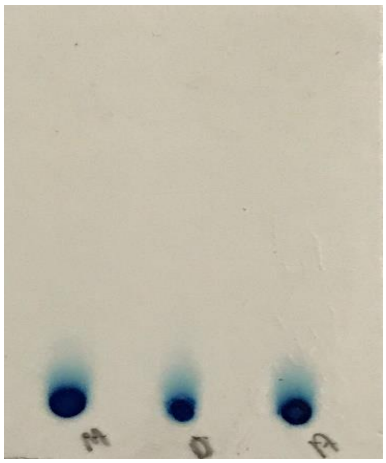
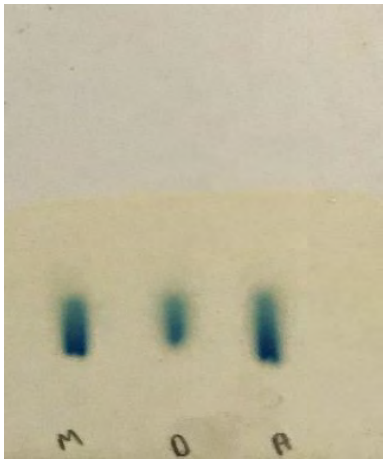
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 5:3:2 chloroform to methanol to trimethylamine
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 5:4:1 chloroform to methanol to petroleum-ether
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 5:4:4 chloroform to methanol to acetonitrile

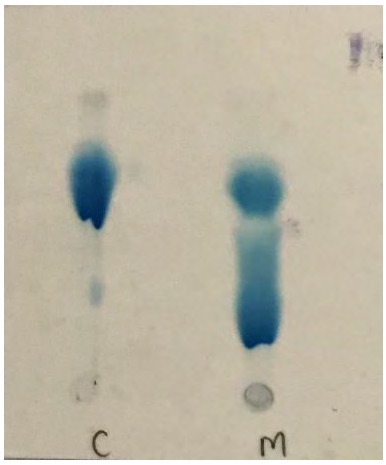
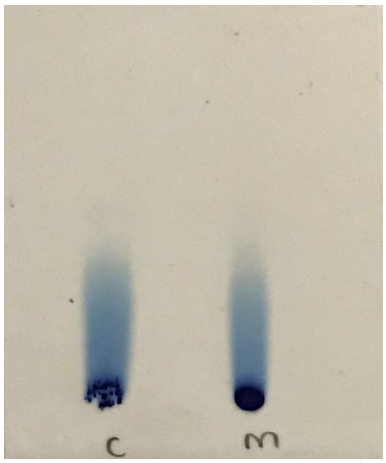
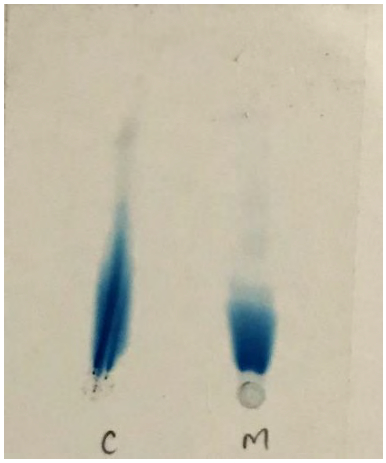
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) Acetonitrile (right) • MP: 7:3:1:1 chloroform to methanol to ethylene-di-amine-tetra-acetic-acid to petroleum-ether
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 6:3:1 chloroform to methanol to tetrahydrofuran
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 3:2:3:2 methanol to ammonium acetate to dichloromethane to ethylene-di-amine-tetra-acetic-acid

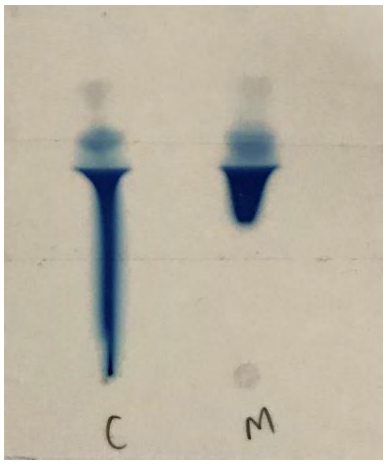
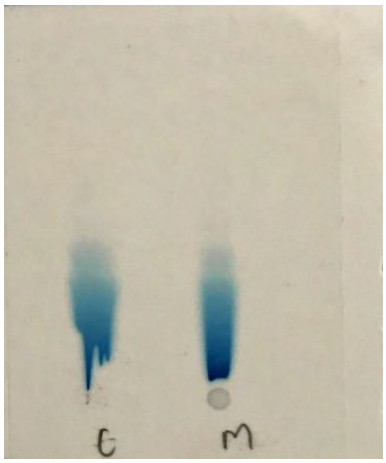
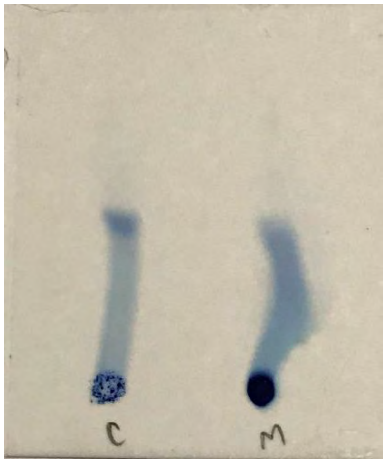
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 4:3:1 tri-ethylamine to ethylene-di-amine-tetra-acetic-acid to ammonium acetate
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 2:2:2:3 tetrabuthyl-ammonium-hydrogensulfate to ethylene-di-amine-tetra-acetic-acid to ammonium acetate to methanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 5:5:3 chloroform to methanol to tetrabuthyl-ammonium-hydrogensulfate

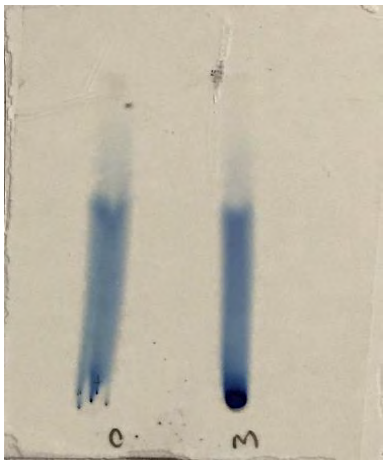
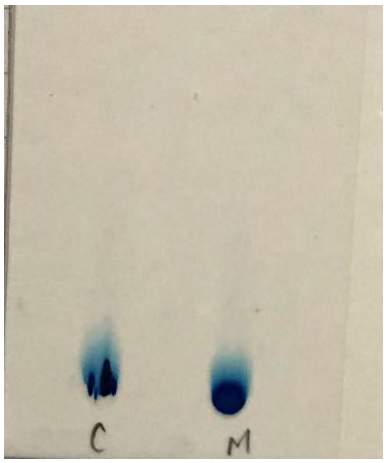
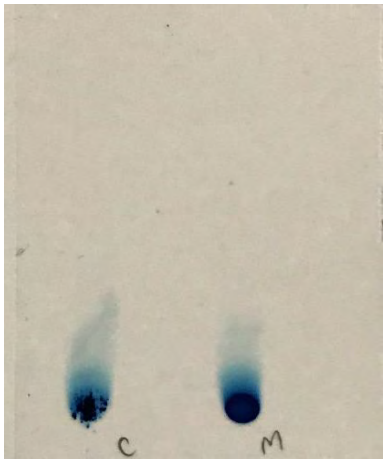
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 2:5:3 octanol to chloroform to methanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 2:5:3 benzene to chloroform to methanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 2:5:3 di-ethyl-ether to chloroform to methanol

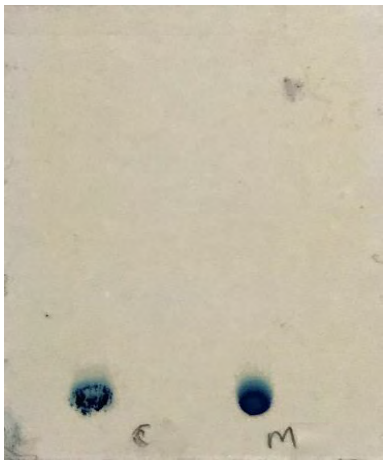
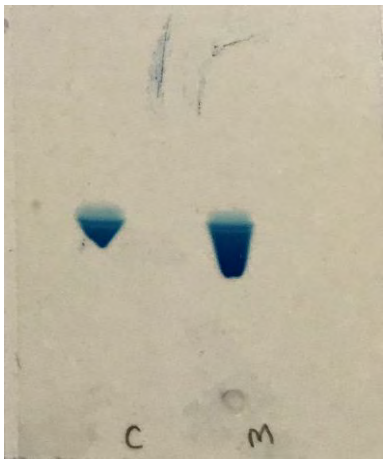
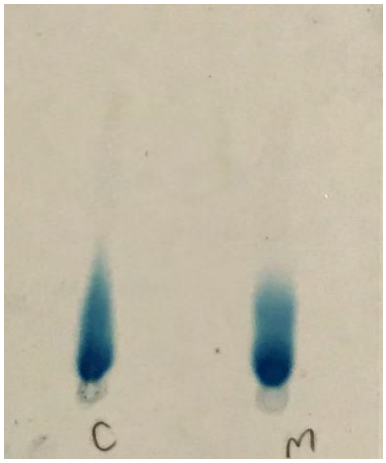
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 1:8:3:1:1 benzene to chloroform to methanol to tetrahydrofuran to octanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 5:2 chloroform to octanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 5:2:1:1 chloroform to octanol to dichloromethane to ethyl-acetate

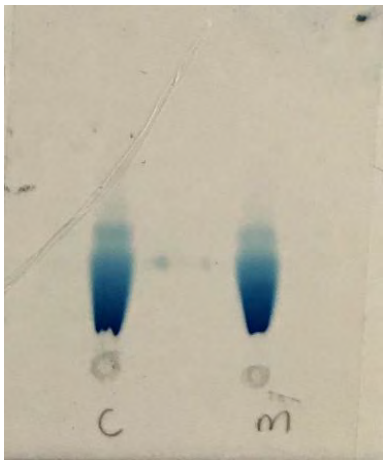
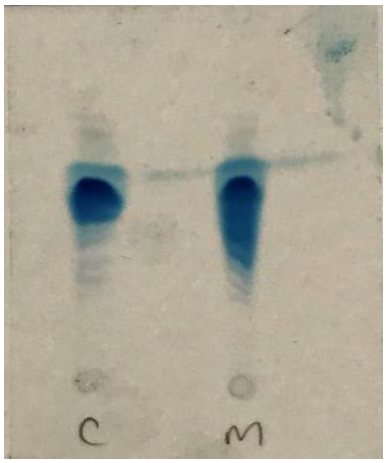
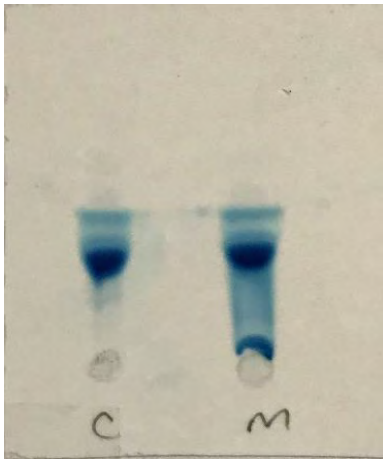
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 5:1:3:1:1 chloroform to octanol methanol to di-ethyl-ether to trimethylamine
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: 3:3:3:2 tetrabuthyl-ammonium-hydrogrensulfate to ammonium acetate to ethylene-di-amine-tetra-acetic-acid to methanol
	• Sample: MB • Solvent: methanol (left) dichloromethane (centre) acetonitrile (right) • MP: tetrabuthyl-ammonium-hydrogrensulfate to ammonium acetate to ethylene-di-amine-tetra-acetic-acid to methanol to trimethylamine to tetrahydrofuran in equal quantities

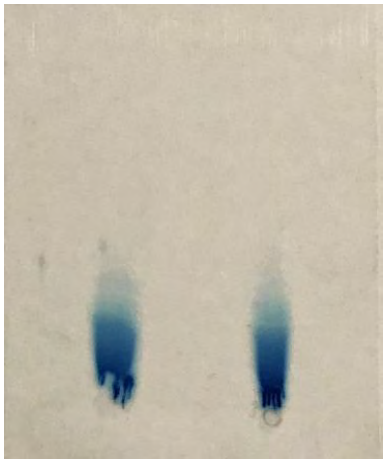
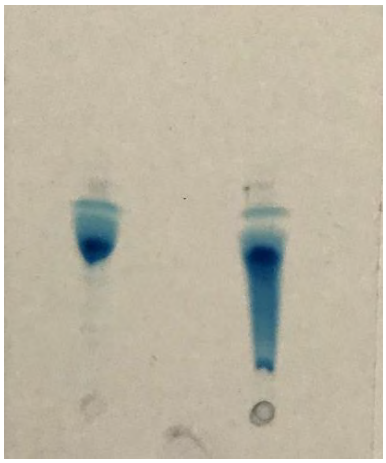
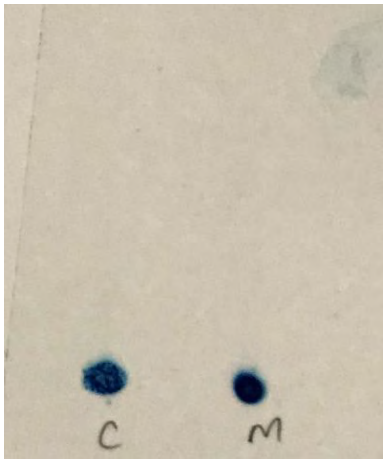
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 7:4 chloroform to methanol
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 2.5:1:2.5 methanol to tri-ethyl-amine to propanol
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 4:3 methanol to dichloromethane

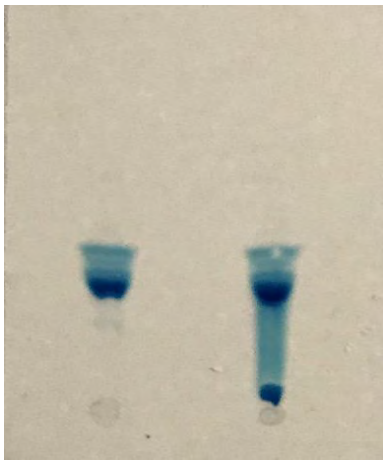
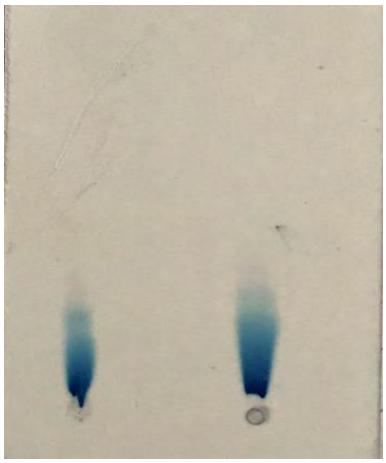
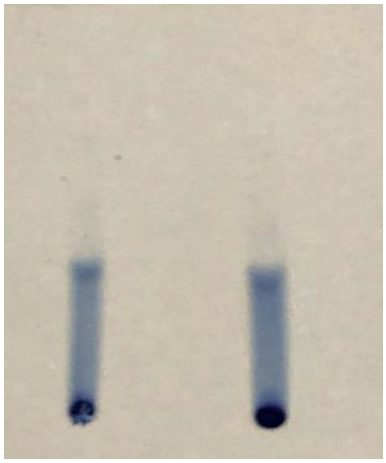
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 2.5:2:1 acetonitrile to ammonium acetate to trimethylamine
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 4:1 methanol to ammonium acetate
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 2:3:2:1:1.5 tri-ethylamine to chloroform to methanol to ammonium acetate to ethyl-acetate

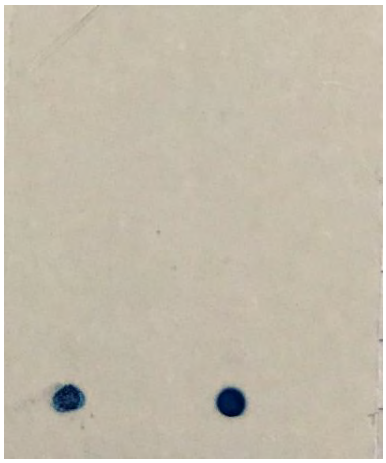
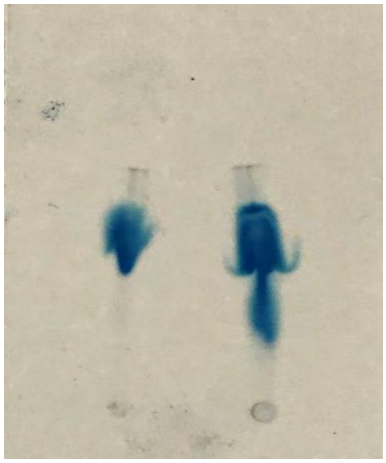
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 3:2:1 chloroform to methanol to tri-ethyl-acetate
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 2.5:1:2.5 chloroform to methanol to benzene
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 4:1:1:1 chloroform to methanol to petroleum-ether to ethylene-di-amine-tetra-acetic-acid

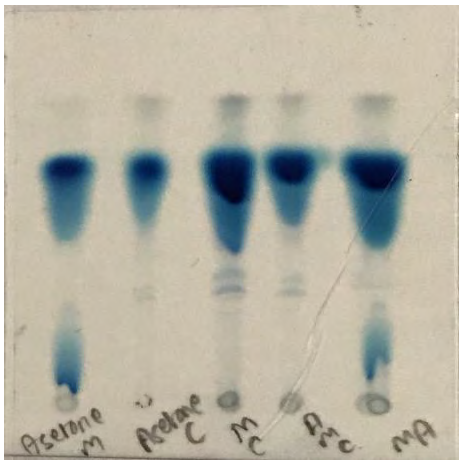
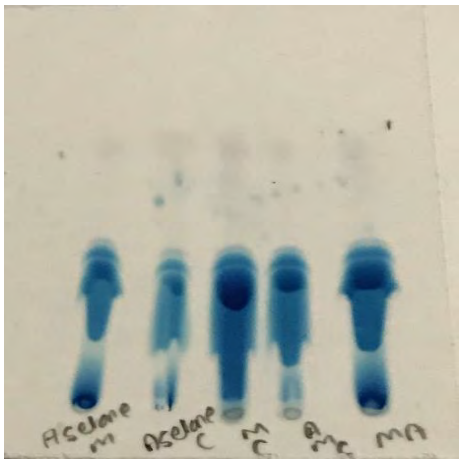
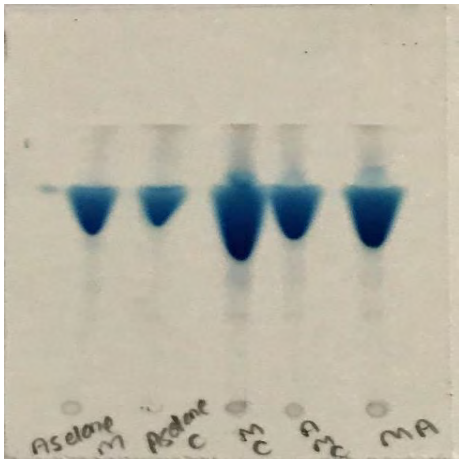
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 1:3:1:1:1 tri-ethylamine to chloroform to tetrahydrofuran to benzene to di-ethyl-ether
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 3:1:1 chloroform to methanol to tetrahydrofuran
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 3:4:2 methanol to dichloromethane to acetonitrile

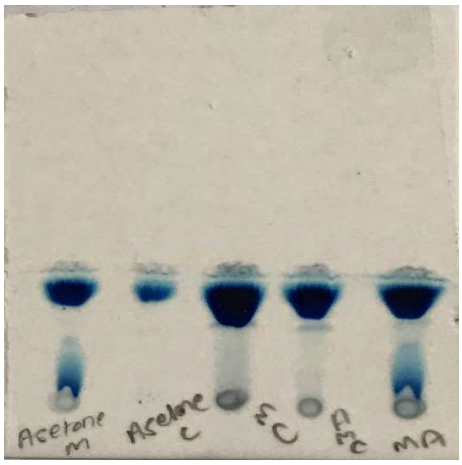
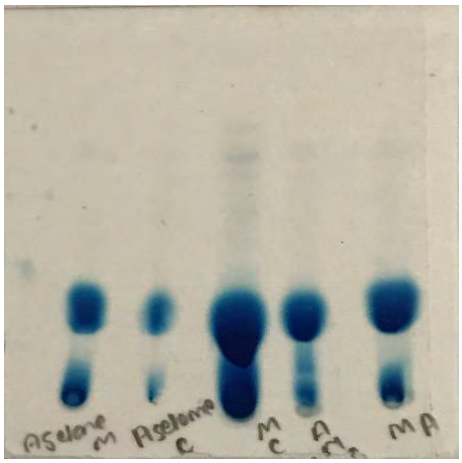
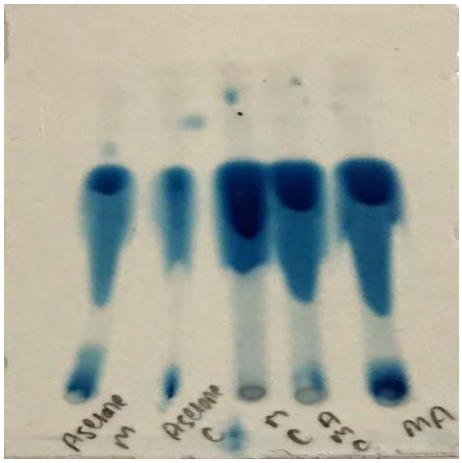
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:3:1 methanol to dichloromethane to ammonium acetate
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:1 chloroform to methanol to dichloromethane
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:2:2 chloroform to methanol to acetonitrile to dichloromethane

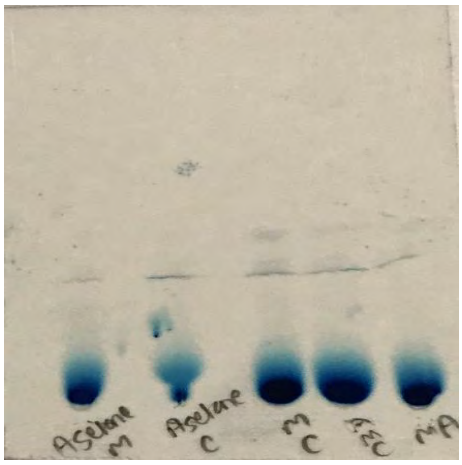
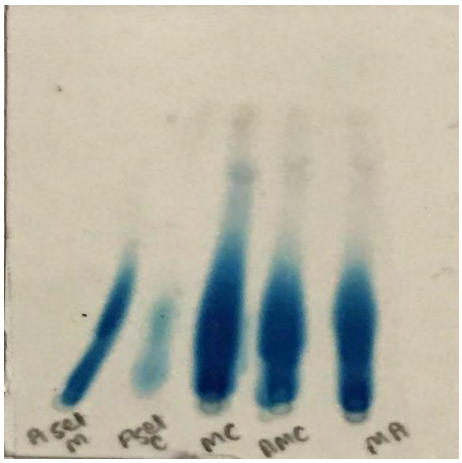
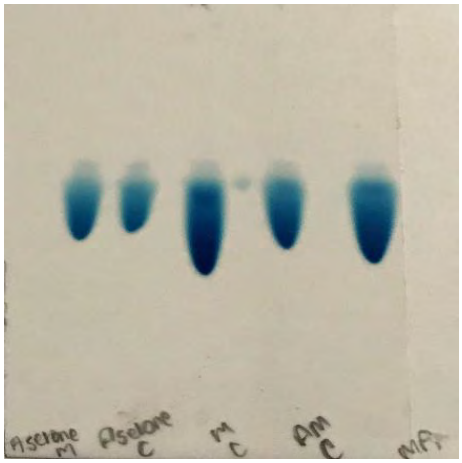
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:2 chloroform to methanol to tetrabuthyl-ammonium-hydrogensulfate
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:2 chloroform to methanol to toluene
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:2:2 chloroform to dichloromethane to acetonitrile to toluene

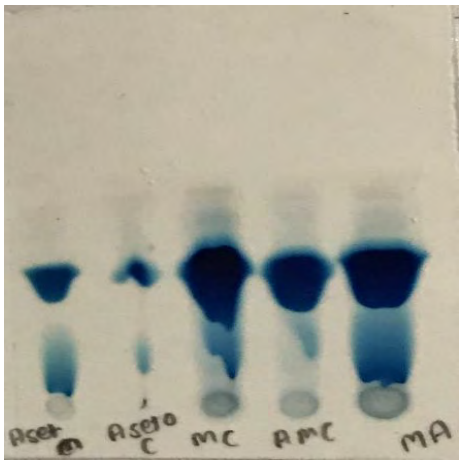
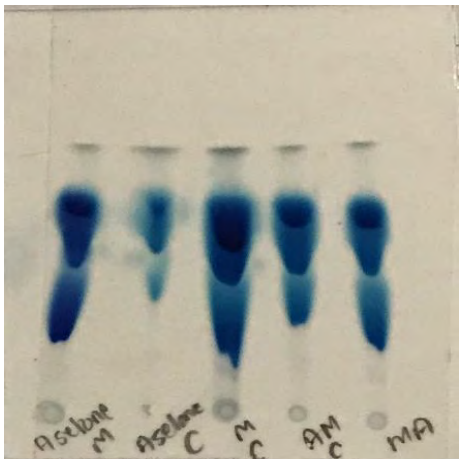
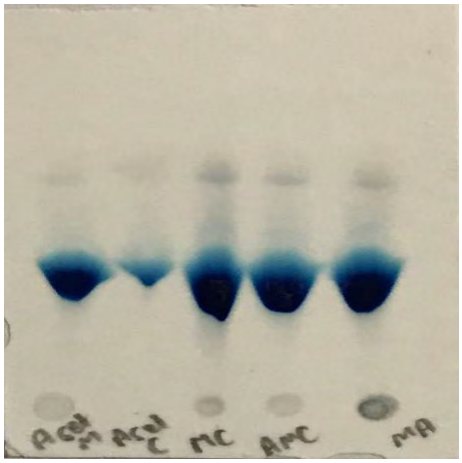
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:2:2 chloroform to methanol to acetonitrile to toluene
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 6:1:3 toluene to ammonium acetate to methanol
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:1:1:1 chloroform to methanol to toluene to trimethylamine

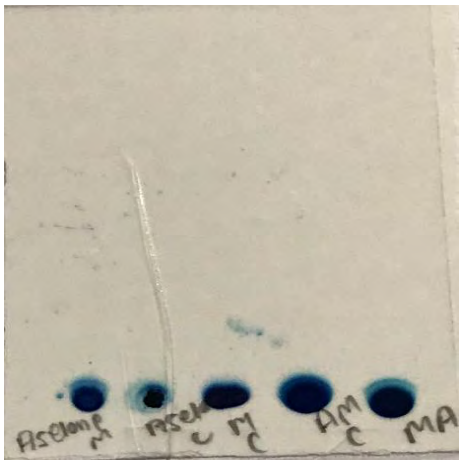
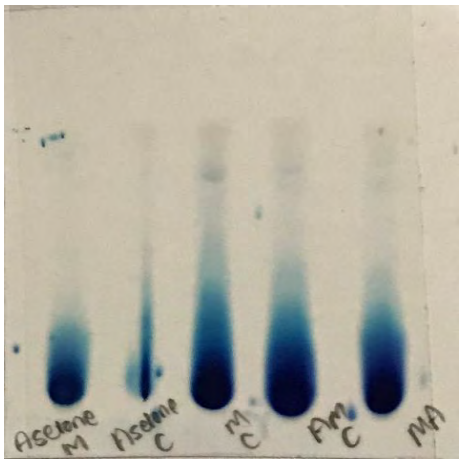
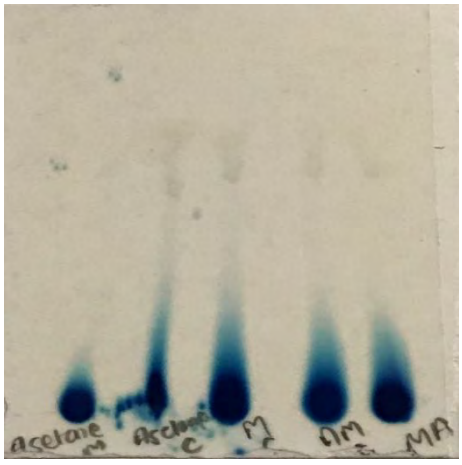
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 5:2:1:1:1 chloroform to methanol to toluene to benzene to tetrahydrofuran
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 6:3:1:1 chloroform to methanol to toluene to ethylene-di-amine-tetra-acetic-acid
	• Sample: MB • Solvent: chloroform (left) methanol (right) • MP: 7:3:1 dichloromethane to methanol to toluene

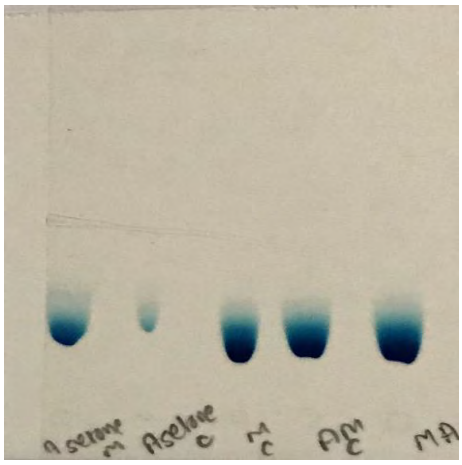
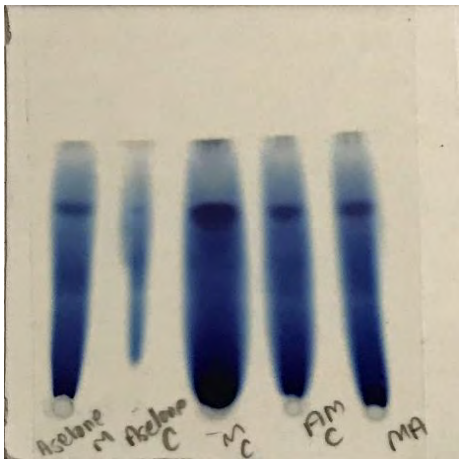
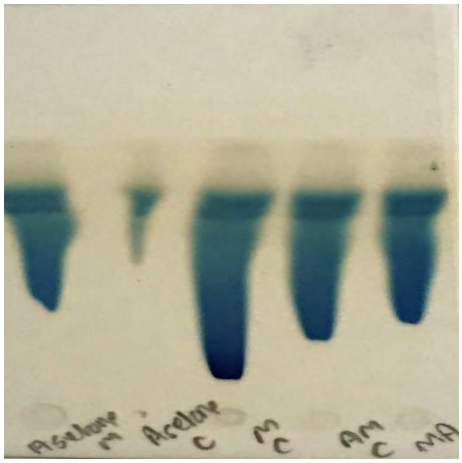
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 7:3 chloroform to methanol
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 6:3:1.5 chloroform to methanol to propanol
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 6:3:1 chloroform to methanol to ammonium acetate

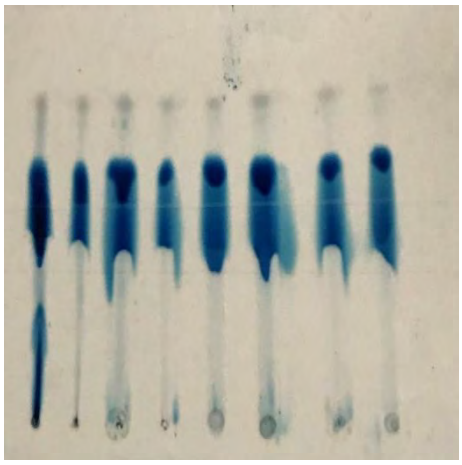
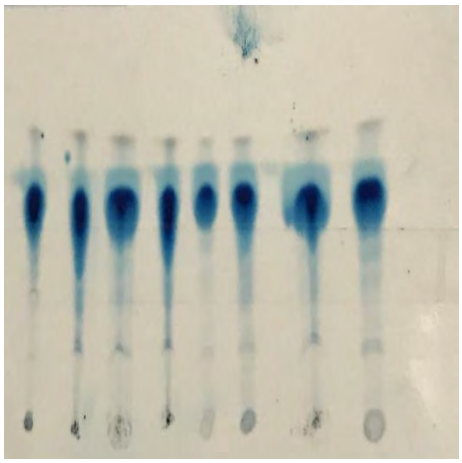
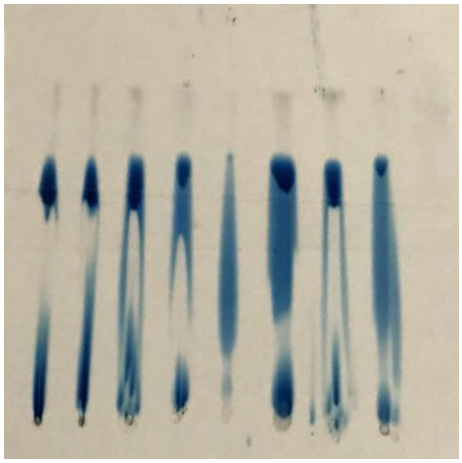
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 7:2:2 chloroform to methanol to toluene
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 6:2:2 chloroform to methanol to acetone
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:2:2 chloroform to methanol to acetonitrile

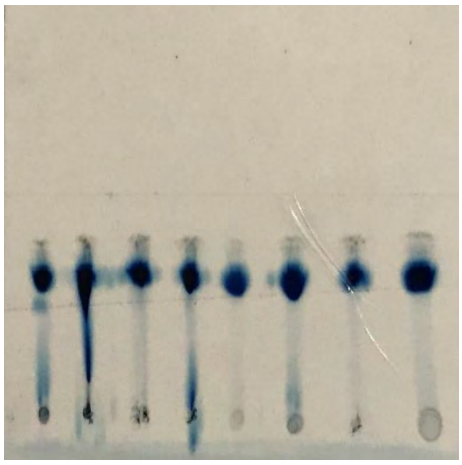
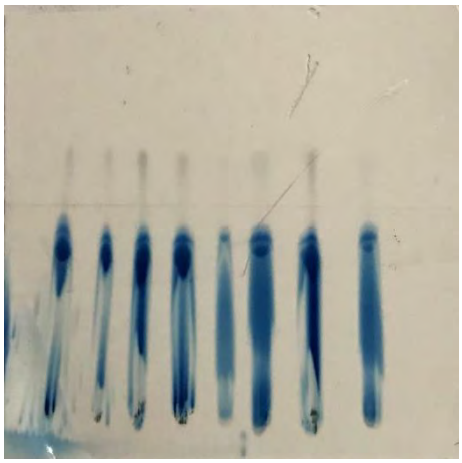
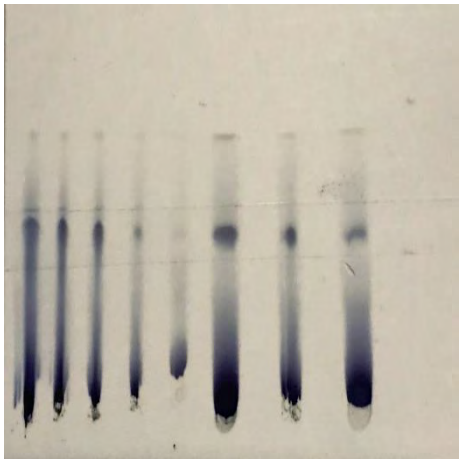
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:2:1:2 chloroform to methanol to ethylene-di-amine-tetra-acetic-acid to toluene
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 1:1 dichloromethane to methanol
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:3:1 chloroform to methanol to di-ethyl-ether

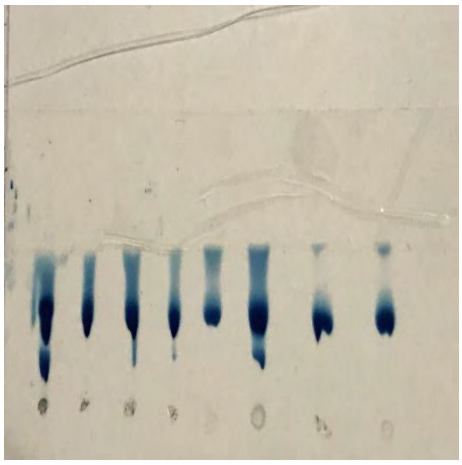
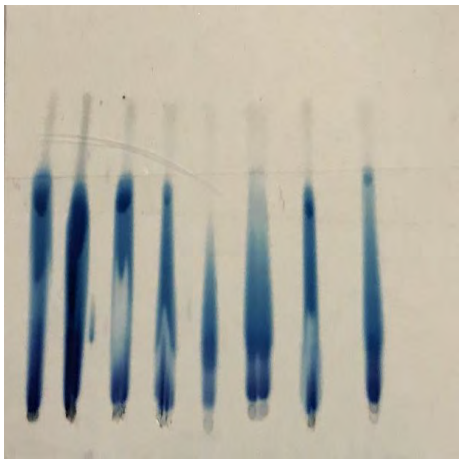
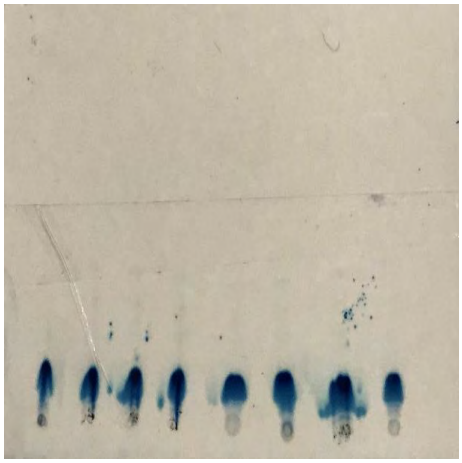
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:3:2 chloroform to methanol to benzene
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:3:2 chloroform to methanol to petroleum-ether
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 6:2:1 chloroform to methanol to dichloromethane

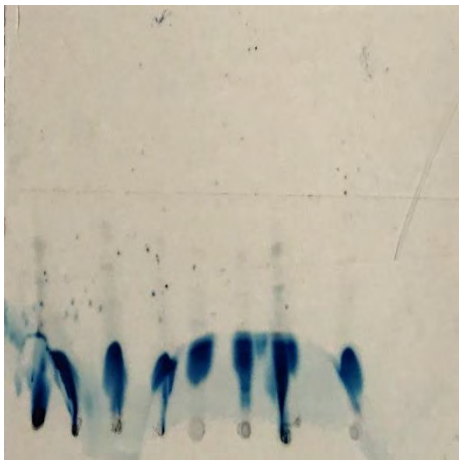
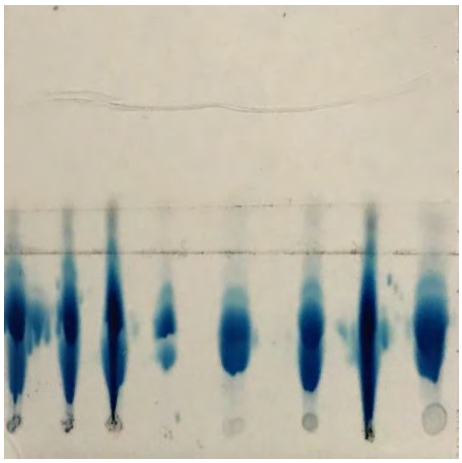
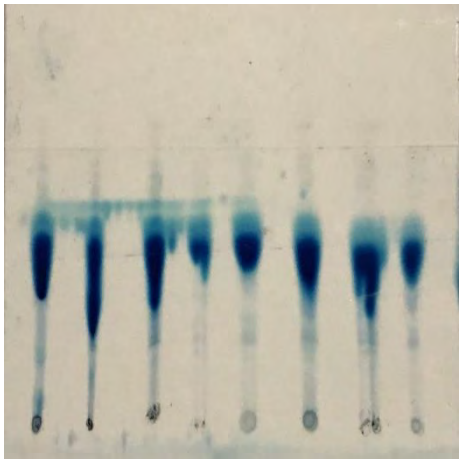
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:3 chloroform to acetonitrile
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 7:4:1 methanol to dichloromethane to acetone
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 7:2:2 methanol to dichloromethane to acetonitrile

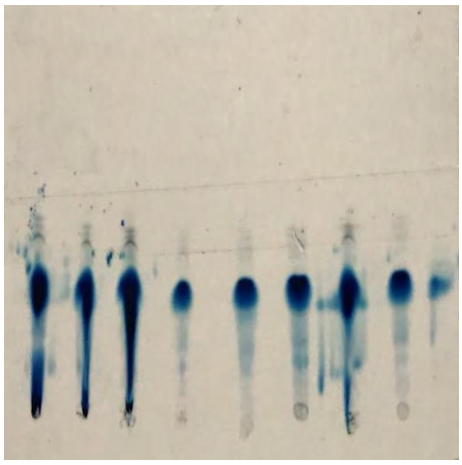
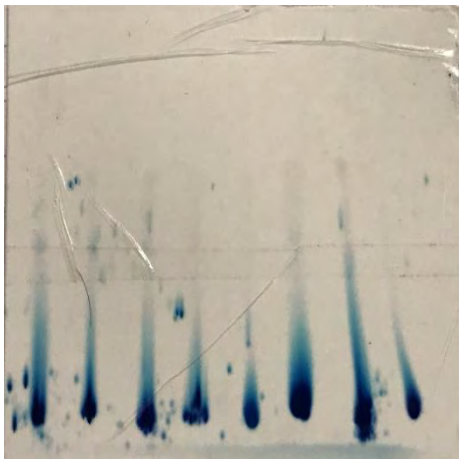
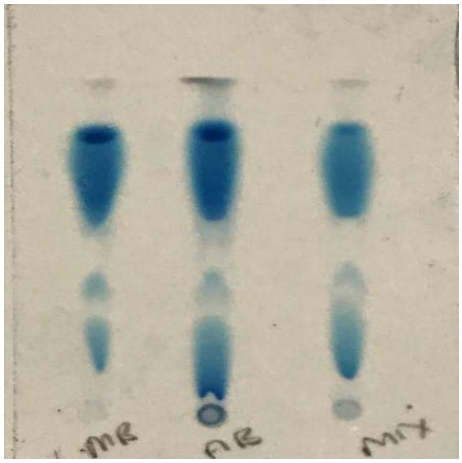
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:2:2:1 chloroform to methanol to ammonium acetate to di-ethyl-ether
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 7:3:2 chloroform to methanol to tri-ethyl-acetate
	• Sample: MB • Solvent: 1. Ac + MeOH 2. Ac + Chl 3. MeOH + Chl 4. Aceto + MeOH + Chl 5. MeOH + Aceto • MP: 5:3:2:1 chloroform to methanol to trimethylamine to tetrahydrofuran

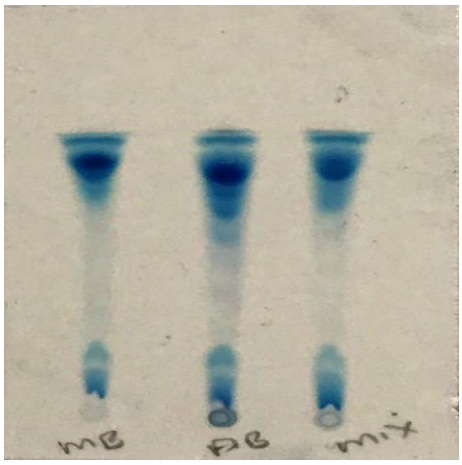
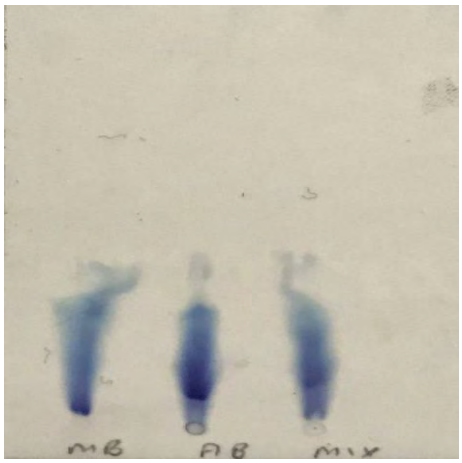
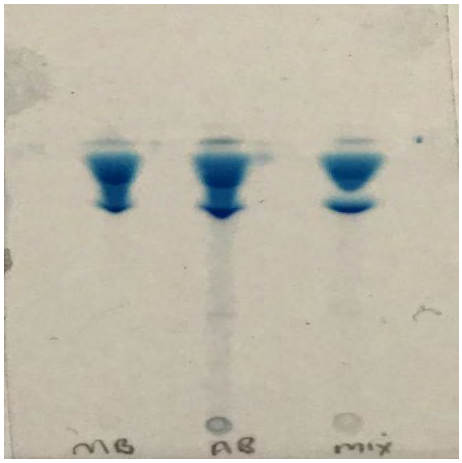
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 7:3 chloroform to methanol
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 6:2:2 chloroform to methanol to dichloromethane
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 5:4:2 chloroform to methanol to acetonitrile

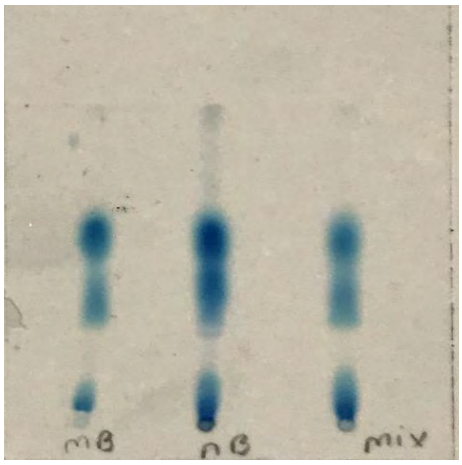
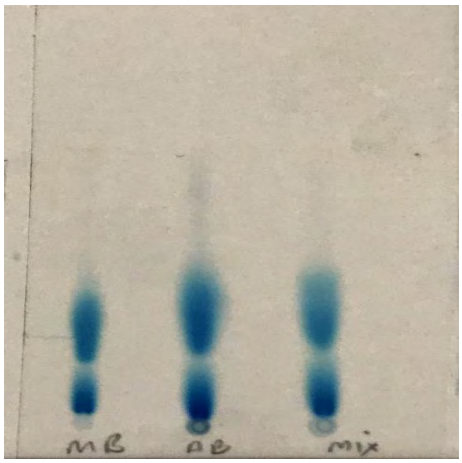
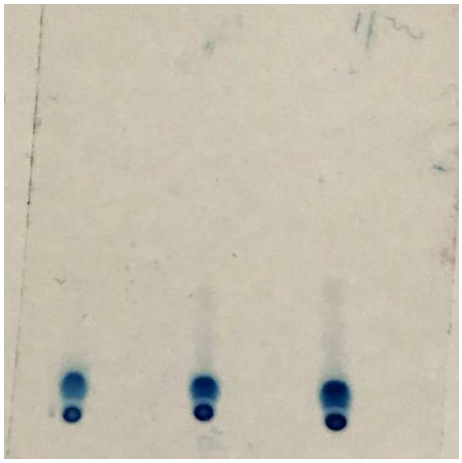
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 5:2:2 chloroform to methanol to toluene
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 5:2:2 chloroform to methanol to propanol
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 5:2:1 chloroform to methanol to tri-ethyl-acetate

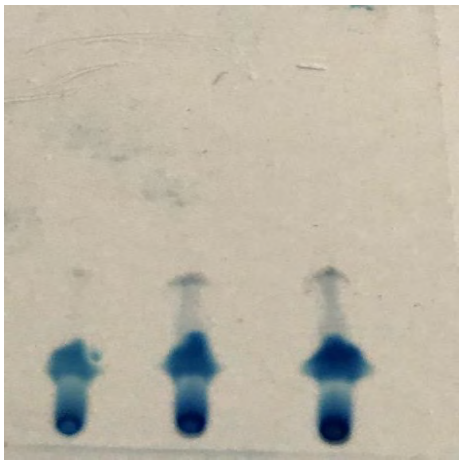
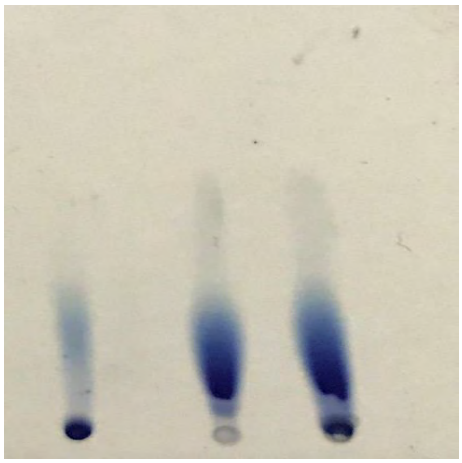
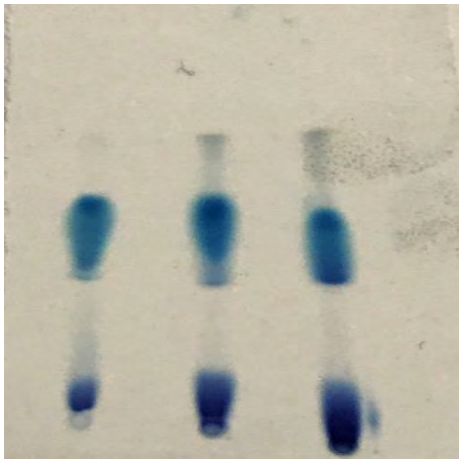
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 5:2:1 chloroform to methanol to tetrahydrofuran
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 1:1 dichloromethane to methanol
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 5:3:1 chloroform to methanol to ammonium acetate

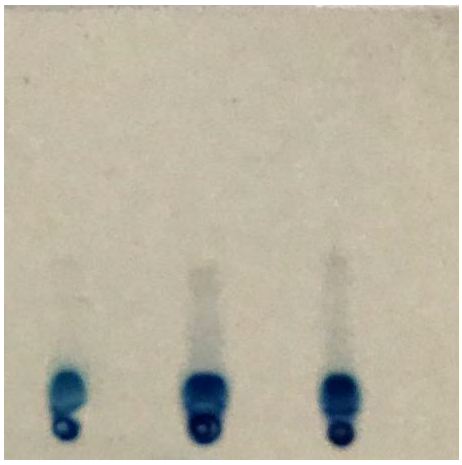
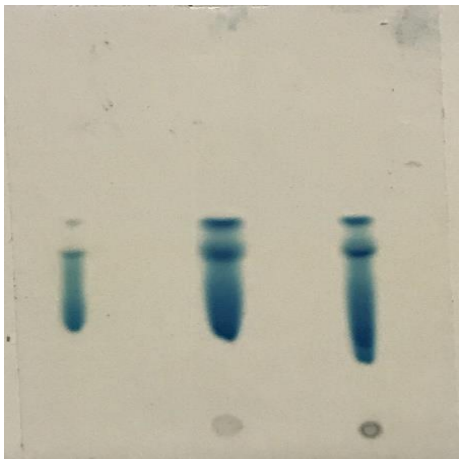
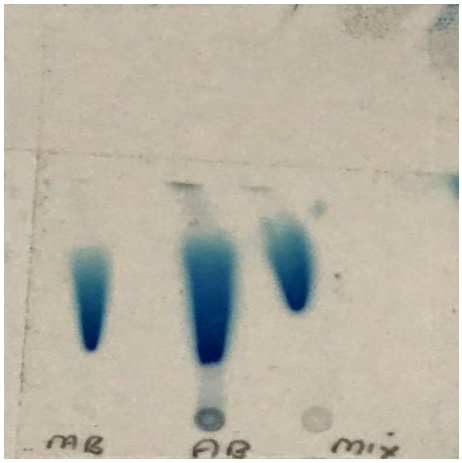
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 6:2:2 chloroform to methanol to ethylene-di-amine-tetra-acetic-acid
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 6:3:1 chloroform to methanol to benzene
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 6:2:2:2 chloroform to methanol to dichloromethane to acetonitrile

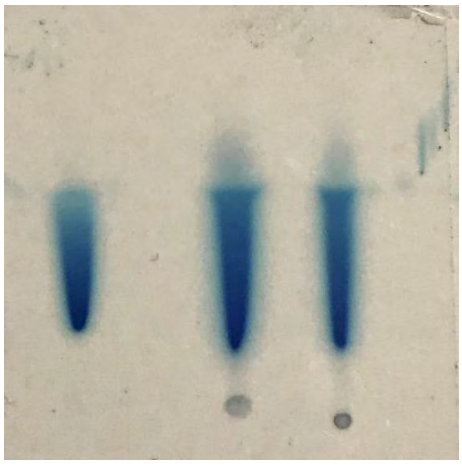
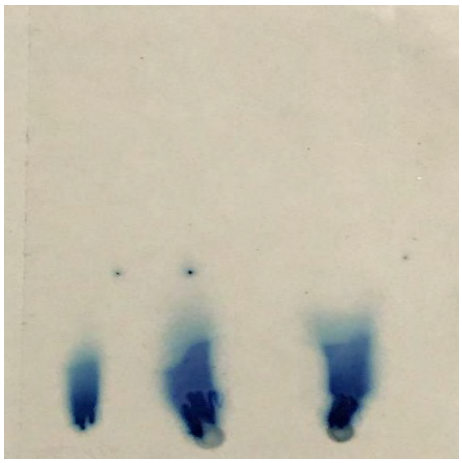
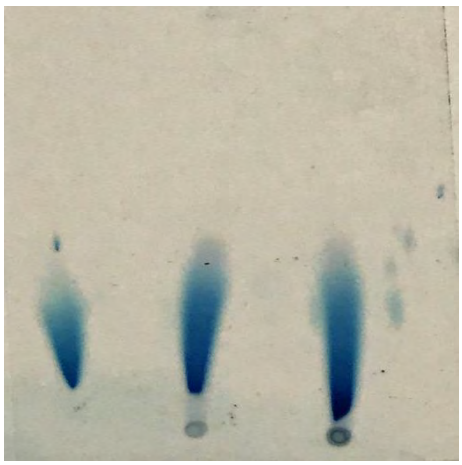
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 6:2:1 chloroform to methanol to tetra-buthyl-ammonium-hydrogensulfate
	• Sample: MB • Solvent: 1. Toluene 2. Ethyl-acetate 3. Tol + Chl 4. EA + Chl 5. MeOH + EA 6. Tol + EA + Chl 7. Tol + EA + MeOH • MP: 7:3:1 acetonitrile to methanol to benzene
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 7:3 chloroform to methanol

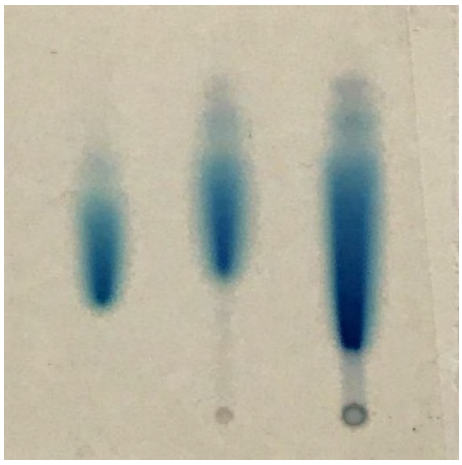
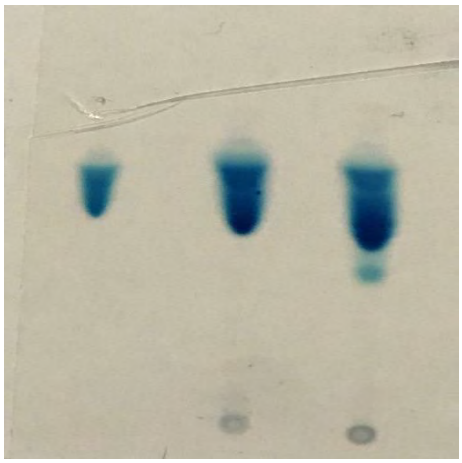
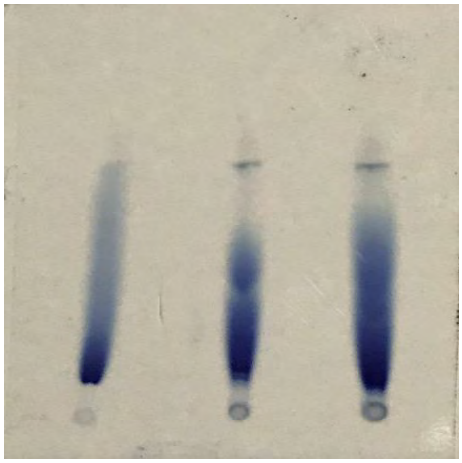
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 6:2:2 chloroform to methanol to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 6:2:2 chloroform to methanol to tri-ethyl-acetate
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 6:2:2 chloroform to methanol to acetonitrile

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 6:2:2 chloroform to methanol to petroleum-ether
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:3 methanol to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 8:1:1 chloroform to methanol to toluene

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:3:5 chloroform to methanol to toluene
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 methanol to tri-ethyl-amine
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 5:4:1 chloroform to methanol to benzene

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 8:1:1 chloroform to methanol to benzene
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 4:5:1 chloroform to methanol to tetrahydrofuran
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 5:2:2:1 chloroform to methanol to acetonitrile to dichloromethane

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 4:2:2:0.5 chloroform to methanol to acetonitrile to trimethylamine
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 6:2:2 chloroform to methanol to tetra-buthyl-ammonium-hydrogensulfate
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 5:1:1:1:2 methanol to ammonium acetate to tetra-buthyl- ammonium-hydrogensulfate to toluene to acetonitrile

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 5:1:1:3 methanol to ammonium acetate to tetra-buthyl- ammonium-hydrogensulfate to dichloromethane
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 5:3:1:1:1 chloroform to methanol to tetrahydrofuran to benzene to toluene
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:5:1:1:1 chloroform to methanol to trimethylamine to dichloromethane to benzene

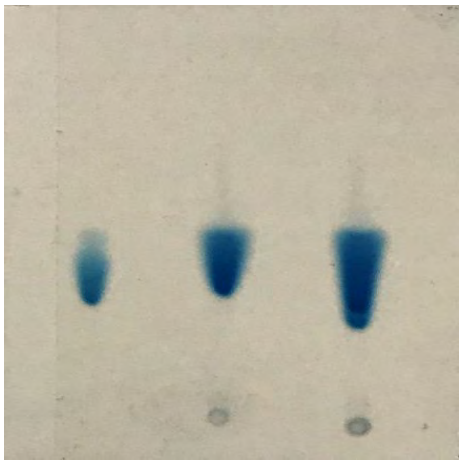
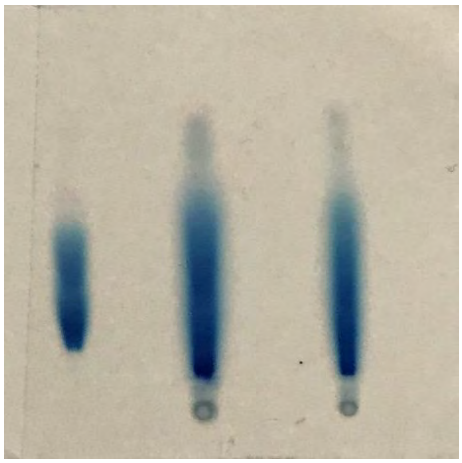
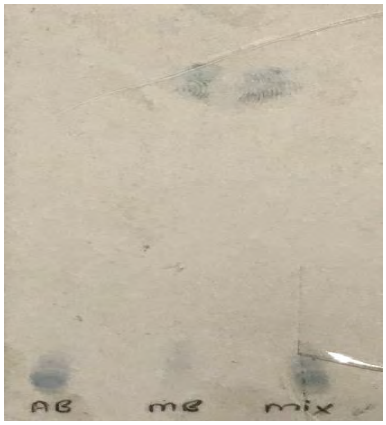
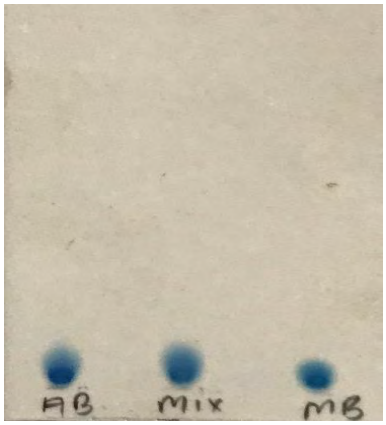
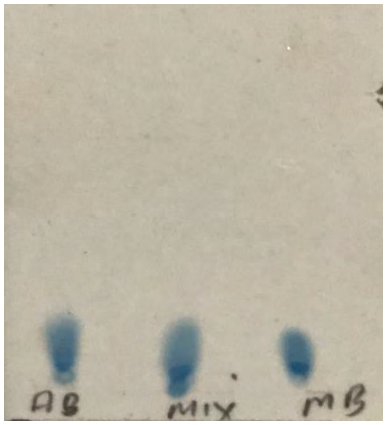
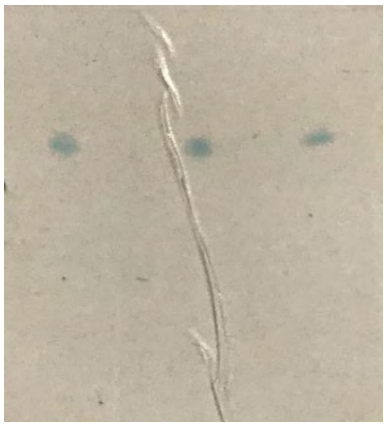
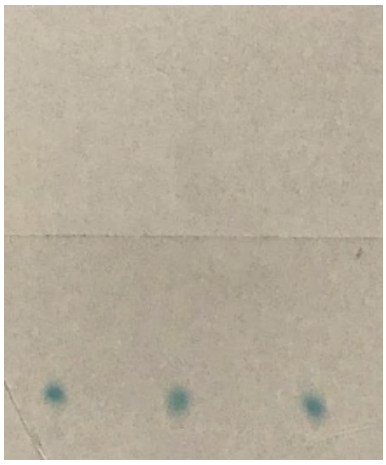
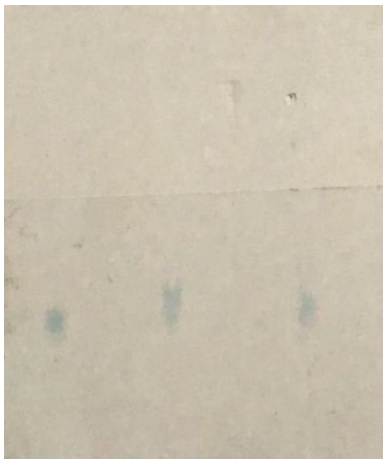
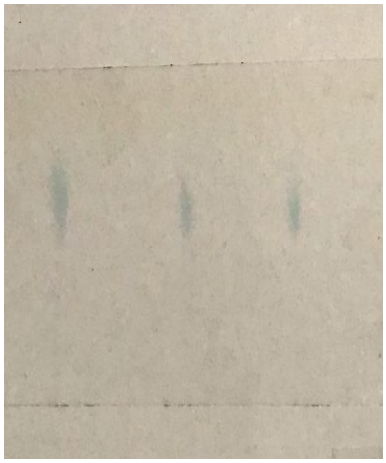
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:5:1:1:1 chloroform to methanol to tetrahydrofuran to toluene
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:5:1:2 chloroform to methanol to tetrahydrofuran to dichloromethane

Table 7.2: Reverse phase TLC (known concentration of 20 µg/ml).

Chromatogram	Description
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:1 5mM ammonium acetate in 10:90 water-methanol to acetonitrile
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:1 5mM ammonium acetate in 10:90 water-methanol to 50:50 acetonitrile-methanol
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 2:3 5mM ammonium acetate in 10:90 water-methanol to acetonitrile

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 chloroform to methanol
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 chloroform to 50:50 acetonitrile-methanol
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 5mM ammonium acetate in 10:90 water-methanol to 50:50 acetonitrile-methanol

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1:1 5mM ammonium acetate in 10:90 water-methanol to 50:50 acetonitrile-methanol to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 5mM ammonium acetate in 10:90 water-methanol to chloroform
	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 dichloromethane to 50:50 acetonitrile-methanol

	• Sample: 1. MB 2. AB 3. MB + AB • Solvent: methanol • MP: 1:1 dichloromethane to chloroform

ADDENDUM B

Methylene blue (primary product)

	Analytical Values:									
Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
9,69		2048409	2004834	2042345	2035041	2016469	2032376	2029912,333	18244,405	0,899
48,45		9915260	10066222	10008656	9955811	9985403	9953834	9980864,333	52423,553	0,525
96,90		20988606	20977521	20885950	20887339	20907965	20879311	20921115,333	49060,298	0,235
242,25		51515975	51495403	51604915	51529932	51465487	51469496	51513534,667	51384,692	0,100
484,50		104100000	104400000	104100000	104200000	103700000	103600000	104016666,667	306050,105	0,294

CONTROL STANDARD

Theor Conc	(µg/ml)	484,50		**Calc Conc**	(µg/ml)	485,10

Name	***Value***	***Conc***	***Avg (Value)***	***SD***	***%RSD***	***%Recovery***
Control 1-1	104100000	485,48	104016666,67	306050,1048	0,29	**100,35**
Control 1-2	104400000	486,88				
Control 1-3	104100000	485,48				
Control 1-4	104200000	485,95				
Control 1-5	103700000	483,62				
Control 1-6	103600000	483,16				

SUMMARY OUTPUT

Regression Statistics

Multiple R	0,999978898
R Square	0,999957796
Adjusted R Square	0,999943728
Standard Error	311806,7652
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	6,91066E+15	6,91066E+15	71080,15649	1,16366E-07
Residual	3	2,9167E+11	97223458819		
Total	4	6,91095E+15			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*	*Upper 95.0%*
Intercept	-193515,1142	199092,9597	-0,971983713	0,402749881	-827117,7682	440087,5398	-827117,7682	440087,5398	422032,942
X Variable 1	214824,0158	805,765453	266,6086204	1,16366E-07	212259,7105	217388,3211	212259,7105	217388,3211	92791,41726

Methylene blue 1

Analytical Values:										
Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
9,85		4362518	4396313	4395000	4376656	4339200	4337532	4367869,833	26050,881	0,596
98,50		19912606	19927087	19879302	19908088	19867041	19877486	19895268,333	23854,910	0,120
492,50		121900000	121800000	120900000	121500000	121400000	121300000	121466666,667	361478,446	0,298

Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
9,85		1972593	1973315	1955522	1978826	1938600	1957639	1962749,167	15023,518	0,765
98,50		20166231	19975516	20126602	20134876	20088080	20092019	20097220,667	66281,593	0,330
492,50		103600000	101900000	103700000	103400000	103100000	103400000	103183333,333	661563,804	0,641

Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
10,44		2002619	1989190	1990398	1981513	1979258	1965757	1984789,167	12428,031	0,626
104,41		19964591	19877167	19863665	19937927	19785608	19772464	19866903,667	77757,045	0,391
522,05		101500000	101300000	101300000	101100000	101100000	101100000	101233333,333	163299,316	0,161

Average of averages

Conc.	Average
10,05	2771802,72
100,47	19953130,89
502,35	108627777,78

CONTROL STANDARD `

Theor Conc	(µg/ml)	502,35		**Calc Conc**	(µg/ml)	503,48

Name	***Value***	***Conc***	***Avg (Value)***	***SD***	***%RSD***	***%Recovery***
Control 1-1	109000000	505,19	108627777,78	217477,1167	0,20	**100,22**
Control 1-2	108333333	502,12				
Control 1-3	108633333	503,50				
Control 1-4	108666667	503,66				
Control 1-5	108533333	503,04				
Control 1-6	108600000	503,35				

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,999766921
R Square	0,999533897
Adjusted R Square	0,999067794
Standard Error	1734508,9
Observations	3

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	6,45162E+15	6,45162E+15	2144,448177	0,01374532

Residual	1	3,00852E+12	3,00852E+12
Total	2	6,45463E+15	

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*	*Upper 95.0%*
Intercept	-491624,1323	1384554,988	-0,355077362	0,782791126	-18084063,28	17100815,02	-18084063,28	17100815,02	422032,942
X Variable 1	216731,4993	4680,198334	46,30818693	0,01374532	157263,9411	276199,0575	157263,9411	276199,0575	92791,41726

Methylene blue 2

	Analytical Values:									
Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
10,40		1990436	1988412	1979929	1997647	1987587	2003076	1991181,167	8136,915	0,409
104,00		20915813	20896962	20892929	20960206	20819709	20818772	20884065,167	55597,052	0,266
520,00		110600000	110600000	110500000	110700000	110100000	110800000	110550000,000	242899,156	0,220

Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
10,60		2091600	2074880	2085839	2057558	2079049	2078905	2077971,833	11626,989	0,560
106,00		22631953	22547061	22185351	22478133	22424988	22558423	22470984,833	156905,869	0,698
530,00		114400000	114300000	114300000	114200000	114300000	114000000	114250000,000	137840,488	0,121

Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**

10,00		2154884	2144608	2121586	2131568	2123050	2126849	2133757,500	13261,822	0,622
100,00		20893455	20883681	20726307	20792197	20683998	20678407	20776340,833	96031,949	0,462
500,00		103300000	103600000	103400000	103000000	103100000	103000000	103233333,333	242212,028	0,235

Average of averages

Conc.	Average
10,33	2067636,83
103,33	21377130,28
516,67	109344444,44

CONTROL STANDARD

Theor Conc	(µg/ml)	502,35		**Calc Conc** (µg/ml)	516,87

Name	*Value*	*Conc*	*Avg (Value)*	*SD*	*%RSD*	*%Recovery*
Control 1-1	109433333	517,29	109344444,44	122323,1906	0,11	**102,89**
Control 1-2	109500000	517,60				
Control 1-3	109400000	517,13				
Control 1-4	109300000	516,66				
Control 1-5	109166667	516,03				
Control 1-6	109266667	516,50				

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,999993004
R Square	0,999986009
Adjusted R Square	0,999972018
Standard Error	302487,5409
Observations	3

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	6,53971E+15	6,53971E+15	71473,2967	0,002381257
Residual	1	91498712384	91498712384		
Total	2	6,53981E+15			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*	*Upper 95.0%*
Intercept	-314059,6805	241457,7599	-1,300681662	0,41726753	-3382071,413	2753952,052	-3382071,413	2753952,052	422032,942
X Variable 1	212159,7947	793,5808501	267,3449021	0,002381257	202076,394	222243,1955	202076,394	222243,1955	92791,41726

Azure A

	Analytical Values:									
Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
9,69		260737	264641	258363	260495	265035	264647	262319,667	2816,649	1,074
48,45		1310456	1330046	1312461	1319359	1309676	1333471	1319244,833	8529,772	0,647
96,90		2602628	2600673	2597153	2635302	2659403	2633694	2621475,500	27291,781	1,041
242,25		6975721	7041824	7028859	7019827	7004699	6987436	7009727,667	25399,489	0,362
484,50		13207666	13138130	13253301	13559206	13441397	13449869	13341594,833	174714,843	1,310

CONTROL STANDARD

Theor Conc	(µg/ml)	484,50		**Calc Conc**	(µg/ml)	480,40

Name	***Value***	***Conc***	***Avg (Value)***	***SD***	***%RSD***	***%Recovery***
Control 1-1	13207666	475,57	13341594,83	165026,7004	1,24	**97,90**
Control 1-2	13138130	473,06				
Control 1-3	13253301	477,22				
Control 1-4	13559206	488,25				
Control 1-5	13441397	484,00				
Control 1-6	13449869	484,31				

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,999579301

R Square	0,999158779
Adjusted R Square	0,998878372
Standard Error	179755,3331
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1,15136E+14	1,15136E+14	3563,245218	1,03577E-05
Residual	3	96935939381	32311979794		
Total	4	1,15232E+14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*	*Upper 95.0%*
Intercept	20714,24133	114776,2823	0,180474928	0,868282913	-344555,1141	385983,5968	-344555,1141	385983,5968	422032,942
X Variable 1	27728,58764	464,5205095	59,69292436	1,03577E-05	26250,27606	29206,89922	26250,27606	29206,89922	92791,41726

Azure B

	Analytical Values:								
Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**
9,69		812323	794051	814062	805951	797167	802194	804291,333	8034,270
48,45		4048603	4043234	4067835	4042111	4050259	4064214	4052709,333	10335,461
96,90		8104712	8092596	8078435	8101520	8070102	8099175	8091090,000	14867,612
242,25		21210365	21436180	21402498	21362141	21326191	21256302	21332279,500	87125,427
484,50		40325926	40052439	40423393	41609436	41086366	41088394	40764325,667	634848,283

CONTROL STANDARD

Theor Conc	(µg/ml)	484,50		**Calc Conc**	(µg/ml)	480,72

Name	***Value***	***Conc***	***Avg (Value)***	***SD***	***%RSD***	***%Recovery***
Control 1-1	40325926	475,54	40764325,67	589602,1902	1,45	**97,82**
Control 1-2	40052439	472,31				
Control 1-3	40423393	476,69				
Control 1-4	41609436	490,71				
Control 1-5	41086366	484,53				
Control 1-6	41088394	484,55				

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,999661403
R Square	0,99932292
Adjusted R Square	0,999097227
Standard Error	492104,7967
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1,07227E+15	1,07227E+15	4427,79204	7,47889E-06
Residual	3	7,26501E+11	2,42167E+11		
Total	4	1,07299E+15			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*	*Upper 95.0%*
Intercept	85480,73117	314215,7624	0,272044695	0,803236138	-914494,061	1085455,523	-914494,061	1085455,523	422032,942
X Variable 1	84620,25219	1271,688394	66,54165643	7,47889E-06	80573,17216	88667,33222	80573,17216	88667,33222	92791,41726

Thionin

	Analytical Values:									
Conc.		**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Value 6**	**Average**	**SD**	**%RSD**
9,69		344304	350553	349653	346767	346351	346480	347351,333	2321,031	0,668
48,45		1729331	1720200	1734800	1717226	1718372	1726409	1724389,667	7698,377	0,446
96,90		3437632	3431523	3420756	3396730	3422221	3397868	3417788,333	15612,152	0,457
242,25		8695036	8756578	8736379	8729050	8719124	8687993	8720693,333	22641,127	0,260
484,50		15879060	15890371	15991435	16225305	16171649	16208821	16061106,833	159657,956	0,994

CONTROL STANDARD

Theor Conc	(µg/ml)	484,50		**Calc Conc**	(µg/ml)	477,64

Name	*Value*	*Conc*	*Avg (Value)*	*SD*	*%RSD*	*%Recovery*
Control 1-1	15879060	472,16	16061106,83	160091,2379	1,00	**97,49**
Control 1-2	15890371	472,50				
Control 1-3	15991435	475,54				
Control 1-4	16225305	482,58				
Control 1-5	16171649	480,97				
Control 1-6	16208821	482,09				

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,99904424
R Square	0,998089394
Adjusted R Square	0,997452525
Standard Error	324670,5448
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1,65198E+14	1,65198E+14	1567,182195	3,54646E-05
Residual	3	3,16233E+11	1,05411E+11		
Total	4	1,65514E+14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*	*Upper 95.0%*
Intercept	196649,7525	207306,662	0,948593502	0,412809487	-463092,5681	856392,0731	-463092,5681	856392,0731	422032,942
X Variable 1	33214,34892	839,0078017	39,58765205	3,54646E-05	30544,25165	35884,4462	30544,25165	35884,4462	92791,41726