University of Stavanger

FACULTY OF SCIENCE AND TECHNOLOGY

MASTER'S THESIS

Study programme: Master of Science in Biological Chemistry	Spring semester, 2020 Open
Author: Marianne Meling Kvamsøe	
Programme coordinator: Cathrine Lillo Supervisor(s): Cato Brede	
Title of master's thesis:	Development of LC-MS/MS method for analysing immunosuppressants in whole blood for the purpose of TDM, with the novel application of SALLE in the sample preparation
Credits: 60	
Keywords: Therapeutic drug monitoring Salting-out assisted liquid-liquid extraction Liquid chromatography coupled with tandem mass spectrometry	Number of pages: 70 + supplemental material/other: 8 appendices Stavanger, July 5th / 2020 date/year

ACKNOWLEDGEMENTS

The work for this project started in September 2019 at Stavanger University Hospital, Department of Medical Biochemistry (Avdeling for medisinsk biokjemi) and was finished in July 2020. This thesis was done on behalf of the Faculty of Science and Technology at University of Stavanger as a part of the curriculum Master of Science in Biological Chemistry.

I want to first express gratitude and give thanks to my supervisor Cato Brede for great patience, guidance, and engagement throughout this project. This year has been a fantastic learning experience.

I would also like to thank my colleagues in the laboratory for letting me use their equipment despite their busy work schedule, making me a part of the team, and for contributing to the project both physically and academically.

Thank you to the researchers and PhD candidates in the research laboratory for creating a welcoming environment, delightful coffee breaks and interesting conversations.

Thank you to family and friends who have given me moral support and advice.

ABSTRACT

The laboratory for Medical Biochemistry at Stavanger University Hospital (SUS) needed a new method for analysing tacrolimus, sirolimus, everolimus and cyclosporin A. A method was developed using the analytical principles of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), with a sample preparation based on the principles of salting-out assisted liquid-liquid extraction (SALLE). LC-MS/MS was preferred because of the potential efficiency and selectivity. SALLE was predicted to be a good sample preparation method due to the extraction solvent's compatibility with the mobile phase of LC-MS/MS.

The instrumental method was first programmed by tuning the mass spectrometer for detection of the most prominent molecular ion adducts and MRM-transitions with the strongest signals. Then the liquid chromatographic (LC) separation was optimised by finding the best elution solvent and elution gradient. The tuning of the mass spectrometer was done by injecting pure solutions of the analytes and their internal standards to eliminate interference. Molecular ions with Na^+ adducts were the most prevalent for tacrolimus, sirolimus, and everolimus. Cyclosporin A, however, had molecular ions with both Na^+ and H^+ adducts. Methanol in the mobile phase procured the best LC-separation and highest signal intensity of the three solvents (methanol, acetonitrile, and acetone).

Optimisation experiments were performed with a saturated NaCl solution (5 M), and several water miscible solvents in various concentrations, as well as one experiment with PPT, to compare the effects. Solvents included in the experiments were methanol, acetonitrile, acetone, tetrahydrofuran. The final optimised sample preparation method was SALLE with NaCl (5 M, 100 µL) and 12.5 % (v/v) methanol in acetonitrile.

The most important performance characteristics measured for the new method were limit of detection (LOD), limit of quantification (LOQ), relative spike recovery (%), repeatability and reproducibility. The LOD and LOQ for each analyte was: tacrolimus (0.1 ng/mL, 0.3 ng/mL), sirolimus (0.2 ng/mL, 0.5 g/mL), everolimus (0.03 ng/mL, 0.1 ng/mL), and cyclosporin A (3.5 ng/mL, 6.4 ng/mL). The relative spike recovery was in the range between 100 - 108 % (± 1 – ± 7), and the repeatability across all analytes and quality control levels was acceptable with a CV in the range of 2.0 – 5.6 %. The reproducibility CV across the different quality control

levels was somewhat high and varied significantly between the analytes. The range for the reproducibility CV for each analyte was: tacrolimus (5.6 – 9.3 %), sirolimus (11.5 – 14.0 %), everolimus (8.0 – 13.4 %), and cyclosporin A (6.2 – 12.3 %). The range of the reproducibility CV was however lower when only including the results from one specific lot of calibration standards. The range of the reproducibility CV for each analyte was then: tacrolimus (2.1 – 4.9 %), sirolimus (4.7 – 6.8 %), everolimus (2.4 – 6.0 %), and cyclosporin A (3.8 – 6.4 %).

The new method was also compared separately to both an established immunoassay-based method used in the laboratory for Medical Biochemistry at Stavanger University Hospital, and an established LC-MS/MS method used in the Department of Pharmacology at Oslo University Hospital (OUS). The relative mean difference (%) with upper and lower limit of agreement (LoA) was calculated for both method comparisons by plotting the results into a Bland-Altman plot. The new method had a relative mean difference of -28 % for tacrolimus and -20 % for cyclosporin A compared to the immunoassay-based method. This meant there was a poor correlation between the two methods, and the new method measured the concentration to be much lower than in the immunoassay-based method. When the new method was compared to the LC-MS/MS method, the correlation for tacrolimus and cyclosporin A was much better, with a mean relative difference of 10 % for tacrolimus and 2 % for cyclosporin A. The correlation for sirolimus and everolimus was however not as good, with a mean relative difference of -12 % for sirolimus and -17 % for everolimus.

The work done in conjunction with this thesis gave the laboratory for Medical Biochemistry a new method for analysing immunosuppressant in blood with a unique sample preparation applying the extraction principles of SALLE, which improved the sensitivity of the analysis, reduced the ion suppression, and increased the efficiency of the analytical process.

ABBREVIATIONS

ACN	Acetonitrile
APC	Antigen-presenting cell
C18	Octadecyl silica
Conc.	Concentration
CV (%)	Correlation coefficient
EDTA-WB	EDTA whole blood
ESI	Electrospray ionisation
ESI+	Positive electrospray ionisation
HSP	Hansen Solubility Parameters
IPT	Immunosuppressant proficiency testing scheme
ISTD	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LLE	Liquid-liquid extraction
LoA	Limit of agreement
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MHC	Major histocompatibility complex
MRM	Multiple reaction monitoring
MS	Mass spectrometry/mass spectrometer
mTOR	Molecular-target-of-rapamycin
NBCS	New-born calf serum
OUS	Oslo University Hospital
PBS	Phosphate buffered saline
PP	Polypropylene
PPT	Protein precipitation
QC	Quality control
SALLE	Salting-out assisted liquid-liquid extraction

SD	Standard deviation
SPE	Solid phase extraction
SUS	Stavanger University Hospital
TCR	T-cell receptor
TDM	Therapeutic drug monitoring
THF	Tetrahydrofuran
TIC	Total ion chromatogram
tR	Retention time

TABLE OF CONTENTS

1 INTRODUCTION – PURPOSE OF THESIS

The laboratory for Medical Biochemistry at Stavanger University Hospital (SUS) needed a new method for analysing immunosuppressants in whole blood for the purpose of therapeutic drug monitoring (TDM). There was already an established routine analysis of tacrolimus and cyclosporin A using an immunoassay-based method developed by and purchased from Abbott Laboratories. One of the reasons for why there was a need for a new method was that the old method was ineffective in the sense that the analytes could only be analysed separately, making the analysis a time-consuming process. Developing a method using the principles of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for analysing immunosuppressants in whole blood would mean several analytes could be analysed simultaneously. This was an advantage as there was a desire to also add two more drugs to the analysis; sirolimus and everolimus. Another reason for developing a new method was that several studies have shown immunoassay-based analytical methods are vulnerable to interferences from drug metabolites, which is important to distinguish from the drugs themselves when it comes to TDM.

Although there were already plenty of published methods for analysing immunosuppressants using LC-MS/MS there was a potential for exploring a new sample preparation method for this specific purpose. No published articles were found to have applied salting-out assisted liquid-liquid extraction (SALLE) as opposed to protein precipitation, liquid-liquid extraction, or online solid phase extraction. With a novel sample preparation, there would be a need to optimise it in order to find the conditions giving highest extraction efficiency and minimum interference in the instrumental analysis. Regardless of the novelty of the sample preparation method, a new analytical method would also have to be validated before it could be implemented into the laboratory's routine analysis of real patient samples.

This thesis will discuss the whole process of developing a new method for TDM of immunosuppressants in whole blood with the use of LC-MS/MS principles, the results of the optimisation and validation, and the observations made along the way.

2 THEORY

2.1 Organ transplant rejection

An allograft transplant is the transplant of an organ or tissue from a human donor to a human recipient. Without medication the transplanted organ or tissue would be rejected by the recipient's immune system. Organ rejection is the result of an advanced immunologic response to the transplanted organ. Both the innate and the adaptive immune response is involved in rejection. This happens because the immune system is originally constructed to identify and protect against pathogens in the form of bacteria, viruses, and parasites. When an organ or tissue is rejected, it is because the immune system recognises the tissue's antigens as being foreign to the body's own antigens and attacks it the same way it attacks pathogens [1].

The immune system's response to foreign antigens has been organized into a three-signal model, or alternatively three-cell model [2, 3]. The first signal is defined as the interaction between an antigen-presenting cell (APC) and a T-cell receptor (TCR), in which APC presents antigens from digested foreign bodies to the T-cell receptor in the form of major histocompatibility complex (MHC) class II [3]. T-cell activation is also dependent on signal 2, which is the co-stimulation of the transmembrane protein CD28 [2, 4]. Signal 1 and 2 is necessary for the expression of interleukin 2 (IL-2). Signal 3 is then activated by these IL-2 cytokines, which triggers the activation of molecular target- of-rapamycin (mTOR), ultimately activating the T-cell proliferation [3].

Organ rejection can be divided into different categories. Hyperacute rejection, which can happen as quickly as minutes after reperfusion and usually happens due to human leukocyte antigen or ABO incompatibility [5]; acute cellular rejection, which is the T-cell mediated rejection that occurs within days after transplantation if the immune system is not suppressed and is characterised by lymphocytic infiltrate [1]; acute antibody-mediated rejection, which is similar to acute cellular rejection but does not necessarily include lymphocytic infiltrate, and is usually more severe [6]; chronic rejection, which is the gradual deterioration of organ function most patients will experience over the years due to a

suppressed, but still functioning immune response [1]. Acute cellular rejection and antibody-mediated rejection are the kind of organ rejections that can be prevented with medication.

2.2 Immunosuppression after solid organ transplant

After a patient has undergone organ transplantation, medication is needed to prevent the patient's immune system from rejecting the new organ. There is a variety of commonly used medications containing one or several active compounds. Each compound targets specific signals or cellular interactions in the immune response. These compounds are categorised into different groups, primarily based on their structure and target. The primary focus of this thesis will be the use of small molecule drugs, specifically tacrolimus, cyclosporin A, sirolimus and everolimus. In addition to small molecule drugs, there are also protein-based drugs that include depleting and nondepleting antibodies (polyclonal and monoclonal antibodies), as well as fusion proteins [7]. The last category of commonly used immunosuppressant drugs are steroid based drugs, such as azathioprine and glucocorticoids [2, 7].

2.2.1 Tacrolimus

Tacrolimus, alternatively known as FK506, was first isolated from a strain of *Streptomyces* later classified as *Streptomyces tsukubensis* [8]. It was discovered in 1987 by a research team from Fujisawa Pharmaceutical Company. The name of this strain comes from the fact that it was isolated from soil samples collected in the area around the foot of Mount Tsukuba [9].

Tacrolimus works as an immunosuppressant by binding to FK506-binding protein 12 (FKBP12) [10] creating a complex that inhibits the function of calcineurin phosphatase [7]. Calcineurin phosphatase is involved in the activation of transcription factors in T-cells and is a part of the first signal in the three-signal model of the immune response [2]. During clinical trials it was discovered that an overdose of tacrolimus could have toxic effects on the pancreas and kidneys [9, 10]. Other reported side effects also include hypertension, hyperlipidaemia, post transplantation diabetes mellitus, etc [7, 11].

Tacrolimus is a 23 membered macrolide lactone with a molecular weight of 804 g/mol. The molecular formula is $C_{44}H_{69}NO_{12}$ [12, 13]. Figure 2-1 depicts the molecular structure of tacrolimus. Tacrolimus is soluble in polar protic and aprotic organic solvents such as

methanol (MeOH), ethanol, acetonitrile (ACN), acetone, etc., dissolves poorly in non-polar solvents such as hexane and petroleum ether, and is insoluble in water [8].

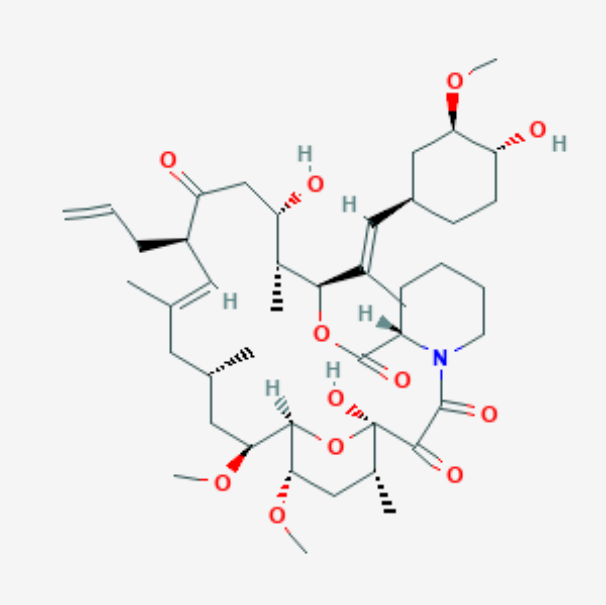


Figure 2-1 Molecular structure of tacrolimus. (Courtesy of PubChem database: PubChem Identifier: ***CID 445643****, URL: https://pubchem.ncbi.nlm.nih.gov/compound/445643#section=2D-Structure (downloaded 20.06.2020))[13]*

2.2.2 Sirolimus (rapamycin) and everolimus

Sirolimus was first isolated in 1975 by a research team from the Department of Microbiology, Ayerst Research Laboratories in Montréal, Canada from a strain of *Streptomyces* later classified as *Streptomyces hygroscopicus.* Sirolimus is alternatively known as rapamycin, which is a name that is inspired by the island where it was found in soil samples, Rapa Nui (Easter Island). Upon discovery, the research team primarily focused on its antifungal properties, especially against the yeast *Candida albicans* [14]. The immunosuppressive properties were only discovered later [15].

Everolimus is a derivative of sirolimus and was first made in 1998 by a research team from Novartis Pharma AG in Basel Switzerland. The goal of their research was to modify sirolimus with the purpose of making a compound that had fewer or less severe side effects, while still keeping its immunosuppressive abilities. Synthesis of everolimus is done with the alkylation of the C40-hydroxyl group on the sirolimus molecule [16].

Similarly to tacrolimus, sirolimus and everolimus also bind to FKBP12, but instead of inhibiting calcineurin phosphatase the complexes inhibit the molecular-target-of-rapamycin (mTOR) [7]. The mTOR is involved in the third signal in the three-signal model of the immune

response, which is activated by several interleukin signal molecules and other cytokines. But sirolimus and everolimus is especially involved in the inhibition of interleukin 2 (IL-2). This in turn inhibits cell proliferation in T-lymphocytes, B-lymphocytes, etc [15]. The side effects associated with sirolimus and everolimus include, but are not limited to hyperlipidaemia, thrombocytopenia and leukopenia, and nephrotoxicity [7, 17].

Sirolimus is a lipophilic macrocyclic lactone [15] with molecular weight of 914.2 g/mol. The molecular formula is $C_{51}H_{79}NO_{13}$[1] [18, 19], and its structure is depicted in Figure 2-2. It dissolves well in polar aprotic and protic solvents, dissolves poorly in non-polar solvents and is virtually insoluble in water [19].

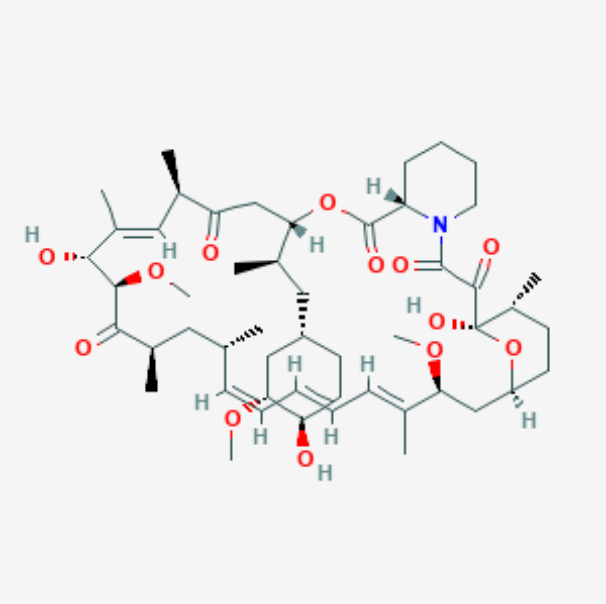


*Figure 2-2 Molecular structure of rapamycin (sirolimus). (Courtesy of PubChem database: PubChem identifier: **CID 5284616**, URL: https://pubchem.ncbi.nlm.nih.gov/compound/5284616#section=2D-Structure (downloaded 20.06.2020)) [18]*

Everolimus is as mentioned above a derivative of sirolimus, and therefore structurally and chemically similar. The molecular formula is $C_{53}H_{83}NO_{14}$ and the molecular weight is 958.2 g/mol [20]. Figure 2-3 depicts the molecular structure of everolimus.

[1] The molecular formula for sirolimus was reported as being $C_{56}H_{89}NO_{14}$ in the first published article describing the structure, but database sources report the molecular structure to be $C_{51}H_{79}NO_{13}$.

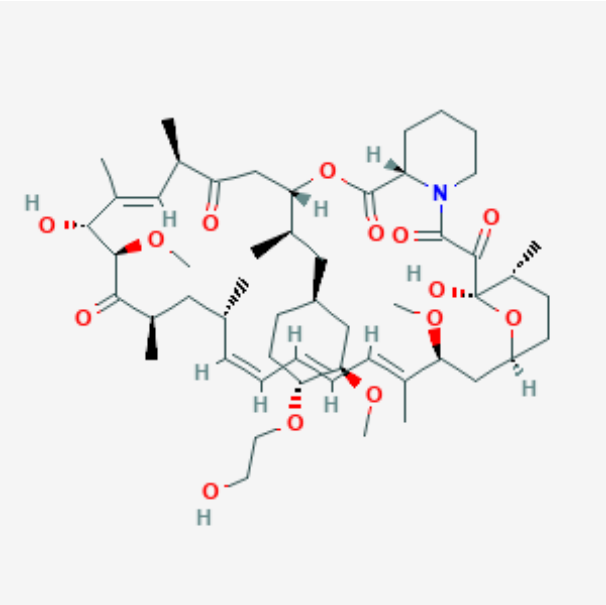


*Figure 2-3 Molecular structure of everolimus. (Courtesy of PubChem database: PubChem identified: **CID 6442177**, URL: https://pubchem.ncbi.nlm.nih.gov/compound/6442177#section=2D-Structure (downloaded 20.06.2020))[20]*

2.2.3 Cyclosporin A

The immunosuppressive effect of cyclosporin A was discovered in 1971-1972 when a research team from Sandoz Limited were treating mice with fermentation products from a strain of *Tylopocladium inflatum* discovered in soil samples from Norway. Cyclosporin A was however not properly identified before 1973 when the fermentation products from *T. inflatum* was purified and observed to mainly consist of this molecule [21]. The name of cyclosporin A comes from its cyclic structure, and from the fact that the molecule was found in the spores of *T. inflatum* [22].

Similarly to tacrolimus, cyclosporin A is also a calcineurin phosphatase inhibitor. But instead of binding to FKBP12, the molecule binds to the protein cyclophilin. This means the immunosuppressive function of cyclosporin A is the same as in tacrolimus. Both inhibit the proliferation of T-cells [11]. The side effects are also similar but in most cases more severe with cyclosporin A compared to tacrolimus. Side effects include nephrotoxicity, hypertension, hyperlipaemia, neurotoxicity, etc [7, 10, 11].

Cyclosporin A consists of 11 amino acids arranged in a cyclic peptide. This cyclic structure is depicted very clearly in Figure 2-4. The molecular formula is $C_{62}H_{111}N_{11}O_{12}$, and the molecular weight is 1202.6 g/mol [22, 23].

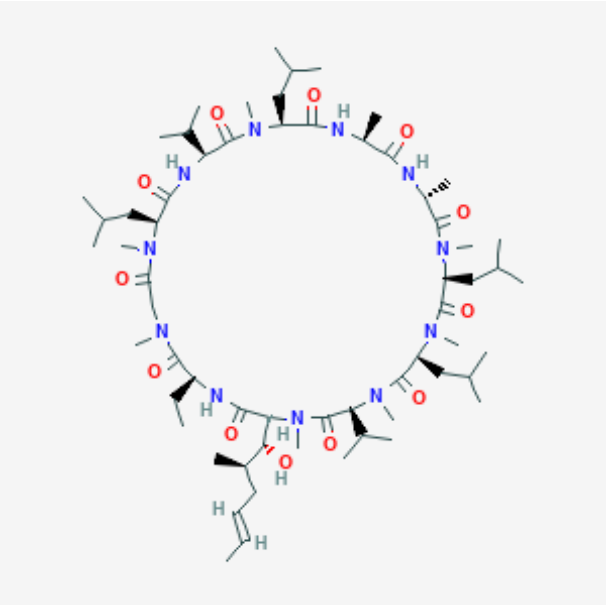


*Figure 2-4 Molecular structure of cyclosporin A (Courtesy of PubChem database: PubChem identifier: **CID 5284373**, URL: https://pubchem.ncbi.nlm.nih.gov/compound/5284373#section=2D-Structure (downloaded 20.06.2020))[23]*

2.3 Therapeutic drug monitoring

The issue with immunosuppressant drugs is that the therapeutic window is very narrow. The goal is to treat transplant patients with enough medicine to prevent organ rejection, but not enough to cause severe side effects. The treatment is reliant on both the pharmacokinetics of the drug, i.e. how fast the drug moves through the body, and the pharmacodynamics of the drug, i.e. how the body responds to the drug. Because these vary from patient to patient, the therapeutic window is determined by the concentration of the drug in the blood rather than the dose given to the patient. The concentration of drugs in the patient's blood is controlled through therapeutic drug monitoring (TDM). TDM is the coordination between an analysing laboratory's results and the physician's prescription of drugs when interpreting the results [24-26].

Plasma or serum has historically been the most common sample matrix for TDM of immunosuppressant drugs. However, this thesis will only focus on the analysis of immunosuppressants in whole blood. The reasoning behind this is that recent studies have shown that whole blood is the preferred matrix [27]. Some studies [28, 29] suggest that the distribution of tacrolimus and cyclosporin A in plasma or serum is very dependent on such factors as haematocrit and the temperature of the sample during centrifuging. Adaway and

Keevil [27] also point out that because of its hydrophobic properties, cyclosporin A will more readily partition into the red blood cells. Although whole blood does not seem to be a significantly better matrix for the analysis of sirolimus [30], the goal was to find a method for simultaneous sample preparation and analysis of tacrolimus, sirolimus, everolimus and cyclosporin A.

2.4 Instrumental analysis – LC-MS/MS versus immunoassay

The most common methods for analysing immunosuppressive drugs for the purpose of TDM are immunoassays and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Immunoassays and LC-MS/MS are based on very different analysing principles, and both methods have advantages and disadvantages. This thesis is based on the development of a method using LC-MS/MS. One advantage with LC-MS/MS is that it is very easy to develop methods for simultaneous analysis of several drugs, and there have in fact been many such methods developed in recent years, some of them described in articles [31-37].

The advantages with immunoassays are that they are simple, quick, cost effective and sensitive [38], but the largest disadvantage of immunoassays is that they are vulnerable to signal interference caused by cross-reactivity with metabolites of the drugs [39]. This is potentially an issue specifically when it comes to TDM of immunosuppressant drugs because cross reactivity with metabolites could lead to an overestimation of the presence of the active drug [37, 40].

2.4.1 Immunoassay

Immunoassay is an umbrella term that includes a large variety of analysing techniques with different detection methods and reactive mediums that all are based on the interaction between antibodies and antigens. Immunoassays can be quantitative, semi-quantitative or qualitative depending on the sensitivity of the method and the mechanisms involved. Immunoassays can be divided into two groups: competitive and non-competitive. In competitive immunoassays unlabelled antigens and labelled antigens have to compete with each other in order to bind to a limited amount of antibodies. In non-competitive immunoassays there is an abundance of antibodies. Immunoassays are often dependent on

washing steps to remove unbound antigens and/or antibodies before detection. Antigens or antibodies can be immobilised by adsorption to a solid phase. This solid phase can be coated or uncoated walls of the reaction wells or coated polymer particles. Sometimes these particles are magnetisable, making it possible to retain the analytes with magnets during a washing step. Detection is often based on antibodies or antigens labelled with for example radioactive isotopes (radioimmunoassay), fluorescent molecules (fluorimetry), or enzymes. [41].

There are many different immunoassay methods used for analysing immunosuppressants for the purpose of TDM, for example microparticle enzyme immunoassay (MEIA), one step immunoassay with chemiluminescent detection (Chemiflex, Abbott) [42], fluorescent polarization immunoassay (FPIA) [37], and chemiluminescence microparticle immunoassay [32]. These methods are similar in the sense that they are all based on the antibody-antigen interaction mentioned above but have different solid phases and detection methods.

2.4.2 Liquid chromatography coupled with tandem mass spectrometry

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the combination of a separation technique and a detection method. LC-MS/MS instruments are popular due to the wide range of molecules and substances these instruments can analyse, including simple and complex biological molecules, organic, and inorganic substances. These methods are also very sensitive and highly selective [43].

2.4.2.1 *High-performance liquid chromatography*

The instrument used in the development of a method for analysing immunosuppressants with the purpose of TDM discussed in this thesis applied the principle of ultra-performance liquid chromatography (UPLC), which is a modern version of high-performance liquid chromatography (HPLC). The different components of a HPLC instrument can be roughly summed up as: a mobile phase reservoir, sample injector, pump, injector, column, and detector (Figure 2-1). There are many different kinds of detectors, for example UV, diode array detectors, and mass spectrometers. The pump forces the mobile phase and sample material from the loop injector through the column and towards the detector. The separation of substances happens in the column itself [44].

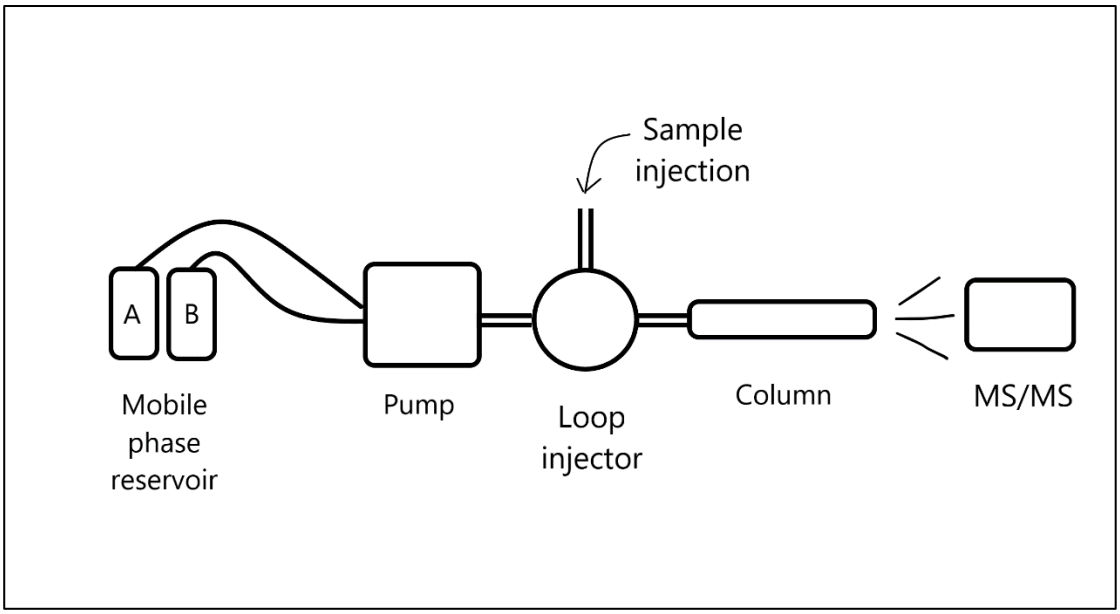


Figure 2-5 Simple illustration of the different components of a HPLC instrument. (Adapted from figure 2.1 in Liquid Chromatography – Mass spectrometry: an introduction by Bob Ardrey [44].)

The basic mechanism of liquid chromatography (LC) is the separation of substances by carrying them through a column with a mobile phase. The column is tightly packed with tiny particles and makes up the stationary phase. The mobile phase is usually a mix of water and a water miscible solvent. Molecules will travel through the column at different speeds depending on the intermolecular interactions between the analytes, the mobile phase, and the stationary phase. The most common type of LC is reversed-phase chromatography, which means the mobile phase is more polar than the stationary phase. Less polar substances will be retained longer by the particles in the column and released, or eluted, much slower than more polar substances [44]. The time it takes for a molecule to be eluted out of a column is called the retention time (tR), and is defined as the time interval from the injection of the sample and when the elution peak is at its highest [43].

Historically LC columns were vertical columns filled with relatively large particles, often larger than 200 µm, to allow the mobile phase travel though the column at a reasonable flow rate (volume/min). In HPLC instruments the columns are usually packed with particles that are around 2 µm in size. This means they can be very tightly packed and create a high chromatographic efficiency due to more equal molecular movement in the column causing less dilution. Because the resistance also increases when the particle size is very small, great pressure is necessary to force the analytes with the mobile phase through the column at a reasonable flow rate, usually over 20 000 psi in the modern UPLC [43].

2.4.2.2 *Tandem mass spectrometry*

One of the most common and advanced detectors for LC are mass spectrometers (MS). The instrument discussed in this thesis have detectors based on the principles of tandem mass spectrometry (MS/MS) specifically, which is an upgraded version of mass spectrometry. The appeal of tandem mass spectrometry is the increased selectivity due to the multiple reaction monitoring (MRM) of both precursor ion and fragmented ion.

The basic principle of MS is the detection of molecular ions. Before analytes are introduced to the detector, the mobile phase has to be sufficiently removed, and the molecules have to be ionized. There are several different ionization techniques ranging from hard ionization through electron impact ionization (EI), to soft ionization including fast atom bombardment (FAB), chemical ionization (CI) and electrospray ionization (ESI). These ionization techniques can produce both positive and negative ions [43].

The ionization technique used in the method development discussed in this thesis was positive electrospray ionization (ESI+). It is a useful ionization technique when analysing larger molecules because it does not cause a lot of fragmentation before detection. The ionization is done under in atmospheric pressure and temperature, and it involves fast injection of the eluted mobile phase through a capillary needle. This needle is encased in highly charged electrodes aimed towards a charged electrostatic lens that only lets positively charged ions pass through. This causes small droplets to form where most of the mobile phase is allowed to evaporate before the positive molecular ions reach electrostatic lens. This is illustrated in Figure 2-6. The formation of droplets is caused by what is called a Coulombic explosion. [43].

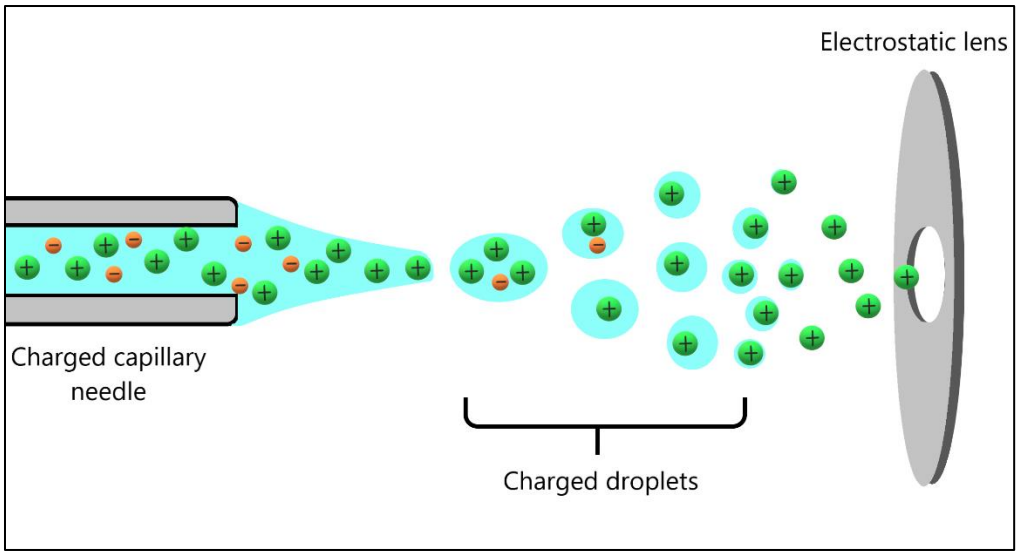


Figure 2-6 Illustration of positive electrospray ionization. Mobile phase containing analytes are injected through a charged capillary needle and aimed towards an electrostatic lens, forming droplets. (Adapted from illustration by Andreas Dahlin "ESI positive mode" [45].)

After ionization, the charged molecules move on towards the mass analyser. The mass analyser both detects and separates ions based on the mass-to-charge ratio (*m/z*). The (*m/z*) is defined as the ratio between the mass (Da) of the molecular ion and its fundamental charge. In ESI+ it is common to detect both single and double charged molecules. [46]. In MS/MS molecular ions are separated and detected twice. After the first detection the molecular ions go through a collision cell where they are subjected to a high voltage, which causes them to fragment. These new fragments will also have a charge and can be separated and detected just like their precursor ions [43].

The instrument discussed in this thesis used a quadrupole mass analyser. A quadrupole mass analyser consists of four metallic rods with two positively charged rods and two negatively charged rods positioned parallel to and opposite of each other (Figure 2-7). This charge creates an electrostatic field that cause molecular or fragment ions to oscillate. Ions with the wrong *m/z* ratio will have an unstable oscillation, causing them to deflect from the quadrupole. Ions with the correct *m/z* will oscillate in a controlled, focused spiral, making an ion beam that can be transmitted towards the detector. The electrostatic field can be manipulated to select for molecular ions with different *m/z* ratios by adjusting the direct-current voltage and radiofrequency applied to the rods [43].

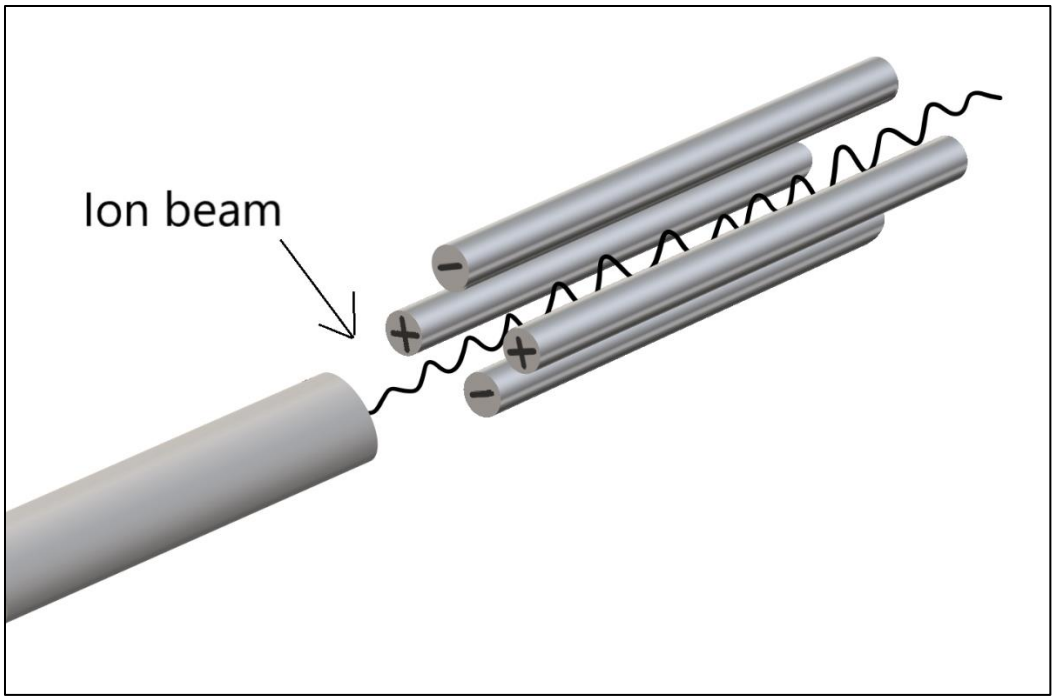


Figure 2-7 Illustration of an oscillating ion beam being lead through a quadrupole mass analyser. (Inspired by Figure 3.13 in Introduction to Spectroscopy, Pavia et.al. [46])

2.5 Sample preparation methods – established and new

There are already many published articles about development of methods for TDM of immunosuppressant drugs. These articles describe a variety of sample preparation methods and different sample matrices, usually plasma, serum, or whole blood. Blood based sample matrices contain a lot of proteins, lipids, cell material, etc., and in order to make the sample material suitable for analysis it may be necessary to either purify it, or to extract the analytes from the solution. A review article by Adaway and Keevil [27] compared several articles describing different sample preparation methods for the purpose of TDM and found that most methods included steps of protein precipitation (PPT). Some methods also included extraction methods such as online solid phase extraction (SPE) and liquid-liquid extractions (LLE). These methods will be described below. While doing research for the purpose of this thesis, no articles were found describing the use of salting-out assisted liquid-liquid extraction (SALLE) in sample preparation for TDM of immunosuppressants. This indicated there was an opening for the development of a novel sample preparation method.

2.5.1 Protein precipitation

Protein precipitation can be done by changing the chemical properties of the proteins in solution. This usually involves adding chemicals such as salts, acids, and solvents to the solution, which will cause the proteins to denature and therefore become more hydrophobic [47]. The most commonly used solvents for TDM methods are methanol and acetonitrile [32-35, 42, 48-51].

2.5.2 Solid phase extraction

The purpose of solid phase extraction (SPE) can both be to remove sample matrix and to increase the concentration of an analyte. The process of SPE can be divided into three steps: retention, washing/rinsing, and elution. There are many different types of solid phases comprising of various compounds that can be categorised into four groups based in the intermolecular interactions: polar (normal phase), non-polar (reversed-phase), weak anion exchange, and strong cation exchange. When a solution is injected into a reversed-phase SPE disk or column, non-polar analytes will be adsorbed to the solid phase. The solid phase can then be washed with a polar solvent (for example water) to flush out any substances with a higher polarity. The analyte is then eluted again with a non-polar solvent, often with a smaller volume than the injected volume [52].

In LC-MS/MS analysis analytes can be extracted through an online-SPE, meaning the SPE is performed in the instrument itself after injection. This process is however rather time consuming [27].

2.5.3 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is the partition of substances between two immiscible solvents of different polarities, usually water (aqueous phase) and a non-polar organic solvent (organic phase). Substances that are sparingly soluble in water can be extracted by mixing the aqueous solution very well with a non-polar solvent in a separatory funnel, creating a momentary emulsion. The hydrophobic substance will then partition into the non-polar solvent before the aqueous phase and the organic phase separates. The aqueous phase can be washed several times with the organic solvent to increase the yield [52, 53].

When LLE is involved in sample preparation for analysis on LC-MS/MS, there is a need for an additional evaporation step because the non-polar organic solvent is not compatible with the mobile phase of most LC-MS/MS systems [27].

2.5.4 Salting-out assisted liquid-liquid extraction

Salting-assisted liquid-liquid extraction (SALLE) work similarly to LLE in the sense that the principle of SALLE is the partition of a substance between two solvents. The difference is that the solvents used in SALLE are miscible, specifically water and polar organic solvents such as MeOH, ethanol, isopropyl alcohol, and ACN. Separation of these two solvents is possible because the aqueous phase is saturated with salt, usually NaCl, and the presence of salt disrupts the solvation forces between the two usually miscible solvents. A solute will then partition into the less polar organic solvent [54].

Analyte extraction through SALLE is very useful when analysing with LC-MS/MS because the organic phase is compatible with the mobile phase. This means there is no need for evaporation and re-dissolving, and the organic phase can be injected directly into the LC.

2.6 Method validation

Eurachem Guide: the Fitness for Purpose of Analytical Methods [55] is a document made with the purpose of guiding laboratories through the process of validating a new method. The document recommends certain performance characteristics for the new method and describes in detail how to execute these measurements and what the purpose behind them are. This document only functions as a guide, and analytical laboratories need to make their own protocol for which criteria need to be fulfilled in order for a method to be validated.

Selectivity:

Selectivity is not so much a quantifiable measurement as it is a gauge of how well a method is able to distinguish an analyte from its matrix without being affected by interference from other substances. Some constant interference will always be present and will be considered during analysis, but a method is most vulnerable to coincidental interference that may occur in different amounts between each analysis. Such interference may contribute to a method bias, which is desirable to keep as low as possible. The selectivity may be estimated by

analysing reference materials from an established method. Samples containing suspected interferences may also be analysed to see how much they affect the results.

Limit of detection (LOD) and limit of quantification (LOQ):

LOD and LOQ are important estimates for determining whether a real sample with a low measured concentration is in fact significantly different from zero, i.e. the detected signal is not from baseline interference. The difference between LOD and LOQ is that LOD is the lowest concentration that can be measured with a reasonable certainty, and LOQ is the lowest acceptable concentration that can be reported.

There are a few ways to estimate LOD and LOQ depending on which kind of analyte, and most importantly which type of matrix is going to be analysed. Some methods need to be blank corrected while others need not. This really depends on whether it is possible, or feasible to analyse samples with truly no analyte in them while still having the correct sample matrix. An adequate number, usually around 10, of blank samples, samples with very low concentrations, or reagent blanks, are analysed with the method, and the standard deviation (SD) is calculated. The method for calculating SD is different depending on the type of sample blank that is measured. Then the LOD is usually calculated by adding 3 x SD to the mean signal value, and LOQ is calculated by adding 10 x SD to the mean signal value.

Working range:

Working range is usually defined as the interval between the lowest and the highest quantity the method can measure with a specific certainty. This is estimated based on the linearity of the calibration curve. The lower end of the working range is usually the LOQ and the upper end of the working range is usually the estimated limit of linearity.

Analytical sensitivity:

This is not considered an important characteristic and not necessary for all methods, but it can for example be useful in spectrophotometric measurements. The analytical sensitivity is defined as “the change in instrument response which corresponds to a change in the measured quantity” by Eurachem Guide [55, p. 30].

Trueness – bias and recovery:

The purpose of trueness is to reflect how close the measured value is to the reference value. The theoretical definition would be the mean value of an infinite amount of identical samples, but that is of course impossible to measure. The trueness can therefore be substituted with a method's analytical bias. The bias can be determined in a number of ways, through either analysing reference material, or by spiking random samples and measuring the recovery. The bias and recovery can either be reported as the absolute bias or recovery, relative bias (%), or recovery (%). Bias can be calculated as the difference between the mean measured value and the reference value, while recovery can be measured as the percent of the mean measured value over the spiked value.

Precision – repeatability and reproducibility:

Eurachem guide defines precision as "a measure of how close results are to one another" [55, p. 35]. Identical samples, ideally 6-15, can be analysed over a short time span (for example the same day), with the same laboratory equipment, and the same analysist. The CV (%) between the measured values for these samples reflect the repeatability, i.e. the analytical method's ability to give similar results with the same analytical settings. Then, the CV (%) of the results for identical samples, ideally 6-15, analysed over a larger time scale can be calculated. This reflects the reproducibility precision.

Ruggedness (robustness):

Ruggedness is a gauge of how stable a method's results are when deliberate changes are made to the procedure. This is preferably done by estimating how the results may vary and measuring standardised reference material.

3 MATERIALS AND METHOD

3.1 Sample material

3.1.1 Samples used for testing pipetting method on Tecan Freedom EVO

TDM-mix 190708 (125 µL) was added to fresh EDTA whole blood (EDTA-WB) (25 mL) with no known concentration of the analytes. The resulting solution was mixed carefully, aliquoted, and kept frozen (-80 °C) to induce haemolysis.

3.1.2 Samples for testing limit of detection and limit of quantification

TDM LOD LOQ 990000:

Fresh EDTA-WB with no known concentration of the analytes was flash frozen (-80 °C) to induce haemolysis and aliquoted into 20 tubes, each tube containing 1 mL blood.

3.1.3 Quality controls

TDM QC (99999506):

TDM-mix 190708 (500 µL) was added to fresh EDTA-WB (100 mL) with no known concentration of the analytes. The resulting solution was mixed carefully and aliquoted into 100 labelled tubes, kept overnight in a refrigerator (4 °C) before being stored in a deep freeze (-80 °C) until use. The calculated theoretical concentration of each analyte was: tacrolimus (8.56 ng/mL), sirolimus (8.36 ng/mL), everolimus (8.31 ng/mL) and cyclosporin A (97.0 ng/mL).

TDM QC Low (99999941):

TDM-mix 190708 (50 µL) was added to fresh EDTA-WB (30 mL) with no known concentration of the analytes. The resulting solution was mixed carefully and aliquoted into 25 labelled tube and kept frozen (-80 °C) until use. The calculated theoretical concentration of each analytes was: tacrolimus (2.85 ng/mL), sirolimus (2.78 ng/mL), everolimus (2.77 ng/mL) and cyclosporin (32.6 ng/mL).

TDM QC Medium (99999942):

TDM-mix 190708 (200 µL) was added to fresh EDTA-WB (30 mL) with no known concentration of the analytes. The resulting solution was mixed carefully and aliquoted into 25 labelled tubes and kept frozen (-80 °C) until use. The calculated theoretical concentration of each analytes was: tacrolimus (11.4 ng/mL), sirolimus (11.1 ng/mL), everolimus (11.1 ng/mL) and cyclosporin A (131 ng/mL).

TDM QC High (99999943):

TDM-mix 190807 (550 µL) was added to fresh EDTA-WB (30 mL) with no known concentration of the analytes. The resulting solution was mixed carefully and aliquoted into 25 labelled tubes and kept frozen (-80 °C) until use. The calculated theoretical concentration of each analytes was: tacrolimus (31.4 ng/mL), sirolimus (30.6 ng/mL), everolimus (30.5 ng/mL) and cyclosporin A (359 ng/mL).

TDM QC Level 1 (99999401):

Fresh EDTA-WB (45 mL) with no known concentration of the analytes was frozen in order to induce haemolysis. The haemolysed EDTA-WB was then thawed and diluted with PBS (60 mL), making haemolysed EDTA-WB diluted with PBS (105 mL), hereafter called “Lys-FB-PBS”. TDM-mix 200115 (60 µL) was added to a 50 mL volumetric flask and diluted up to the mark with Lys-FB-PBS. The resulting solution was mixed thoroughly, aliquoted into 50 labelled tubes and kept frozen (-80 °C) until use. The calculated theoretical concentration of each analytes was: tacrolimus (3.50 ng/mL), sirolimus (2.27 ng/mL), everolimus (1.80 ng/mL) and cyclosporin A (38.4 ng/mL).

TDM QC Level 2 (99999402):

TDM-mix 200115 (600 µL) was added to a 50 mL volumetric flask and diluted up to the mark with Lys-FB-PBS. The resulting solution was mixed thoroughly, aliquoted into 50 labelled tubes and kept frozen (-80 °C) until use. The calculated theoretical concentration of each analytes was: tacrolimus (35.0 ng/mL), sirolimus (22.7 ng/mL), everolimus (18.0 ng/mL) and cyclosporin A (384 ng/mL).

3.1.4 Samples used for optimisation of sample preparation

TDM-mix 190708 (250 µL) was added to a 50 mL volumetric flask and diluted to the mark with fresh EDTA-WB with no known concentration of the analytes. The resulting solution was

mixed thoroughly, aliquoted into 25 labelled tubes, and kept frozen (-80 °C) until use. The calculated theoretical concentration of each analyte was: tacrolimus (8.56 ng/mL), sirolimus (8.36 ng/mL), everolimus (8.31 ng/mL) and cyclosporin A (97.0 ng/mL).

3.1.5 Samples used for recovery testing

Blood samples (950 µL) from 10 random individuals were spiked with TDM-mix 190708 (50 µL), and then flash frozen (-80 °C) to induce haemolysis. The calculated theoretical concentration of each analyte was: tacrolimus (8.56 ng/mL), sirolimus (8.35 ng/mL), everolimus (8.31 ng/mL) and cyclosporin A (97.9 ng/mL).

3.1.6 Anonymised patient samples from Stavanger University Hospital

A total of 100 anonymized samples were collected from the laboratory of Medical Biochemistry in Stavanger University Hospital (SUS) after being analysed for tacrolimus and cyclosporin A. They were frozen and stored in temperatures of -20 °C until use. The analysis of these samples for the purpose of comparing the new method with an existing method without patient consent was approved by the Regional Ethics Committee (REK vest, reference number 80982). This document is included in

3.1.7 Anonymised patient samples from Oslo University Hospital

Anonymised patient samples were received from Oslo University Hospital (OUS). There were 30 samples per analyte (tacrolimus, sirolimus, everolimus and cyclosporin A), making a total of 120 samples. The use of these samples without patient consent for the purpose of comparing sample preparation and analysis was approved by the Regional Ethics Committee (REK vest, reference number 80982).

3.2 Calibration standards

3.2.1 Calibration standards in methanol (lot: 2019-001)

TDM-mix 190708 (100 µL) was added to a 25 mL volumetric flask and diluted with methanol (MeOH), mixed well, and transferred to 25 mL glass vials. A serial dilution was then made by transferring 10 mL solution to 25 mL volumetric flasks in succession. Concentrations of each analyte can be found in Table 3-1.

Table 3-1 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in MeOH calibration standard STD# 0 - STD# 6.

STD#	Contains:	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
STD# 0	Blank	0	0	0	0
STD# 1	STD# 2 (10 mL)	0.0701	0.0684	0.0680	0.802
STD# 2	STD# 3 (10 mL)	0.175	0.171	0.170	2.01
STD# 3	STD# 4 (10 mL)	0.438	0.428	0.426	5.01
STD# 4	STD# 5 (10 mL)	1.10	1.07	1.06	12.5
STD# 5	STD# 6 (10 mL)	2.74	2.67	2.66	31.3
STD# 6	TDM-mix (0.1 mL)	6.85	6.68	6.65	78.3

3.2.2 Calibration standards in new-born calf serum (TDM NBCS STD#0-7).

Calibration standards TDM NBCS were made by adding different volumes of TDM-Mix 190708 to 25 mL volumetric flasks and diluting up to the mark with new-born calf serum. The volume (µL) of TDM-mix 190708 added to each standard level is described in Table 3-1, along with the calculated concentration (ng/mL) for each analyte.

Table 3-2 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM NCBS STD calibration standard STD# 0 – STD# 7.

	TDM-mix 190708 (µL)	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
TDM NCBS STD#0	0	0	0	0	0
TDM NCBS STD#1	50	3.42	3.34	3.32	39.1
TDM NCBS STD#2	75	5.12	5.00	4.97	58.6
TDM NCBS STD#3	100	6.82	6.66	6.62	78.0
TDM NCBS STD#4	200	13.6	13.3	13.2	155
TDM NCBS STD#5	300	20.3	19.8	19.7	232
TDM NCBS STD#6	500	33.6	32.8	32.6	384
TDM NCBS STD#7	1500	96.9	94.6	94.1	1108

3.2.3 Calibration standards in EDTA whole blood (TDM WB STD#0-7)

Calibration standards TDM WB were made by adding different volumes of TDM-Mix 190708 to 10 mL volumetric flasks and diluting up to the mark with EDTA-WB. The volume (µL) of TDM-mix 190708 added to each standard level is described in Table 3-3, along with the calculated concentration (ng/mL) for each analyte.

Table 3-3 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM WB STD calibration standard STD# 0 – STD# 7.

	TDM-mix 190708 (µL)	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
TDM WB STD#0	0	0	0	0	0
TDM WB STD#1	15	2.57	2.51	2.49	29.4
TDM WB STD#2	30	5.13	5.01	4.99	58.7
TDM WB STD#3	50	8.56	8.36	8.31	97.9
TDM WB STD#4	80	13.7	13.4	13.3	157
TDM WB STD#5	160	27.4	26.7	26.6	313
TDM WB STD#6	320	54.8	53.5	53.2	627
TDM WB STD#7	640	110	107	106	1253

3.2.4 Calibration standards in EDTA whole blood diluted with phosphate buffered saline (TDM WB-PBS STD#0-7)

Fresh EDTA-WB was diluted to a ratio of 1:2 with phosphate buffered saline (PBS), making EDTA-WB with PBS (EDTA-WB-PBS). Calibration standards TDM WB-PBS were made by adding different volumes of TDM-Mix 190708 to 10 mL volumetric flasks and diluting up to the mark with EDTA-WB-PBS. The volume (µL) of TDM-mix 190708 added to each standard level is described in Table 3-4, along with the calculated concentration (ng/mL) for each analyte.

Table 3-4 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM WB-PBS STD calibration standard STD# 0 – STD# 7.

	TDM-mix 190708 (µL)	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
TDM WB-PBS STD#0	0	0	0	0	0
TDM WB-PBS STD#1	15	2.57	2.51	2.49	29.4
TDM WB-PBS STD#2	30	5.13	5.01	4.99	58.7
TDM WB-PBS STD#3	50	8.56	8.36	8.31	97.9
TDM WB-PBS STD#4	80	13.7	13.4	13.3	157
TDM WB-PBS STD#5	160	27.4	26.7	26.6	313
TDM WB-PBS STD#6	320	54.8	53.5	53.2	627
TDM WB-PBS STD#7	640	110	107	106	1253

3.2.5 Calibration standards in pre-haemolysed EDTA whole blood diluted with phosphate buffered saline (TDM WB-PBS-Lys STD#0-7)

Fresh EDTA-WB was frozen (-20°C) to induce haemolysis. This haemolysed EDTA-WB was then diluted with PBS in a ratio of 1:2, making haemolysed EDTA-WB with PBS (Lys-EDTA-WB-PBS). Calibration standards TDM WB-PBS-Lys were made by adding different volumes of TDM-Mix 190708 to 10 mL volumetric flasks and diluting up to the mark with Lys-EDTA-WB-PBS. The volume (µL) of TDM-mix 190708 added to each standard level is described in Table 3-5 along with the calculated concentration (ng/mL) for each analyte.

Table 3-5 Concentrations of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM WB-PBS-Lys STD calibration standard STD# 0 – STD# 7.

	TDM-mix 190708 (µL)	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
TDM WB-PBS-Lys STD#0	0	0	0	0	0
TDM WB-PBS-Lys STD#1	15	2.57	2.51	2.49	29.4
TDM WB-PBS-Lys STD#2	30	5.13	5.01	4.99	58.7
TDM WB-PBS-Lys STD#3	50	8.56	8.36	8.31	97.9
TDM WB-PBS-Lys STD#4	80	13.7	13.4	13.3	157
TDM WB-PBS-Lys STD#5	160	27.4	26.7	26.6	313
TDM WB-PBS-Lys STD#6	320	54.8	53.5	53.2	627
TDM WB-PBS-Lys STD#7	640	110	107	106	1253

3.2.6 Calibration standards in pre-haemolysed EDTA whole blood diluted with phosphate buffered saline (TDM Lys-WB-PBS STD#0-7)

Fresh EDTA-WB was frozen (-20°C) to induce haemolysis. This haemolysed EDTA-WB was then diluted with PBS in a ratio of 1:2, making haemolysed EDTA-WB with PBS (Lys-EDTA-WB-PBS). Calibration standards TDM Lys-WB-PBS were made by adding different volumes of TDM-Mix 190708 to 10 mL volumetric flasks and diluting up to the mark with Lys-EDTA-WB-PBS. The volume (µL)of TDM-mix 190708 added to each standard level is described in Table 3-6**Error! Reference source not found.**, together with the calculated concentration (ng/mL) for each compound.

Table 3-6 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM Lys-WB-PBS STD calibration standard STD# 0 – STD# 7.

	TDM-mix 190708 (µL)	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
TDM Lys-WB-PBS STD#0	0	0	0	0	0
TDM Lys-WB-PBS STD#1	15	2.57	2.51	2.49	29.4
TDM Lys-WB-PBS STD#2	30	5.13	5.01	4.99	58.7
TDM Lys-WB-PBS STD#3	50	8.56	8.36	8.31	97.9
TDM Lys-WB-PBS STD#4	80	13.7	13.4	13.3	157
TDM Lys-WB-PBS STD#5	160	27.4	26.7	26.6	313
TDM Lys-WB-PBS STD#6	280	47.9	46.8	46.5	548
TDM Lys-WB-PBS STD#7	500	85.6	83.5	83.1	979

3.2.7 6PLUS1® Multilevel Whole Blood Calibrator set, Mass*Tox*® Immunosuppressants in whole blood (MassTox STD#0-6)

This was a calibrator set purchased from ChromSystems. It comprised of lyophilised human whole blood with different concentrations of tacrolimus, sirolimus, everolimus and cyclosporin A. One kit included a set of 6 calibration levels with one blank. The calibrator set was prepared according to the manufacturer's instructions. The concentration (ng/mL) of each analyte is described in Table 3-7.Table 3-7 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in MassTox calibration standard.

Table 3-7 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in MassTox calibration standard.

	Tacrolimus (ng/mL)	Sirolimus (ng/mL)	Everolimus (ng/mL)	Cyclosporin A (ng/mL)
MassTox STD#0	0	0	0	0
MassTox STD#1	1.44	2.46	1.92	23
MassTox STD#2	5.66	6.62	4.8	126
MassTox STD#3	11.5	12.9	8.91	314
MassTox STD#4	16.7	19.4	13.2	511
MassTox STD#5	22.9	28.9	18.4	791
MassTox STD#6	39.4	47.8	32.6	1003

3.3 Solutions

3.3.1 TDM-Mix 190708

Stock solutions of tacrolimus, rapamycin (sirolimus), everolimus and cyclosporin A were made respectively by the weighing the compounds and dissolving in MeOH in 25 mL volumetric flasks. The different compounds came with analytical certificates guaranteeing a specific purity for each compound. The purity was used to accurately calculate the concentration of the resulting solutions. The mass (mg), volume (mL), purity (%), and adjusted concentration (ng/mL) are described in Table 3-8.

Table 3-8 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in stock solution of each analyte.

	Tacrolimus monohydrate	**Rapamycin (sirolimus)**	**Everolimus**	**Cyclosporin A**
M.W. (g/mol)	822.03	914.17	958.22	1202.61
Amount (mg)	2.3	2.16	2.63	2.45
Vol. (mL)	25	25	25	25
Purity (%)	95.1	96.7	79	99.9
Mole ratio	0.97808	1	1	1
Conc. (ng/mL)	0.092	0.0864	0.1052	0.098
Adjusted conc. (ng/mL)	0.0856	0.0835	0.0831	0.0979

TDM-mix 190708 was made by adding the stock solutions in different volumes to one 25 mL volumetric flask and diluting the mix up to the mark with MeOH. The volume (µL) of stock solution and the resulting concentrations (ng/mL) are described in Table 3-9.

Table 3-9 Concentration of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM-mix 190708.

	Volume (µL)	**Concentration (ng/mL)**
Tacrolimus	500	1711
Sirolimus	500	1671
Everolimus	500	1662
Cyclosporin	5000	19580

3.3.2 TDM-mix 200115

Stock solutions of tacrolimus, rapamycin (sirolimus), everolimus and cyclosporin A were made respectively by weighing the compounds and dissolving them in MeOH in 25 mL volumetric flasks. The different compounds came with analytical certificates that guaranteed a specific purity of each compound. The purity was used to accurately calculate the

concentration on the resulting solutions. The mass (mg), volume (mL), purity (%), and adjusted concentration (ng/mL) are described in Table 3-10.

Table 3-10 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in stock solution

	Tacrolimus monohydrate	Rapamycin (sirolimus)	Everolimus	Cyclosporin A
M.W. (g/mol)	822.03	914.17	958.22	1202.61
Amount (mg)	4.9	3.06	2.96	10
Vol. (mL)	25	25	25	25
Purity (%)	95.1	96.7	79	99.9
Mole ratio	0.97808	1	1	1
Conc. (ng/mL)	0.196	0.1224	0.1184	0.4
Adjusted conc. (ng/mL)	0.182311	0.118360	0.093536	0.3996

TDM-mix 200115 was made by adding different volumes of the stock solutions to one 25 mL volumetric flask and diluting the mix up to the mark with MeOH. The volume (µL) of stock solution and the resulting concentration (ng/mL) are described in Table 3-11**Error! Reference source not found.**.

Table 3-11 Concentration (ng/mL) of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM-Mix 200115

	Volume (µL)	Concentration (ng/mL)
Tacrolimus (190705)	400	2917
Sirolimus (190705)	400	1894
Everolimus (190705)	400	1497
Cyclosporin (190705)	2000	31968

3.3.3 TDM internal standard stock solutions

Internal standard (ISTD) stock solutions of tacrolimus (^{13}C, D_2), rapamycin (sirolimus, D_3), everolimus (D_4) and cyclosporin A (D_4) were made respectively by weighing each compound and dissolving them in MeOH (25 mL). The mass (mg) of the compounds and the resulting concentration (mg/mL) are described in Table 3-12.

Table 3-12 Concentration (ng/mL) of tacrolimus (13C, D2), sirolimus (D3), everolimus (D4) and cyclosporin A (D4) stock solutions

	Mass (mg)	Volume (mL)	Concentration (mg/mL)
Tacrolimus (13C, D2) ISTD	1	25	0.04
Sirolimus (D3) ISTD	1	25	0.04
Everolimus (D4) ISTD	1	25	0.04
Cyclosporin A (D4) ISTD	1	25	0.04

3.3.4 TDM ISTD user solution 190509

An ISTD user solution was made by transferring a specific volume of the different stock solutions to a bottle (250 mL) and diluting with MeOH to the mark. The volume (µL) of stock solution added and resulting concentration (ng/mL) are described in Table 3-13.

Table 3-13 Concentration (ng/mL) of ISTD of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM ISTD user solution 190509

	Volume stock solution (µL)	**Concentration (ng/mL)**
Tacrolimus ISTD	250	40
Sirolimus ISTD	250	40
Everolimus ISTD	250	40
Cyclosporin ISTD	250	40

3.3.5 TDM ISTD user solution 191206

An ISTD user solution was made by adding the different stock solutions to a 250 mL laboratory bottle and diluting with MeOH. The volume (µL) of stock solution added and resulting concentration (ng/mL) are described in Table 3-14.

Table 3-14 Concentration (ng/mL) of ISTD of tacrolimus, sirolimus, everolimus and cyclosporin A in TDM ISTD user solution 191206

	Volume stock (µL)	**Concentration (ng/mL)**
Tacrolimus ISTD	250	40
Sirolimus ISTD	250	40
Everolimus ISTD	250	40
Cyclosporin ISTD	500	80

3.3.6 Saturated sodium chloride solution (5 M, 250 mL)

NaCl (73.1 g) was added to a 250 mL borosilicate bottle and dissolved in deionised water (250 mL).

3.3.7 Zinc sulphate solution (0.1 m, 30 mL)

$ZnSO_4$ (0.86 g) was added to a 30 mL test tube and dissolved in deionised water (30 mL).

3.3.8 Magnesium sulphate solution (2.7 M, 30 mL)

$MgSO_4 \cdot 7\ H_2O$ (20 g) was added to a 30 mL test tube and dissolved in deionised water (30 mL).

3.3.9 Phosphate buffered saline (PBS)

The contents of BupH™ Phosphate Buffered Saline Pack were added to a 1 L laboratory bottle and filled with deionised water (500 mL), giving a concentration of sodium phosphate (0.1 M) and NaCl (0.15 M).

3.3.10 ELGA water

“ELGA water” is ultra-purified water made with the water treatment system ELGA PURELAB® Ultra Genetic. Properties of the treated water were inorganics (18.2 MΩ-cm), total organic carbon (TOC) (<1 ppb), pH (effectively neutral), bacteria (<0.1 CFU/mL).

3.3.11 Formic acid solution (0.2 %)

ELGA water (2000 mL) and formic acid (4 mL) was added to a glass laboratory bottle. Used in the mobile phase.

3.4 Chemicals:

3.4.1 Analytes and ISTD

Table 3-15 List of commercially purchased immunosuppressants and internal standards, with their chemical purity and manufacturer

Analyte	CAS	Manufacturer	Product number	Purity	Isotopic impurity
FK506 (Tacrolimus)	104987-11-3	Sigma-Aldrich	PHR1809-150MG	95.1 %	-
Sirolimus (Rapamycin)	53123-88-9	Sigma-Aldrich	37094-10MG	96.7 %	-
Everolimus	159351-69-6	Sigma-Aldrich	94687-10MG	79 %	-
Cyclosporin A	59865-13-3	Sigma-Aldrich	32425-100MG	99.9 %	-
FK506 (Tacrolimus) 13C, D2	1356841-89-8	Toronto Research Chemicals	F370002	> 85 %	(not specified)
Rapamycin (Sirolimus) D3	392711-19-2	Toronto Research Chemicals	R124002	Technical grade	6.05 %[2]
Everolimus D4	1338452-54-2	Toronto Research Chemicals	E945402	>85 %	0 %
Cyclosporin A D4	-	Toronto Research Chemicals	C988902	95 %	0.07 %

3.4.2 Other chemicals

Table 3-16 List of other chemicals, their purity and manufacturer

Chemical	Manufacturer	Purity
Acetone	VWR Chemicals	HPLC grade
Acetonitrile (ACN),	Rathburn	LC-MS grade
Methanol (MeOH),	Fisher Scientific	>= 99.9 %, LC-MS grade
Tetrahydrofuran (THF)	VWR Chemicals	99.70 &
Formic acid (HCOOH)	VWR Chemicals	LC-MS grade
Sodium chloride (NaCl)	Merck	99.8 %
Magnesium sulphate ($MgSO_4 \cdot 7\ H_2O$)	Merck	≥ 99.5 %
Zinc sulphate ($ZnSO_4 \cdot 7\ H_2O$)	Merck	≥ 99.0 %
BupH™ Phosphate Buffered Saline Pack	Thermo Scientific	(not specified)

[2] The manufacturer reports in the analytical certificate that the compound contains approximately 6.05 % of the unlabelled rapamycin (sirolimus).

3.5 Laboratory equipment

Table 3-17 List of laboratory equipment with manufacturer and purpose.

Item	Manufacturer	Purpose
VACUETTE®EDTA (K_2EDTA), 6 mL / 9 mL	Greiner Bio-One GmbH	Collection of whole blood for making quality controls and calibration standards
Micro tube 1.5 mL, PP	Sarstedt	Reaction tubes for optimisation experiment
Nunc™ 96-well microplate, MicroWell™, PP	Thermo Fisher	Sample container for use in LC-MS/MS instruments
Nunc™ 96-well storage plate, DeepWell™ 1.3 mL, PP	Thermo Fisher	Sample container for use in LC-MS/MS instruments
Nunc™ 96-well cap mat	Thermo Fisher	Protective cap mat for microplates and storage plates.
Capped tube, 7 mL, PP	Sarstedt	Container for quality controls and calibration standards

3.6 Instruments and software

3.6.1 LC-MS/MS instruments

The LC-system used in the development of the analysis method discussed in this thesis was ACQUITY™ Ultra performance LC® system purchased from Waters™. This system is comprised of four modules: sample manager, column manager, sample manager, and binary solvent manager. The product name for the MS detector was Xevo TQ-S, and the ionization source was ZSpray™ (ESI|APCI|EsCi).

The LC-column was an ACQUITY UPLC® BEH C18 column with inner dimensions of 2.1 x 50 mm, and a particle size of 1.7 µm.

3.6.2 Sample preparation – pipetting robot and mixer

The samples were pipetted with a Tecan Freedom EVO® 150 pipetting robot purchased from Tecan Trading AG, Switzerland.

Samples, calibrator, and controls were thawed by rotating in an Elmi Rotamix RM1 from GH Zeal Ltd.

3.6.3 Processing software

The results from the LC-MS/MS instrument was processed using the software MassLynx V4.2 SCN986.

Bland-Altman plot analysis was calculated with Analyse-it for Microsoft Excel (ver. 5.3).

3.7 Method development

3.7.1 Instrumentation

3.7.1.1 *Tuning of tandem mass spectrometer for multiple reaction monitoring*

Stock solutions of analytes tacrolimus, sirolimus, everolimus and cyclosporin A, and corresponding internal standards were diluted with MeOH in a ratio of 1:100. This was done to make solutions suitable for tuning for multiple reaction monitoring (MRM) in tandem mass spectrometry (MS/MS). The theoretical mass-to-charge ratio (*m/z*) for proton (H^+), sodium (Na^+), and ammonium (NH_4^+) adducts, or combinations of these, were calculated prior to tuning. These calculations can be found in Appendix 2.

The detector was switched to the positive electrospray ionization mode (ESI+). The flowrate was set to 0.005 mL/min, and the mobile phase was set to an isocratic gradient with 75 % MeOH (solvent B) and 25 % formic acid (0.2 %) (solvent A). For the sake of simplicity and compatibility, the capillary voltage was set to 2 kV and the cone voltage was set to 20 V. Neither were changed during tuning.

The diluted solutions of each analyte and internal standard were injected directly into the instrument via reservoir channels. An initial scan (MS1 scan) in MS mode detected molecular ions. The *m/z* of these ions were recorded and compared with the calculated *m/z* to identify which type of ion adducts were the most prevalent. A daughter scan in MS/MS mode detected fragments from the precursor ions found in MS1 scan. The scan was done by starting out with a lower collision energy and gradually increasing until several fragments with different *m/z* could be detected. The collision energy was adjusted until one or more fragments had a high and narrow enough signal to be isolated from the surrounding noise. The *m/z* of these fragments the collision energy (eV) was recorded.

3.7.1.2 *Development of inlet method for LC-separation*

TDM-mix 190708 (50 µL) was diluted with MeOH (200 µL) and pipetted into a well on a polypropylene (PP) microplate. This solution was analysed several times in order to find the optimal solvent for the mobile phase, and to optimise the elution gradient.

The first series of analyses were done to compare how well the different organic solvents were able to separate and elute the analytes. A linear gradient was made by injecting the sample into the column at a flow rate of 0.500 mL/min and increasing the ratio of solvent A to B from 1 % to 99% over the course of 10 min.

Further experiments were performed to find the optimal step gradient for good separation and even elution. The solvent gradient was adjusted by programming the pumps in the LC-instrument to change the ratio (%) between organic solvent and water at specific time intervals.

When the optimal solvent gradient was found, the solvent A to solvent B ratio (%) and timing (min) were programmed as parameters into the inlet file for the finished instrumental analysis method.

3.7.2 Optimisation of sample preparation

The design of the optimisation experiments was based on the hypothesis that SALLE in sample preparation would make a solution with high concentrations of analytes suitable for injection into the LC-MS/MS. The purpose of this experiment was to compare sample preparation with SALLE to an existing sample preparation method. While searching for published articles about analysis for immunosuppressants drugs on LC-MS/MS instruments, many were found to describe methods involving PPT with either MeOH [32-35, 50, 51], ACN [48, 49], or a combination of both [34, 42, 56]. Some of these articles also included the addition of $ZnSO_4$ solution as a clearing agent. To simplify the process, one sample preparation experiment involving PPT with MeOH and $ZnSO_4$ (0.1 M) was designed to represent the methods in these articles.

3.7.2.1 *Hansen solubility parameters*

Hansen solubility parameter (HSP) was used to predict which solvents or combinations of solvents would give the highest solubility of cyclosporin A. Only water miscible solvents

available in the lab were included in the search. There were no HSP data available for the other analytes at the time of research. The HSP values for cyclosporin A, acetone, ACN, MeOH and tetrahydrofuran (THF) are described in Table 3-18.

Table 3-18 Hansen solubility parameters for cyclosporin A, acetone, ACN, MeOH and THF

Compound	δD (dispersion)	δP (polarity)	δH (hydrogen bonding)
Cyclosporine A	20.0	8.8	6.6
Acetone	15.5	10.4	7
ACN	15.3	18	6.1
MeOH	14.7	12.3	22.3
THF	16.8	5.7	8

3.7.2.2 *Optimisation experiments*

All optimisation experiments were prepared by transferring sample material (described in paragraph 3.1.4) (50 µL) to polypropylene (PP) vials and adding various reagents before mixing well and centrifuging at 4000xG for 10 min. These reagents included saturated NaCl (5 M), $MgSO_4$ (2.7 M), $ZnSO_4$ (0.1 M), MeOH, ACN, acetone and THF. Each experiment was repeated with three parallels.

After centrifugation, supernatant (200 µL) was transferred from the vial into a well on a microplate, regardless of the volume of the supernatant or the organic phase. ISTD user solution 190506 (50 µL) was also added to each sample on the microplate.

Some experiments were quantified by including a solvent calibration standard (described in paragraph 3.2.1) in the analysis. The comparison of these experiments was based on concentration (ng/mL) and ISTD area. Some experiments did not include a solvent calibration standard and were instead compared through response and ISTD area.

The MRM transitions used for these experiments were: tacrolimus $[M+Na]^+$ *m/z* 826.5 > 616.2, tacrolimus 13C, D2 $[M + Na]^+$ *m/z* 829.5 > 619.2, sirolimus $[M + Na]^+$ *m/z* 936.5 > 409.2, sirolimus D3 $[M + Na]^+$ *m/z* 939.5 > 409.2, everolimus $[M + Na]^+$ *m/z* 980.5 > 389.2, everolimus D_4 $[M + Na]^+$ *m/z* 984.5 > 393.2, cyclosporin A $[M + H]^+$ *m/z* 1202.7 > 156.1, and cyclosporin A D_4 $[M + H]^+$ *m/z* 1206.8 > 156.1.

The solvent gradient used for these experiments is described in Table 4-2.

3.7.2.3 *Extraction efficiency*

The extraction efficiency was measured in some of the sample preparations that achieved phase separation. The samples involved are described in paragraph 4.3.3. After supernatant (200 µL) had been transferred to a microplate for analysis, any leftover supernatant was volumetrically measured. This was done by collecting the organic phase in different volumes using a pipette until there was no organic phase left.

Quantification of these analyses were based on a solvent calibration standard (described in paragraph 3.2.1).

3.7.3 Method validation

The hospital laboratory has its own procedures for validating a new method introduced to the routine. However, the validation of the experiments done in this thesis were based on the requirements described in Eurachem Guide: The Fitness for Purpose of Analytical Methods [55].

The final sample preparation was based on the results from the optimisation experiments. The difference between the sample preparation in the final method and in the optimisation experiments was that the ISTD was added to the samples together with the rest of the reagents. This replaced the MeOH in the optimized SALLE method with 12.5 % (v/v) MeOH in ACN. The MRM transitions for these experiments were the same in the optimisation experiments and are described in paragraph 3.7.2.2. The mobile phase consisted of MeOH (solvent B) and formic acid (0.2 %) (solvent A), and the elution gradient (%A : %B) is described in Table 4-2.

3.7.3.1 *Limit of detection and limit of quantification*

19 blank EDTA-WB sample material (described in paragraph 3.1.2) (50 µL), NaCl (5 M, 100 µL), ACN (350 µL) and TDM ISTD user solution 191206 (described in paragraph 3.3.5) (50 µL) was pipetted into polypropylene 96-well storage plates, mixed well by shaking for 15 seconds, and centrifuged at 4000xG for 10 min. The analysis was calibrated with MassTox calibration standard (described in paragraph 3.2.7).

3.7.3.2 *Trueness – determination of relative recovery*

10 spiked EDTA-WB samples (described in paragraph 3.1.5) were analysed to calculate the recovery bias. Spiked blood sample (50 µL), NaCl (5 M, 100 µL), ACN (350 µL) and TDM ISTD user solution 190509 (described in paragraph 3.3.4) (50 µL) were transferred to PP 96-well storage plates, mixed well by shaking for 15 seconds, and centrifuged at 4000xG for 10 min. The analysis was calibrated with TDM WB-PBS-Lys (described in paragraph 3.2.5).

3.7.3.3 *Precision – repeatability*

10 aliquots of TDM QC Low (99999941), TDM QC Medium (99999942) and TDM QC High (99999943) (described in paragraph 3.1.3), and one calibration standard set were mixed until the vials reached room temperature. Sample material (50 µL), NaCl (5 M, 100 µL), ISTD user solution 190509 (50 µL) and ACN (350 µL) were transferred to a PP 96-well 1.3 mL storage plate, mixed well by shaking for 15 seconds, and centrifuged at 4000xG for 10 min before analysis. The concentration (ng/mL) of the quality controls were calculated with calibration standard TDM NBCS STD#0-7 (described in 3.2.2).

3.7.3.4 *Precision – reproducibility*

Quality controls TDM QC (99999506), TDM QC Low (99999941), TDM QC Medium (99999942) and TDM QC (99999943) (described in paragraph 3.1.3) were analysed 23 times over the course of four months. New quality controls and a new calibration standard set was thawed before each analysis, and then discarded after use.

Calibration standards, quality controls and samples were mixed until the vials reached room temperature. Sample material (50 µL), NaCl (5 M, 100 µL), TDM ISTD user solution (50 µL)[3] and ACN (350 µL) were transferred to a PP 96-well 1.3 mL storage plate, mixed well by shaking for 15 seconds, and centrifuged at 4000xG for 10 min before analysis.

The concentration (ng/mL) of the quality controls were calculated with calibration standard TDM NBCS STD #0-7 (paragraph 3.2.2), TDM WB STD#0-7 (paragraph 3.2.3), TDM WB-PBS STD#0-7 (paragraph 3.2.4), TDM WB-PBS-Lys STD#0-7 (paragraph 3.2.5) and TDM Lys-WB-PBS STD#0-7 (paragraph 3.2.6).

[3] TDM ISTD user solution 190509 was eventually replaced with TDM ISTD user solution 191206.

3.7.4 Method comparison with Abbot Architect immunoassay

A total of 100 anonymised samples (described in paragraph 3.1.6) were analysed over the course of 4 months. The sample preparation was as follows: The sample preparation was as follows: sample material (50 µL), NaCl (5 M, 100 µL), TDM ISTD user solution[3] (50 µL) (paragraph 3.3.5) and ACN (350 µL) pipetted into 1.3 mL PP 96-well storage plates, mixed well by shaking for 15 seconds, and centrifuged at 4000xG for 10 min.

The concentration (ng/mL) was quantified by the inclusion of calibration standard MassTox STD#0-6 (paragraph 3.2.7). TDM QC (99999506), TDM QC Level 1 (99999401) and TDM QC Level 2 (99999402) (paragraph 3.1.3) were also included in the analysis.

The MRM transitions used in the analysis were the same as those used in the optimisation experiments and are described in paragraph 3.7.2.2. The mobile phase consisted of MeOH (solvent B) and formic acid (0.2 %) (solvent A), and the solvent gradient (%A : %B) is described in Table 4-2.

3.7.5 Method comparison with Oslo University Hospital, Department of Pharmacology

120 anonymised samples (described in paragraph 3.1.7) were analysed over the course of three days. The sample preparation was as follows: sample material (50 µL), NaCl (5 M, 100 µL), TDM ISTD user solution 191206 (50 µL) (paragraph 3.3.5) and ACN (350 µL) pipetted into 1.3 mL PP 96-well storage plates, mixed well by shaking for 15 seconds, and centrifuged at 4000xG for 10 min.

The concentration (ng/mL) was quantified by the inclusion of calibration standard MassTox STD#0-6 (paragraph 3.2.7). TDM QC (99999506), TDM QC Level 1 (99999401) and TDM QC Level 2 (99999402) (paragraph 3.1.3) were included in the analysis as well.

The MRM transitions used in the analysis were the same as those used in the optimisation experiments and are described in paragraph 3.7.2.2. The mobile phase consisted of MeOH (solvent B) and formic acid (0.2 %) (solvent A), and the elution gradient (%A : %B) is described in Table 4-2.

4 RESULTS AND DISCUSSION

4.1 MRM transitions for tacrolimus, sirolimus, everolimus and cyclosporin A

Table 4-1 MRM transitions tacrolimus, tacrolimus ISTD (^{13}C, D_2), sirolimus, sirolimus ISTD (D_3), everolimus, everolimus ISTD (D_4), cyclosporin A and cyclosporin A ISTD (D_4). The m/z of the molecular ion and fragment ion, the adduct, and the collision energy (eV) needed to get the strongest signal for fragment ion.

	Molecular ion (m/z)	Adduct	Fragment ion (m/z)	Collision energy (eV)
Tacrolimus	826.3	$[M + Na]^+$	616.2	34
	826.3	$[M + Na]^+$	443.1	45
	826.3	$[M + Na]^+$	505.1	40
Tacrolimus 13C, D2	829.4	$[M + Na]^+$	619.2	34
Sirolimus	936.3	$[M + Na]^+$	409.1	50
	936.3	$[M + Na]^+$	453.1	50
Sirolimus D3	939.5	$[M + Na]^+$	409	50
Everolimus	981.1	$[M + Na]^+$	389.1	56
	981.1	$[M + Na]^+$	409.12	56
Everolimus D4	984.4	$[M + Na]^+$	393.2	50
Cyclosporin A	1225.1	$[M + Na]^+$	646.1	86
			377.1	98
			575.2	88
	602	$[M + 2\ H]^{2+}$	99.9	50
			156	30
			240.9	25
	613	$[M + Na + H]^{2+}$	100	28
			556.5	18
			492.9	20
	624	$[M + 2\ Na]^{2+}$	1224.6	30
			1112.5	35
	1202.7	$[M + H]^+$	156	70
			425.2	50
Cyclosporin A D4	615.2	$[M + Na + H]^{2+}$	100	28
	604	$[M + 2\ H]^{2+}$	100	50
	1228.6	$[M + Na]^+$	377.1	98

Single charged sodium (Na^+) adducts were found in all analytes and internal standards. Cyclosporin A and cyclosporin A (D_4) were also found to have single and double charged proton (H^+) adducts, as well as double charged $Na^+ + H^+$ adducts. Cyclosporin A also had

double charged H^+ adducts. All the molecular ions found for each compound and their corresponding fragments are described in Table 4-1.

Other articles report the use of MRM transitions with NH_4^+ adducts, for example [31, 32, 37, 42]. NH_4^+ adducts are often preferred because they can be fragmented with lower collision energy (eV) compared to for example Na^+ adducts. No NH_4^+ adducts were detected in this tuning experiment. This was not surprising for two reasons. Na^+ adducts are formed very easily [57], and there were no ammonium salts in the mobile phase, therefore not supplying the sample material with sufficient NH_4^+ ions. Na^+ adducts also seem to form more easily than NH_4^+ adducts, and in order to favour the formation of NH_4^+ adducts the whole LC-system needs to be devoid of Na^+.

Tuning attempts had been made prior to the official research for this thesis. In these experiments the mobile phase had consisted of methanol (MeOH) and a weak ammonium acetate solution. NH_4^+ adducts were detected in these experiments and were included in the instrument's MS method for a few experiments with samples of whole blood. The signal appeared however to decrease over time. A decision was also made to change the mobile phase to contain formic acid instead of ammonium acetate for the sake of practicality as the other analyses on the instrument used formic acid in the mobile phase. After the change of mobile phase the NH_4^+ adducts almost completely disappeared.

The MRM transitions programmed into the MS method used for the experiments discussed below were based on tuning experiments made prior to the official start of the thesis. This means the MRM transitions used for the detection of analytes are not identical to the molecular ions and fragment ions found in Table 4-1. Most are however very close in *m/z*. The exception is for the analyte cyclosporin A where the MRM transition in the experiments below are with single charged H^+ adducts which had not been noted in this tuning experiment.

4.2 LC-separation of tacrolimus, sirolimus, everolimus and cyclosporin A

An optimised solvent gradient in the mobile phase is the key to good separation. The goal of this experiment was to find which solvent worked best with formic acid (0.2 %). This was judged both on the solvent's ability to separate the analytes, the elution of analytes, and the intensity of the signals. The MS-method was programmed with 20 MRM channels including MRM transitions described in Table 4-1.

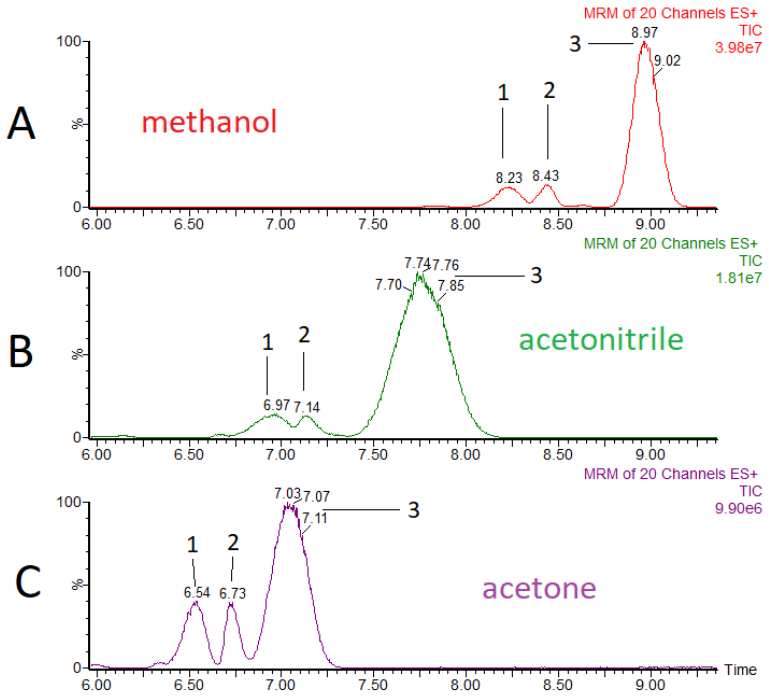


Figure 4-1 Total ion chromatogram (TIC) of linear elution gradients done with three different solvents as solvent B in the mobile phase: MeOH (chromatogram A), ACN (chromatogram B), and acetone (chromatogram C).

Chromatogram A, B and C in Figure 4-1 displays the total ion chromatogram (TIC) for the linear gradient with either MeOH (A), ACN (B) or acetone (C) as solvent B in the mobile phase. The elution of analytes happened at an earlier point in acetone than in ACN and MeOH. MeOH had the latest elution of analytes. This was possibly due the differences in the solvents' intramolecular interactions with the analytes. The hydroxyl group in MeOH gives the solvent stronger polar interactions, and weaker non-polar interactions, compared to acetone and ACN, causing the lipophilic analytes to only elute at higher concentrations of MeOH.

All three solvents were able to separate the molecules into three peaks. It was possible to identify the different molecules through selecting the chromatogram for specific MRM transitions. In all three experiments peak 1 was identified as tacrolimus, peak 2 was identified as sirolimus and everolimus, and peak 3 was identified as cyclosporin A. Because the retention times (tR) for everolimus and sirolimus were so close together, it was difficult to separate them. It is possible these similar tR was caused by the similar structure of the two molecules. This was not a big issue because of the difference in the analytes' molecular weight, which made it possible for the mass spectrometer to distinguish them. Separation is however desirable, and the linear gradient with MeOH gave a better separation between tacrolimus (1), sirolimus + everolimus (2), and cyclosporin A (3). Acetone (chromatogram C) appears to give a better separation between peak 1 and peak 2, but this is only because the relative intensity of the two peaks is higher in the TIC than in chromatogram C than in chromatogram A. The elution peaks were also much narrower in MeOH compared to ACN, and to some degree compared to acetone.

The signal intensity was the strongest in the linear gradient (Figure 4-1) with MeOH than in acetone and ACN. The signal intensity of the TIC in the linear gradient with acetone and ACN was only about 25 % and 46 % compared to that of MeOH, respectively. Signal intensity is significant during analysis because it correlates to the sensitivity. A strong signal intensity could raise the sensitivity of the analysis.

The conclusion of the linear gradient experiments was that the best solvent to use in the mobile phase was MeOH. This was due to the stronger signal intensity and the ability to separate the different analytes. As discussed earlier, the analytes eluted later with MeOH than with ACN and acetone, but that was potentially an advantage. The hypothesis was that more polar molecules pertaining either to the sample matrix or the reagents that may have been left in the injection volume would have ample time to elute before the analytes, therefore potentially reducing interference.

The linear reagent gave an indication of which concentration of solvent B would elute the analytes. The following was the estimated approximate concentration of solvent B (%) that would elute each analyte: tacrolimus A (~79 %,), sirolimus + everolimus (~82 %), and cyclosporin A (~87.5 %). This estimation was helpful when designing the optimised elution gradient. The goal was to start with a concentration that was high enough to elute or flush

out hydrophilic molecules but low enough to still retain all the analytes, then gradually raising the concentration slowly enough to separate the analytes, and then end the injection with a high concentration of solvent B to flush out anything still left in the column. This concentration had to be 99 % solvent B to prevent the C18 chains in the column from collapsing.

The solvent ratio (%A : %B) was adjusted multiple times until the separation and run time was satisfactory. The optimised step gradient was then programmed into the instrument's inlet method. The solvent gradient is described in Table 4-2 as the ratio of solvent A (%) and solvent B (%) at given time intervals (min).

Table 4-2 Solvent gradient of mobile phase defined as ratio of solvent A (%) and solvent B (%) over time (min), including the flow rate.

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
0.00	0.500	70	30
0.01	0.500	40	40
0.40	0.500	15	85
0.50	0.500	1	99
1.00	0.500	1	99
1.01	0.500	70	30
1.10	0.500	70	30

4.3 Optimised sample preparation of EDTA whole blood for TDM of immunosuppressants for analysis on LC-MS/MS

The main objective when developing a new sample preparation method was the use of salting-out assisted liquid-liquid extraction (SALLE). This was based on the hypothesis that an extraction method would potentially raise the analytical sensitivity, as well as reduce interference compared to sample preparation with protein precipitation (PPT). Traditional liquid-liquid extraction (LLE) was not a viable option due to the fact that hydrophobic solvents would not be compatible with the LC-MS/MS instrument. In SALLE it is possible to use water miscible solvents such as simple alcohols and ACN. The optimisation experiments involved testing different water miscible solvents, or combinations of these, and comparing the results. Each experiment was made with three parallels to correct for internal CV (%).

The different sample preparations were evaluated based on analytical factors such as the concentration of the analytes in the top phase or supernatant and the intensity of ion suppression, as well as pre- and post-analytical factors like the visual appearance of the vials and the general ease of result processing. The analytical factors were important to estimate the sensitivity and efficiency of the sample preparation, while the pre-analytical factor based on visual appearance was important to eliminate uneven extraction.

4.3.1 Preanalytical observations

There were a lot of visible and notable differences in appearance between the samples during sample preparation. As expected, some experiments did not have a separation of two phases, while others had a clean separation. However, in experiments with phase separation the aqueous phase would vary in volume. In most cases this was because the different experiments had different volumes of aqueous solution added. But in some experiments the aqueous phase was smaller than expected. This was possibly due to incomplete phase separation and there was still a considerable amount of water left in the organic phase.

A few experiments had a noticeable discoloration in the supernatant or organic phase. This had the potential to increase the ion suppression because the injected solution possibly contained a lot of cell debris and proteins. Some samples were so severely discoloured that they were not included in the analysis. There was a concern that unprecipitated proteins would give strong interferences, and possibly clog the LC-instrumentation. Such strong discolouration was taken into consideration when deciding which preparation method was suitable for the finished method. Other samples had a lot of clumped debris along the sides of the walls. The worry around this was that this clumping of what was likely erythrocytes could lead to less efficient as well as uneven extraction.

In SALLE experiments involving NaCl (5 M) and MeOH in ACN, separation of phases was observed with 0 - 17.5 % (v/v) MeOH in ACN. Increasing the concentration of MeOH caused the phase separation to cease, and the supernatant got discoloured. Samples with no MeOH or lower concentrations of MeOH appeared to have a lot of debris on the sides of the walls. Changing the volume and concentration of NaCl did not have a significant visual effect on the samples. Figure 4-2 demonstrates the visual difference between SALLE experiments with different concentrations of MeOH in ACN.

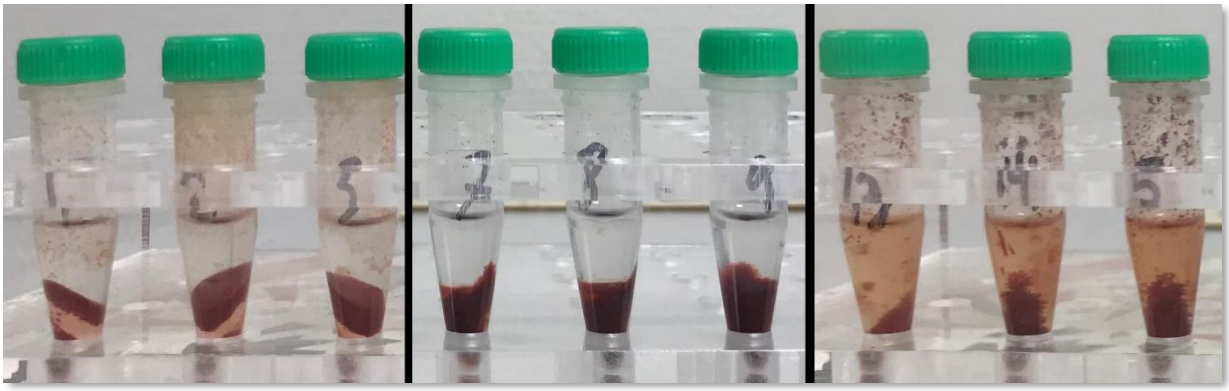


Figure 4-2 Three images demonstrating difference in phase separation. Samples were prepared with NaCl (5 M, 100 µL), MeOH (10 µL) and ACN (390 µL) (left image); NaCl (5 M, 100 µL), MeOH (50 µL) and ACN (350 µL) (middle image); NaCl (5 M, 100 µL), MeOH (100 µL) and ACN (200 µL) (right image).

Similar effects were observed in samples prepared with NaCl (5 M), MeOH and acetone. The organic phase seemed to decrease in volume when increasing the concentration of MeOH, and phase separation ceased with 12.5 % (v/v) MeOH in acetone. The supernatant in experiments with 12.5 % (v/v) MeOH in acetone and higher were also very discoloured.

Two SALLE experiments with 5 % (v/v) MeOH in acetone were prepared with 200 µL and 300 µL NaCl (5 M) instead of 100 µL. The aqueous phase was larger in these samples, which was expected. But the organic phase also appeared to be smaller, as well as slightly discoloured. This is demonstrated in Figure 4-3.

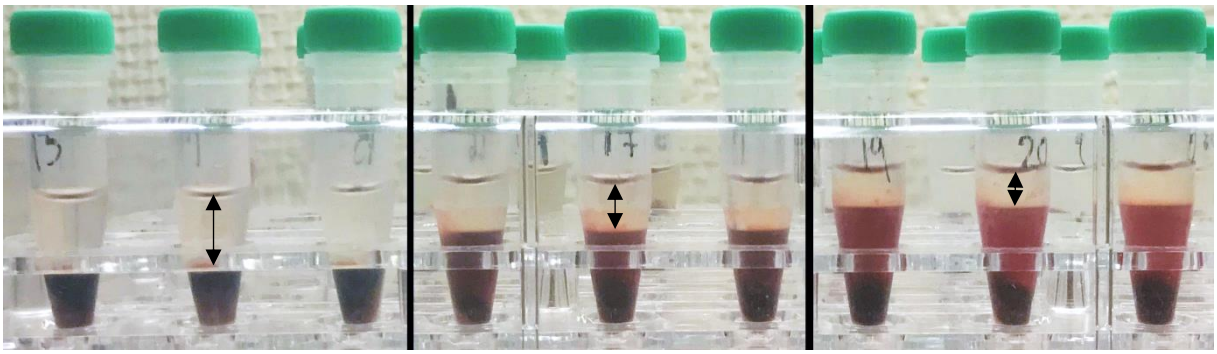


Figure 4-3 Three images of samples prepared with different volumes of NaCl (5 M). All samples were prepared with 5 % (v/v) MeOH in acetone. Samples in left image was prepared with NaCl (5 M, 100 µL), samples in middle image was prepared with NaCl (5 M, 200 µL), and samples in right image was prepares with NaCl (5 M, 300 µL). Arrows emphasise the difference in volume in the organic phase.

As expected, addition of $ZnSO_4$ (0.1 M) in PPT experiments with MeOH made the supernatant clear up. But in SALLE experiments involving 12.5 % (v/v) MeOH in ACN, addition of $ZnSO_4$ (0.1 M) caused a discoloration of the organic phase. Figure 4-4 demonstrates the visual difference in three experiments prepared with $ZnSO_4$.

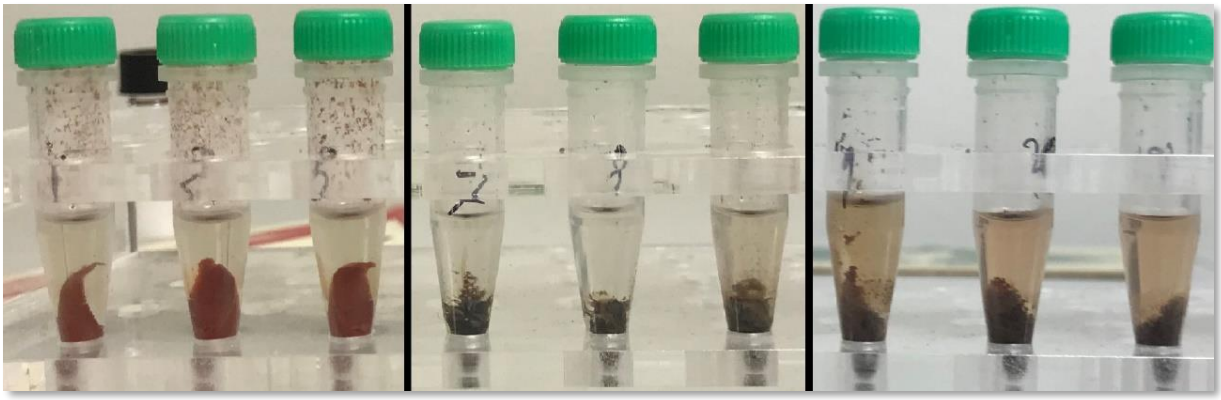


Figure 4-4 Three images demonstrating the effect of adding $ZnSO_4$ to different experiments. The samples were prepared with MeOH (400 µL) and dH_2O (100 µL) (left image); MeOH (400 µL) and $ZnSO_4$ (100 µL) (middle image); MeOH (50 µL), ACN (350 µL) and $ZnSO_4$ (100 µL) (right image).

Adding $MgSO_4$ (2.7 M) to SALLE experiments involving MeOH in ACN had no visible effect on the organic phase. It did however make the aqueous phase clearer and created a band of proteins and cell debris between the two phases, demonstrated in Figure 4-1. Similar effects were observed in SALLE experiments involving MeOH in acetone. Addition of $MgSO_4$ (2.7 M) also facilitated phase separation in SALLE experiments with 12.5 % (v/v) MeOH in acetone, but with a very small aqueous phase and discoloration of the organic phase.

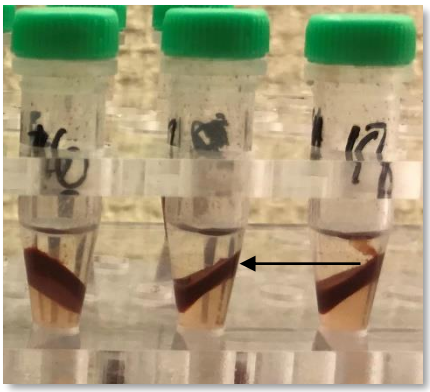


Figure 4-5 Experiment prepared with $MgSO_4$ (2.7 M, 50 µL), NaCl (5 M, 100 µL), MeOH (50 µL) and ACN (350 µL). Arrow points to layer of cell debris between two phases.

SALLE experiments involving MeOH in tetrahydrofuran (THF) appeared to have a clean separation of two phases, regardless of concentration of MeOH in THF. However, there were concerns regarding the stability of the samples over time. After analysis, capped PP vials containing THF had been left out in room temperature for 1-2 days. There was a noticeable resistance when pulling the vials out from the rack. One tube containing only THF had a visible deformation, which is demonstrated in Figure 4-6. This was thought to be caused by absorption of THF into the walls of the PP vial, resulting in swelling. This could potentially

cause problems because the Nunc 96-well plates were also made of PP. This discovery influenced the decision to not include THF in the final sample preparation method.

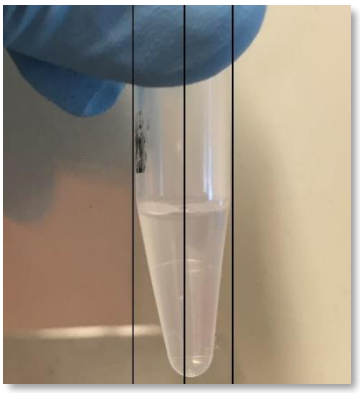


Figure 4-6 Polypropylene vial containing THF showing deformation after two days. Parallel vertical lines have been added to the image to emphasise the deformation.

4.3.2 Analytical results and observations

A variety of experiments were performed, each with different combinations of salt solutions and solvents. Some combinations were even tested multiple times. Included in this section are the results that best reflect the effects of each experiment. This means that the results of some individual experiments will not be included of discussed in detail. The experiments are also not presented chronologically. It is done this way for the sake of practicality and comprehension. However, it is important to point out that each experiment listed below only includes samples prepared and analysed simultaneously because the results would not have been comparable otherwise due to analytical conditions changing over time.

In the optimisation experiments the different methods were either compared through response and ISTD area, or concentration and ISTD area. The response is defined as a factor of analyte area over ISTD area. As the sample material for each experiment was identical, the difference in response or concentration would give an indication of which sample preparation method was most effective in extracting the analytes from the aqueous sample material to the organic phase.

ISTD area was also compared as a way of monitoring ion suppression. A small ISTD area could indicate strong ion suppression. The analytical sensitivity could be significantly reduced with strong ion suppression due to a reduction in detector response.

It was decided that the response, concentration, and ISTD were to be presented as relative response (%), relative concentration (%), and relative ISTD area. This decision was made due to the large difference in concentration between the different analytes, and it made it easier to visualise the difference in efficiency between experiments.

Relative response (%) was defined as:

$$\bar{x}_{all\ parallels}\left(\frac{response_{one\ parallel}}{highest\ reponse\ of\ the\ series}\times 100\right)=relative\ response\ (\%) \quad (1)$$

Relative concentration (%) was defined as:

$$\bar{x}_{all\ parallels}\left(\frac{conc_{one\ parallel}}{highest\ conc\ of\ the\ series}\times 100\right)=relative\ conc\ (\%) \quad (2)$$

Relative ISTD area (%) was defined as:

$$\bar{x}_{all\ parallels}\left(\frac{ISTD\ area_{one\ parallel}}{highest\ ISTD\ area\ of\ the\ series}\times 100\right)=relative\ ISTD\ area\ (\%) \quad (3)$$

All experiments were analysed with MeOH as solvent B and formic acid (0.2 %) as solvent A, with the exception of Experiment 5.2 where solvent B was changed to acetone.

4.3.2.1 *Experiment 1: SALLE with MeOH and ACN:*

The design of these experiments was based on a coincidental discovery that SALLE with ACN and a smaller amount of MeOH made clean separations of two phases. Experiments were then designed to compare the results from samples prepared with varying concentrations of MeOH in ACN. Sample preparation in this experiment was: sample material (50 µL), NaCl (5 M, 100 µL) and solvent combination (400 µL).

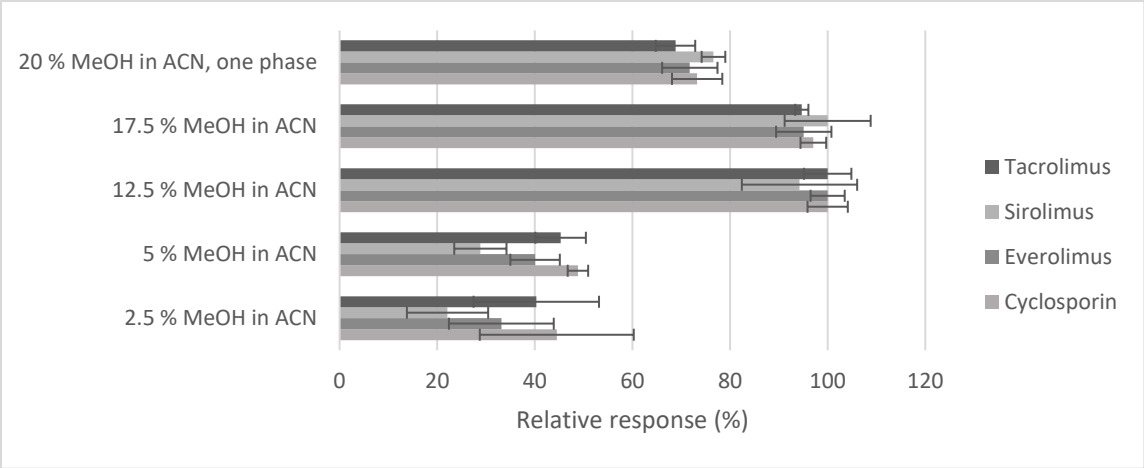


Figure 4-7 Relative analyte response (%) for tacrolimus, sirolimus, everolimus and cyclosporin in experiments involving SALLE with MeOH in ACN.

As shown in Figure 4-7 the relative response (%) was highest in samples with 12.5 – 17.5 % (v/v) MeOH in ACN. There was a small difference between 12.5 % (v/v) and 17.5 % (v/v) MeOH in ACN, with the former being a little higher. However, this was not significant as the standard deviation (SD) overlapped. The relative ISTD area (%) (Figure 4-8) were also similar in these samples, with no significant difference except in cyclosporin A. Cyclosporin A had a larger relative ISTD area (%) in samples prepared with 12.5 % (v/v) MeOH in ACN.

In samples with 2.5 – 5 % (v/v) MeOH in ACN the relative response (%) was significantly lower than in higher concentrations of MeOH in ACN. The relative ISTD area (%) was however similar. This lower response was thought to be caused by poor extraction of the analytes.

As expected, samples prepared with 20 % (v/v) MeOH in ACN had a lower relative response (%) because there was no phase separation. This caused a dilution of sample material rather than extraction of analytes. The relative ISTD area (%) was also smaller than in samples with 12.5 – 17.5 % MeOH in ACN, indicating ion suppression. These samples had a visible discoloration of the supernatant, which could have affected the ion suppression.

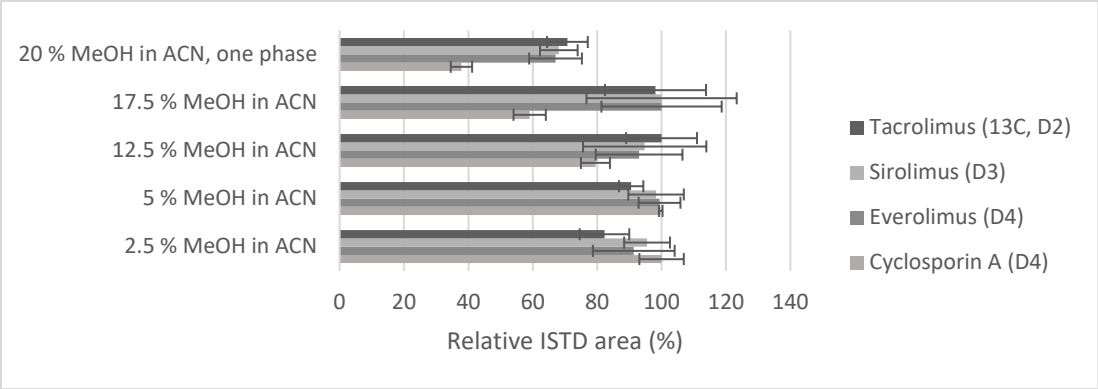


Figure 4-8 Relative ISTD area (%) for tacrolimus (^{13}C, D_2), sirolimus (D_3), everolimus (D_4) and cyclosporin A (D_4) in experiments involving SALLE with and MeOH in ACN.

The conclusion was that the most efficient sample preparation method for this experiment was SALLE with 12.5 % (v/v) MeOH in ACN as opposed to SALLE with 17.5 % (v/v) MeOH in ACN. Even though both sample preparation methods yielded very similar results, the response for cyclosporin A was significantly higher in SALLE with 12.5 % (v/v) MeOH in ACN.

4.3.2.2 *Experiment 2: SALLE with MeOH and ACN, with $MgSO_4$:*

The second experiment was designed to compare the effects of adding $MgSO_4$ to SALLE with MeOH and ACN. The comparison was based on samples prepared with 12.5 % MeOH in ACN because this solvent combination was considered the most efficient in Experiment 1. $MgSO_4$ was chosen as an alternative salt to $ZnSO_4$ to see if the results improve. $ZnSO_4$ is reported to work well with cyclosporin A and tacrolimus, while reduced analyte signal have been reported in sirolimus and everolimus with the addition of $ZnSO_4$ [34]. A saturated solution of $MgSO_4$ (2.7 M) was chosen in order to not dilute the aqueous phase and lose the salting-out effect of SALLE.

Sample preparation in this experiment was: sample material (50 µL), NaCl (5 M, 100 µL), solvent combination (400 µL) and $MgSO_4$ (2.7 M, 25 – 50 µL). Concentration was quantified by the inclusion of a calibration standard (described in paragraph 3.2.1) in the analysis.

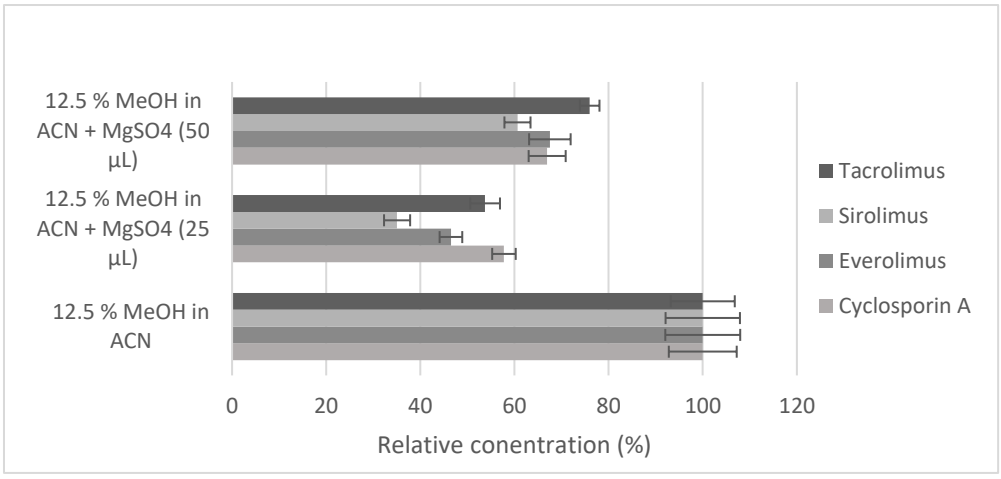


Figure 4-9 Relative analyte concentration (%) for tacrolimus, sirolimus, everolimus and cyclosporin A in SALLE experiments with MeOH in ACN, with an addition of $MgSO_4$.

As shown in Figure 4-9, the relative concentration (%) was significantly higher in samples prepared with 12.5 % MeOH in ACN and no $MgSO_4$ (2.7 M), while the relative ISTD area (%) (Figure 4-10) was similar across all three preparation methods. Addition of $MgSO_4$ (2.7 M) appeared to negatively affect the extraction efficiency of the analytes.

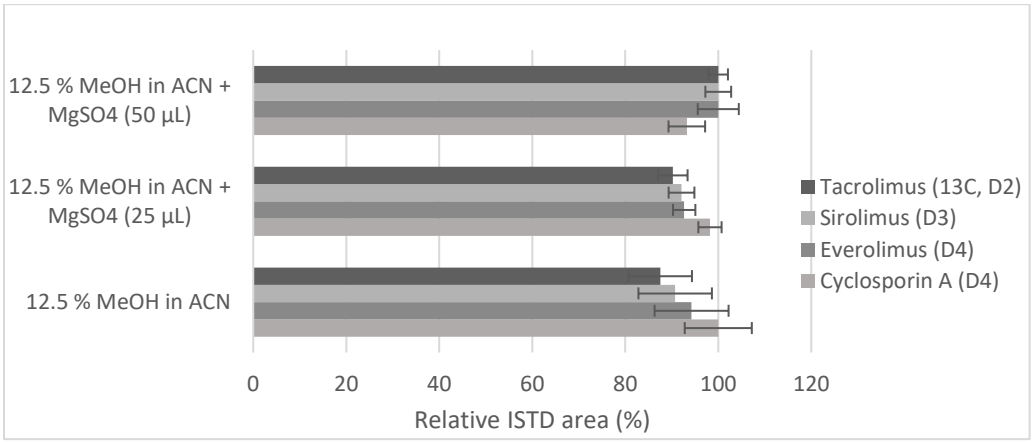


Figure 4-10 Relative ISTD area (%) for tacrolimus (^{13}C, D_2), sirolimus (D_3), everolimus (D_4) and cyclosporin A (D_4) in experiments involving SALLE with MeOH and ACN, with an addition of $MgSO_4$.

4.3.2.3 *Experiment 3: SALLE with MeOH and acetone*

The design of the third experiment was based on the solubility parameters from HSP. The HSP of acetone matched well with those of cyclosporin A. These findings indicated that SALLE with acetone could extract the analytes more efficiently. Samples prepared with SALLE and 12.5 % (v/v) MeOH in ACN were included in the analysis because that was the most effective method in Experiment 1.

In the four sample preparations involving acetone, two sets of samples were prepared with sample material (50 µL), NaCl (5 M, 100 µL) and solvent combination (400 µL). One sample (marked with * in Figure 4-11 and Figure 4-12), was prepared with sample material (50 µL), NaCl (5 M, 200 µL) and 5 % (v/v) MeOH in acetone (400 µL), and one sample (marked with ** in Figure 4-11 and Figure 4-12) was prepared with sample material (50 µL), NaCl (5 M, 300 µL) and 5 % (v/v) MeOH in acetone (400 µL).

These sample preparations were also compared to PPT with MeOH and $ZnSO_4$ based on methods described in other articles [32-35, 50, 51]. These samples were prepared as follows: sample material (50 µL), $ZnSO_4$ (0.1 M, 100 µL) and MeOH (400 µL).

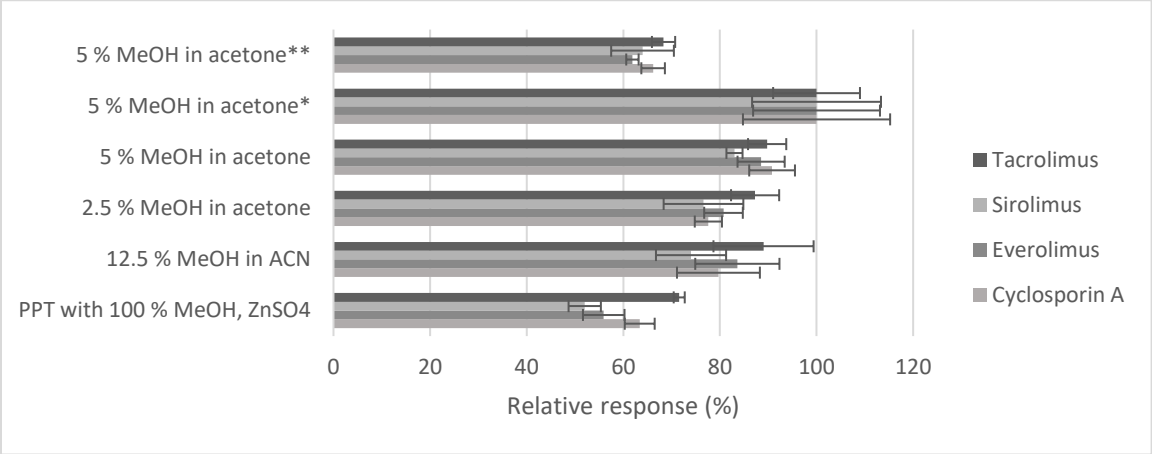


Figure 4-11 Relative response (%) for tacrolimus, sirolimus, everolimus and cyclosporin A in SALLE experiment with NaCl and MeOH in acetone. Including SALLE with 12.5 % (v/v) MeOH in ACN and protein precipitation with MeOH and $ZnSO_4$.

The sample preparation with the highest relative response (%) (Figure 4-11) for all analytes was 5 % (v/v) MeOH in acetone and NaCl (5 M, 200 µL) (5 % MeOH in acetone** in Figure 4-11) , but not significantly higher than samples prepared with 5 % (v/v) MeOH in acetone and NaCl (5 M, 100 µL). The difference in relative response (%) between the three samples prepared with 5 % (v/v) MeOH in acetone, but with different volumes of NaCl (5 M) indicated the ideal volume of NaCl (5 M) was 200 µL. When considering the visual appearance of these samples (demonstrated in Figure 4-3), the higher response could have been caused by a smaller organic phase. But this may not have been the case since the organic phase was even smaller in the samples with 300 µL NaCl (5 M), and the relative response (%) was also lower.

In samples with 12.5 % (v/v) MeOH in ACN the relative response (%) was somewhat lower than in samples with MeOH in acetone. As expected, the relative response (%) in the PPT

sample preparation was lower compared to the other methods. The sample material was diluted with a factor of 8.

Figure 4-12 demonstrates an interesting effect in the PPT samples prepared with MeOH and $ZnSO_4$. The relative ISTD area (%) for sirolimus (D_3) and everolimus (D_4) was greatly reduced when compared to the other sample preparations, while the relative ISTD area (%) for tacrolimus (^{13}C, D_2) and cyclosporin A (D_4) was only a little lower. This effect of a stronger ion suppression of sirolimus and everolimus as opposed to tacrolimus and cyclosporin A had also been reported in a study done by Koster, et al. [58] in 2009.

The relative ISTD area (%) (Figure 4-12) appeared to vary for the different analytes in some of the samples. The largest relative ISTD area (%) for tacrolimus (^{13}C, D_2) was in samples with 5 % (v/v) MeOH in acetone and NaCl (5 M, 200 µL) while the relative ISTD area (%) for the other analytes were significantly lower. The largest relative ISTD area (%) for sirolimus (D_3), everolimus (D_4) and cyclosporin A (D_4) were all found in samples prepared with both 5 % (v/v) MeOH in acetone and NaCl (5 M, 300 µL), and with 12.5 % (v/v) MeOH in ACN and NaCl (5 M, 100 µL).

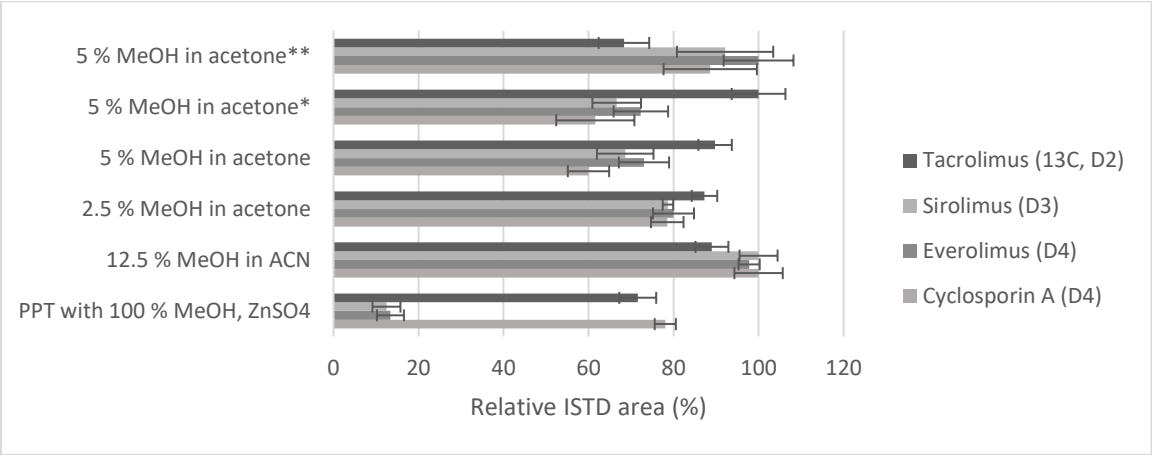


Figure 4-12 Relative ISTD area (%) for tacrolimus (13C, D2), sirolimus (D3), everolimus (D4) and cyclosporin A (D4) in SALLE experiments with NaCl and MeOH in acetone. Including SALLE with 12.5 % (v/v) MeOH in ACN and PP with MeOH and $ZnSO_4$.

The conclusion of this experiment was that sample preparation involving SALLE with 12.5 % (v/v) MeOH in ACN was the most effective. Although the relative response (%) was a little lower, but perhaps not significantly lower, than in samples prepared with 5 % (v/v) MeOH in acetone, the relative ISTD area (%) was large in all four analytes. Hence, 12.5 % (v/v) MeOH in ac produced highest extraction efficiency combined with lowest ion suppression effects.

There was also a concern that, since the boiling point of acetone is much lower than that of acetonitrile, discrepancies could occur during sample preparation because of uncontrolled solvent evaporation. The small gain in analyte response did not outweigh the risk of uncertainty in sample preparation.

4.3.2.4 *Experiment 4: SALLE with MeOH and acetone, with $MgSO_4$*

The fourth experiment was designed with the same intentions as experiment 2. The most efficient sample preparation involving acetone in Experiment 3 was SALLE with 5 % (v/v) MeOH in acetone. That method was included in this experiment together with samples prepared with SALLE and 0 – 12.5 % (v/v) MeOH in acetone, and some experiments with the addition of $MgSO_4$ (2.7 M, 25 µL). Samples with 12.5 % (v/v) MeOH and acetone with $MgSO_4$ (2.7 M, 25 µL) were included because the addition of $MgSO_4$ facilitated a phase separation which was not observed in samples without. The concentration of the analytes was quantified by the inclusion of a calibration standard (described in paragraph 3.2.1).

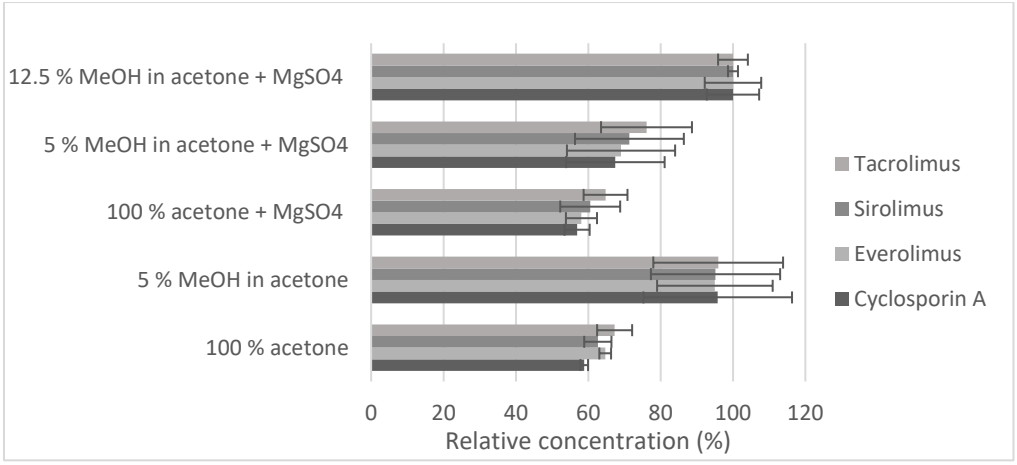


Figure 4-13 Relative concentration (%) for tacrolimus, sirolimus, everolimus and cyclosporin A in SALLE experiments with MeOH in acetone, with an addition of $MgSO_4$ (2.7 M, 25 µL).

As can be seen in Figure 4-1, addition of $MgSO_4$ (2.7 M) to samples prepared with 5 % (v/v) MeOH in acetone lowered the relative concentration (%) of every analyte. The highest relative concentration (%) was observed in samples with 12.5 % (v/v) MeOH in acetone. In the samples prepared with 100 % acetone the relative concentration (%) was comparably the lowest. This was possibly due to poor extraction.

However, this sample preparation had the largest relative ISTD area (%) compared to the other methods, which can be seen in Figure 4-14. In samples with MeOH and/or $MgSO_4$ (2.7 M) there was a gain of concentration, but a loss of almost half of the relative ISTD area (%),

compared to samples with 100 % acetone. This indicated a strong ion suppression that could be caused by the $MgSO_4$ itself.

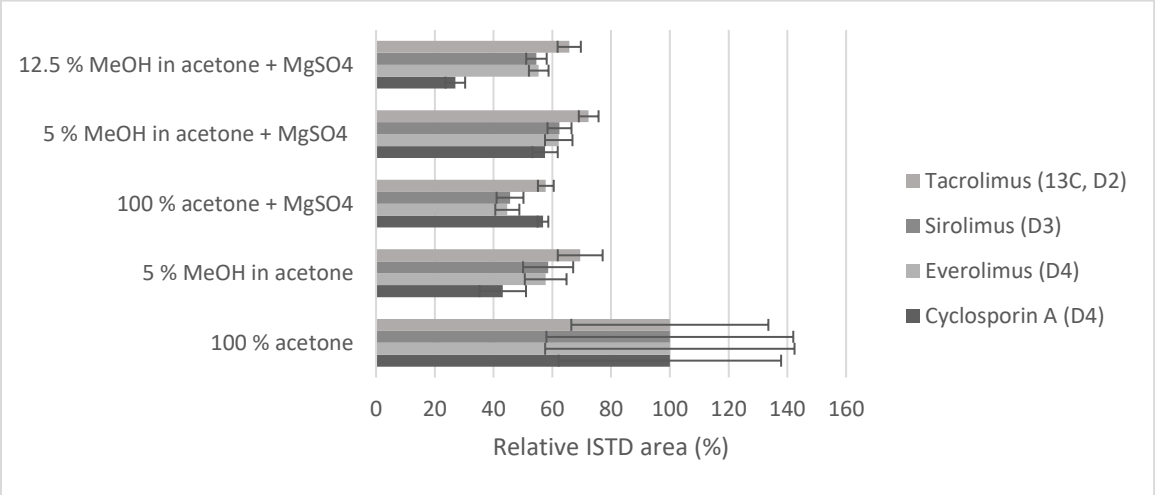


Figure 4-14 Relative ISTD area (%) for tacrolimus (^{13}C, D_2), sirolimus (D_3), everolimus (D_4) and cyclosporin A (D_4) in SALLE experiments with MeOH in acetone, with an addition of $MgSO_4$.

The conclusion of this experiment was that there was no significant benefit gained from adding $MgSO_4$ to SALLE with MeOH in acetone because the ISTD area was greatly reduced.

4.3.2.5 *Experiment 5.1: SALLE with MeOH and THF*

This experiment was designed with the intention of comparing the effects of SALLE with MeOH in THF to SALLE with 12.5 % (v/v) MeOH in ACN. Comparing the HSP of THF to that of cyclosporin A (table found in paragraph 3.7.2.1) suggested this solvent was worth trying out.

Samples were prepared with concentrations of 0 – 12.5 % (v/v) MeOH in THF. The sample preparation was sample material (50 µL), NaCl (5 M, 100 µL) and solvent combination (400 µL). Concentration was quantified by the inclusion of a calibration standard (described in paragraph 3.2.1) in the analysis.

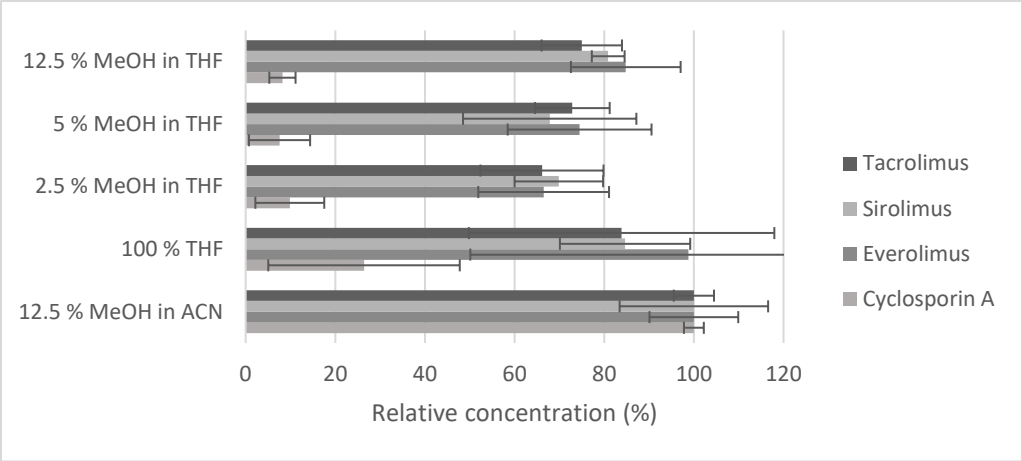


Figure 4-15 Relative concentration (%) for tacrolimus, sirolimus, everolimus and cyclosporin A in SALLE experiments with NaCl and MeOH in THF. Including SALLE with 12.5 % MeOH in ACN.

The relative concentration (%) (Figure 4-1) was lower for tacrolimus, sirolimus and everolimus in samples prepared with THF than the samples prepared with 12.5 % (v/v) MeOH in ACN. In cyclosporin A, there was a large difference in relative concentration (%). In samples with 12.5 % (v/v) MeOH in THF, the relative concentration was only around 10 % compared to 12.5 % (v/v) MeOH in ACN. In samples with 100 % THF, the relative concentration (%) appeared to be a little better, but not significantly so because of the high SD.

The relative ISTD area (%) (Figure 4-16) in samples prepared with THF was only about 40 – 80 % of that of samples prepared with 12.5 % (v/v) MeOH in ACN. This range was due to large variation of effectiveness in the extraction of analytes, but also high SD. As opposed to the relative concentration, there were no apparent difference in relative ISTD area (%) between samples prepared with 2.5 – 12.5 (%) (v/v) MeOH in THF and 100 % THF.

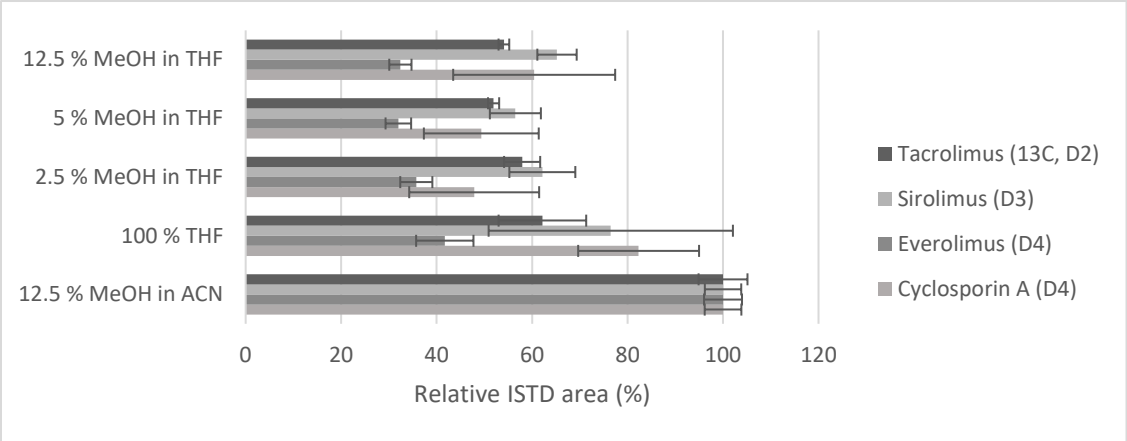


Figure 4-16 Relative ISTD area (%) for tacrolimus (^{13}C, D_2), sirolimus (D_3), everolimus (D_4) and cyclosporin A (D_4) in SALLE experiments with NaCl and MeOH in THF. Including SALLE with 12.5 % (v/v) MeOH in ACN.

The disadvantages of using THF in sample preparation proved to be many. The very low relative concentration of cyclosporin A was surprising because the experiment was designed based on the fact that there was a relatively good match of HSP between THF and cyclosporin A. There was also a strong ion suppression. THF is a strong solvent, so it is possible that there was a lot of other molecules partitioned into the organic phase. As mentioned in paragraph 4.3.1, there was swelling of the PP vials when preparing samples with THF. This swelling effect was not visually noticeable until after 1-2 days but leeching of material from the PP vials could not be ruled out.

The low relative concentration (%) could be explained by poor extraction, but it could also be explained by the build-up of signal interference. Figure 4-17 depicts the chromatograms of tacrolimus [M + Na^+] in a sample prepared with SALLE with 12.5 % (v/v) MeOH in ACN (A), and in a sample prepared with SALLE with 12.5 % (v/v) MeOH in THF. In this particular analysis there was already some interference in samples prepared with 12.5 % (v/v) MeOH in ACN. This interference was thought to be caused by issues pertaining to the LC-column. The column used for this analysis was also regularly used in routine analysis of serum. This interference did not occur in later analyses. As there was a distinct peak at 0.83, which corresponded with the tR of tacrolimus ^{13}C, D_2 [M + Na^+], this was assumed to be the peak of tacrolimus.

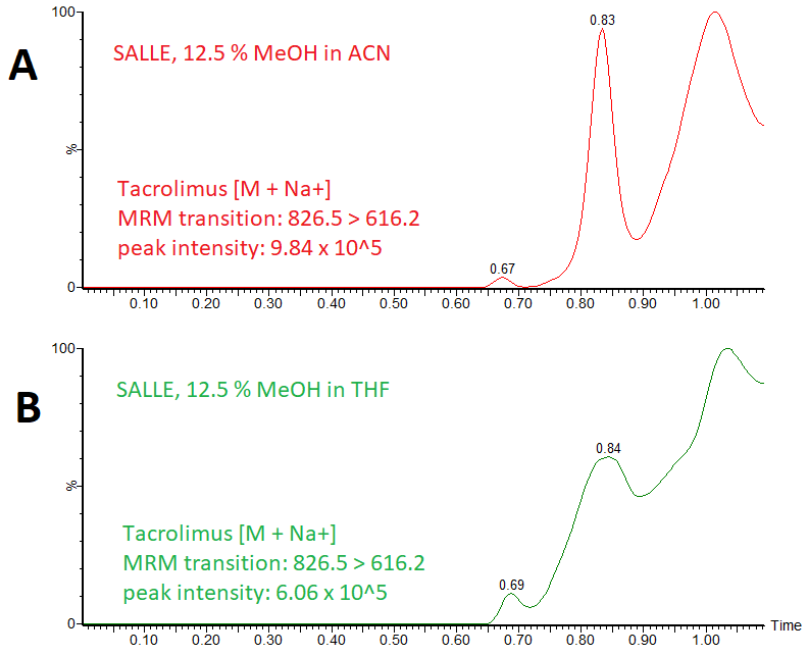


Figure 4-17 Chromatogram of tacrolimus ([M + Na⁺] 826.5 > 616.2 m/z) in one sample prepared with SALLE and 12.5 % (v/v) MeOH in ACN (A), and one sample prepared with SALLE and 12.5 % (v/v) MeOH in THF (B).

Despite there being some interference in the first sample, the adverse effects on the chromatogram from using THF in sample preparation was very clear. Chromatogram B depicts a large increase of interference, and poor separation between the interference and the tacrolimus peak. The tR of tacrolimus in chromatogram B had also been shifted a little, but this was probably because there was a widening of the peak. The intensity of the signal was also lower, but it is uncertain whether this was caused by ion suppression or poor extraction. Proper integration of the analyte peaks was difficult in samples with THF, which possibly contributed to the low calculated concentration in these samples.

Similar effects were observed in the chromatograms of the other analytes as well. The intensity of the interferences in the chromatogram for sirolimus made it nearly impossible to distinguish the sirolimus peak from the signal noise. This signal interference would carry over to several subsequent injections of samples with no THF before being reduced enough to be able to distinguish the analyte peak from the noise.

To summarise, this experiment demonstrated that the use of THF was not suitable for sample preparation because of its ability to swell PP, the low extraction yield, strong ion suppression and strong signal interference.

4.3.2.6 *Experiment 5.2: SALLE with MeOH and THF (acetone as mobile phase).*

The 96-well microplate from Experiment 5.1 was analysed again, but with acetone as solvent B in the mobile phase. This was done to see if the use of acetone would help flush out some of the interferences between each injection.

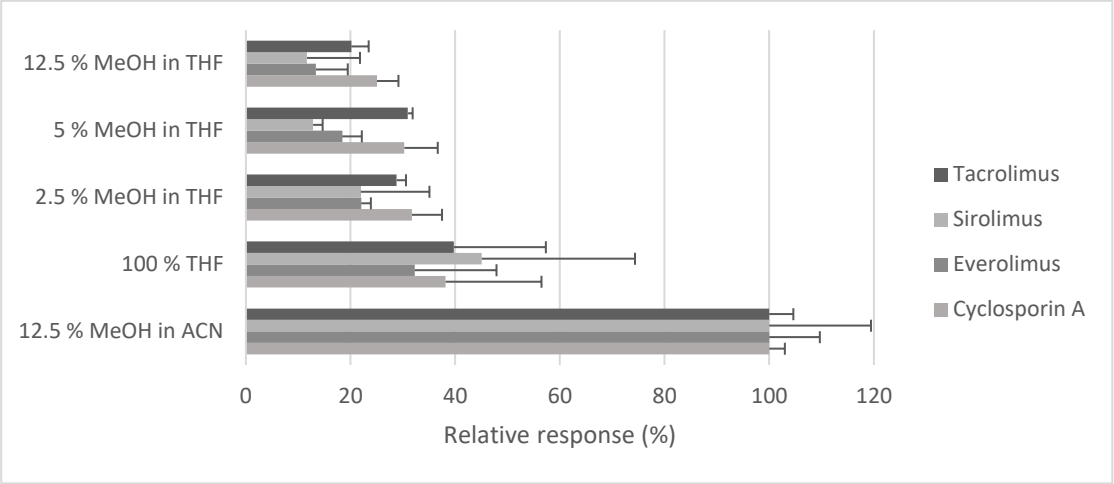


Figure 4-18 Relative concentration (%) for tacrolimus, sirolimus, everolimus and cyclosporin A in SALLE experiments with NaCl and MeOH in THF. Including SALLE with 12.5 % (v/v) MeOH in ACN. Acetone in mobile phase for LC-separation. Minus error bar is excluded.

Even after changing solvent B to acetone, the relative concentration (%) (Figure 4-18) was lower for all the analytes in samples with THF than in samples prepared with 12.5 % (v/v) MeOH in ACN. For the sake of legibility the SD minus bar was excluded from Figure 4-19 because the SD was very high. In some cases higher than the mean value. This high SD was especially present in sirolimus because the signal interference was very strong, and the integration of the analyte peak was very inaccurate.

The relative ISTD area (%) (Figure 4-19) was the largest for tacrolimus, sirolimus and everolimus in samples with 12.5 % (v/v) MeOH in ACN. Interestingly, the relative ISTD area (%) for cyclosporin A in 12.5 % (v/v) MeOH in ACN as only about 50 % of the ISTD area for the sample with 100 % THF. The ISTD area for the rest of the samples with THF were also small, indicating strong ion suppression.

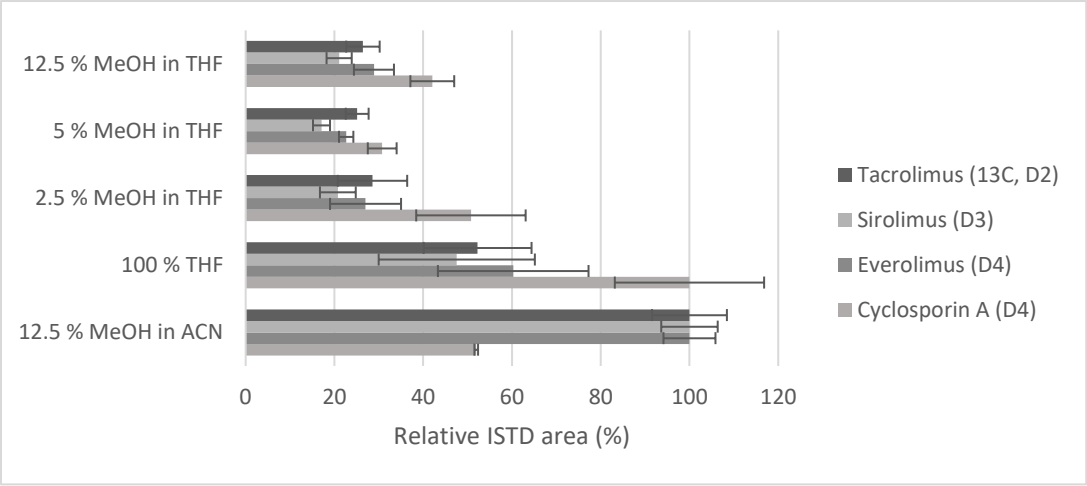


Figure 4-19 Relative ISTD area (%) for tacrolimus (^{13}C, D_2), sirolimus (D_3), everolimus (D_4) and cyclosporin A (D_4) in SALLE experiments with NaCl and MeOH in THF. Including SALLE with 12.5 % (v/v) MeOH in ACN. Acetone in mobile phase for LC-separation.

The chromatograms in Figure 4-20 also shows a similar effect as seen in Experiment 5.1. Changing solvent B in the mobile phase from MeOH to acetone greatly reduced the interference in the samples with 12.5 % (v/v) MeOH in ACN, which can be seen in chromatogram A. However there were no benefits in samples prepared with THF. Chromatogram B shows the chromatogram for tacrolimus [M + Na^+] from an injection that was collected from the same well as in Experiment 5.1. The interference was so strong the peak for tacrolimus was virtually undetectable, and the integration was highly inaccurate.

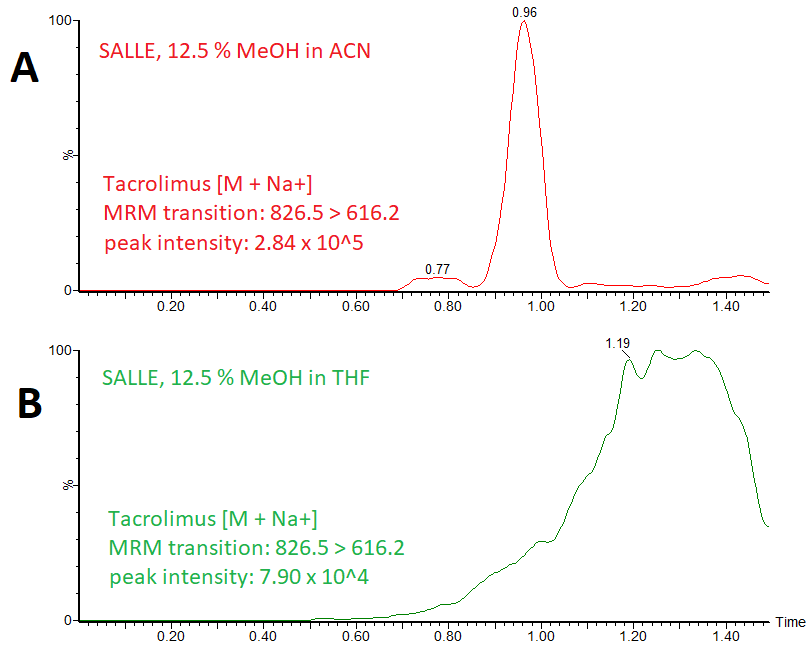


Figure 4-20 Chromatogram of tacrolimus ([M + Na^+] 826.5 > 616.2 m/z) in one sample prepared with SALLE and 12.5 % (v/v) MeOH in ACN (A), and one sample prepared with SALLE and 12.5 % (v/v) MeOH in THF (B). Acetone in the mobile phase for LC-separation.

Changing solvent B in the mobile phase to acetone did not improve the separation of analyte peak and interference in samples prepared with THF. The conclusion of this experiment was the same as in Experiment 5.2; the use of THF was not suitable for sample preparation.

4.3.3 Extraction efficiency

The extraction efficiency was calculated by first calculating the total mass of the analyte in the organic phase for each parallel. This was done using this equation:

$$tot.vol.(mL) \times conc.\left({}^{ng}/_{mL}\right) = tot.m.(ng) \quad (4)$$

The theoretical mass in the sample was also calculated using eq. 4. The total mass for each analyte were: tacrolimus (0.428 ng), sirolimus (0.418 ng), everolimus (0.416 ng) and cyclosporin A (4.90 ng).

The extraction efficiency (%) was then defined as:

$$\left(\frac{tot.m._{sample}\ (ng)}{tot.m._{theoretical}\ (ng)}\right) \times 100 = extraction\ efficiency\ (\%) \quad (5)$$

The extraction efficiency (%) for the different sample preparation methods are presented in Figure 4-21 as the mean value of three parallels.

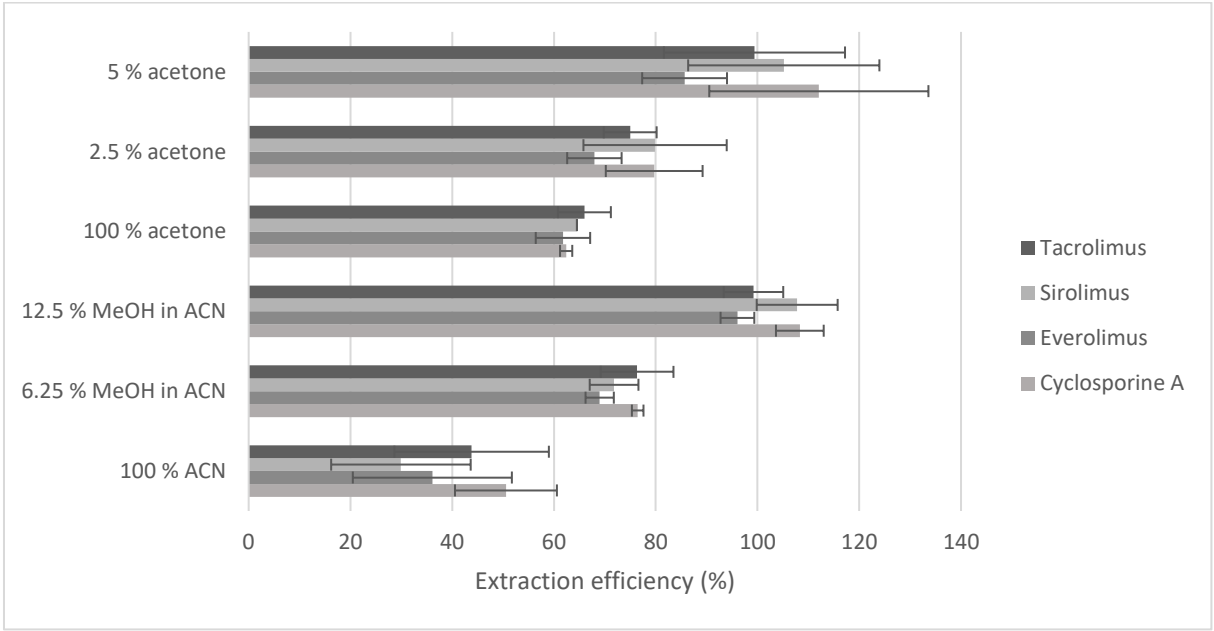


Figure 4-21 Extraction efficiency (%) in samples prepared with SALLE involving different solvent combinations.

Samples prepared with 5 % (v/v) MeOH in acetone and 12.5 % (v/v) MeOH in ACN appeared to be similarly efficient in the extraction of tacrolimus, sirolimus, everolimus and cyclosporin A. The SD for samples prepared with 5 % (v/v) MeOH in acetone was larger than in 12.5 (v/v) MeOH in ACN, and this was thought to be mostly due to large variation in the volume of organic phase measured in the vials.

This method for calculating the extraction efficiency was vulnerable to uncertainty because the volumetric measurement of the organic phase was not particularly accurate. This extraction efficiency experiment had the purpose of giving an estimation of how much of the analyte was actually extracted.

Calculating the extraction efficiency (%) gave an indication of how much of the analyte was actually extracted, independent from the volume in the organic phase.

During the optimisation experiments it was observed that the organic phase, or rather top phase, varied in volume despite being prepared with the same volume of solvent. This was believed to have been caused by incomplete separation of the aqueous solution and the organic solvent. A low concentration in the organic phase could be caused both by incomplete extraction, i.e. some analyte is still left in the aqueous phase, and/or by dilution of the phase due to incomplete phase separation.

In the samples prepared with SALLE and 100% ACN the total measured volume of organic phase was relative consistent. Even when considering the uncertainty associated with the measuring method, there seemed to be a consistent loss of 6 % of solvent in the organic phase. This indicated that some of the ACN was still left in the aqueous phase. The measured total volume of organic phase in samples prepared with 12.5 % (v/v) MeOH in ACN were not as consistent, but the average volume loss was a little lower than in 100 % ACN. Despite this loss of volume, there was no indication that significant amounts of the analytes were still left in the aqueous phase because the calculated extraction efficiency was ~100 % for all analytes.

4.4 Method validation

The method validation experiments quantified the performance of the new method. The working range for tacrolimus, sirolimus and everolimus was determined by the LOQ and the concentration of the highest calibration standard of TDM LYS-WB-PBS STD#0-7, while the working range for cyclosporin A was determined by the LOQ and the concentration of the highest calibration standard of MassTox STD#0-6. These were chosen because they were the calibration curves with the widest range while keeping the linearity, as can be seen with the calibration correlation coefficient (r^2) which was based on the same calibration standards. This good correlation also showed that it was very much possible to make calibration standards on site.

The remaining method performance characteristics are presented in Table 4-3, and the calculation process and results are discussed in more detail in the paragraphs below.

Table 4-3 Method performance characteristics for therapeutic drug monitoring on LC-MS/MS with the use of salting-out assisted liquid-liquid extraction in sample preparation, including limit of detection, limit of quantification, working range, calibration r^2, trueness (recovery (%)), and precision (repeatability and reproducibility).

	Tacrolimus	Sirolimus	Everolimus	Cyclosporin A
LOD (ng/mL)	0.1	0.2	0.03	3.5
LOQ (ng/mL)	0.3	0.5	0.1	6.4
Working range (ng/mL)	0.3 - 86	0.5 - 84	0.1 - 83	6.4 - 1003
Calibration R^2	0.9966	0.9943	0.9965	0.9974
Recovery (%)	101 (± 1)	105 (± 1)	100 (± 1)	108 (± 7)
Repeatability CV (%)				
TDM QC Low	2.0	3.9	2.3	3.3
TDM QC Med	4.1	5.6	4.0	4.5
TDM QC High	1.1	3.0	2.3	3.1
Reproducibility CV (%)				
TDM QC	9.3 (4.6*)	14.0 (6.4*)	11.4 (4.5*)	12.1 (6.4*)
TDM QC Low	6.9 (4.9*)	12.7 (6.8*)	13.4 (6.0 *)	12.3 (8.3*)
TDM QC Med	7.8 (3.7*)	11.5 (5.1*)	9.0 (5.0*)	8.7 (3.8*)
TDM QC High	5.6 (2.1*)	12.0 (4.7*)	8.0 (2.4*)	6.2 (4.1*)

** The CV (%) of QC quantified with the same calibration standard (TDM Lys-WB-PBS STD#0-7, paragraph 3.2.6) (n=10).*

4.4.1 Limit of detection and limit of quantification

Processing the data for the purpose of estimating limit of detection (LOD) and limit of quantification (LOQ) for the method involved generous integration of analyte peaks around the tR defined by tacrolimus (^{13}C, D_2) $[M + Na]^+$, sirolimus (D_3) $[M + Na]^+$, everolimus (D_4) $[M + Na]^+$ and cyclosporin A (D_4) $[M + H]^+$.

Analyte response was defined as:

$$\frac{analyte\ area}{ISTD\ area} = analyte\ response \quad (6)$$

The analyte response for the calibration standards were plotted against the theoretical concentration (ng/mL) in Microsoft Excel and fitted with a linear curve. The slope (a), y-intercept (b) and the correlation coefficient (r^2) for this curve is described in Table 4-4.

Table 4-4 Linear fit for calibration standards. Slope (a), y-intercept (b) and correlation coefficient (r^2) tacrolimus, sirolimus, everolimus and cyclosporin A.

	Tacrolimus	**Sirolimus**	**Everolimus**	**Cyclosporin A**
a	0.016	0.0671	0.0193	0.005
b	0	0.0623	0	0
r^2	0.999	0.9981	0.999	0.992

The fitted curve for sirolimus could not be forced through the origin.

The mean and SD of the analyte response in 20 blank samples were calculated using Microsoft Excel and are described in Table 4-5.

Table 4-5 Response mean and SD for tacrolimus, sirolimus, everolimus and cyclosporin A in 20 blank whole blood samples.

	Tacrolimus	**Sirolimus**	**Everolimus**	**Cyclosporin A**
Mean	0.00125	0.3323	0.00005	0.0116
SD	0.00044	0.0054	0.00023	0.0020

The mean response for sirolimus was much higher than the other analytes. The manufacturer (Toronto Research Chemicals) for the isotopically labelled sirolimus (D_3) reports an isotopic impurity of 6.05 % in the Certificate of Analysis, i.e. the compound contains 6.05 % of the unlabelled sirolimus (also described in Table 3-15). This unlabelled

sirolimus would be detected by the LC-MS/MS as the analyte, which could explain a portion of the elevated analyte response.

Estimation of LOD was defined as:

$$\bar{x}\, response_{blank} + (3 \times SD) = response_{LOD} \quad (7)$$

Estimation of LOQ was defined as:

$$\bar{x}\, response_{blank} + (10 \times SD) = resopnse_{LOQ} \quad (8)$$

Because the fitted curve for sirolimus could not be forced through the origin, the mean response was substituted with the y-intercept when calculating LOD and LOQ.

The concentration (ng/mL) of LOD was calculated using this equation:

$$\frac{response_{LOD} - b}{a} = conc_{LOD}\ (ng/mL) \quad (9)$$

The concentration (ng/mL) of LOQ was calculated using this equation:

$$\frac{response_{LOQ} - b}{a} = conc_{LOQ}\ (ng/mL) \quad (10)$$

The estimated concentration (ng/mL) LOD and LOQ are described in Table 4-3.

4.4.2 Trueness – determination of relative recovery

The relative spike recovery (%) (apparent recovery) was defined by Magnusson, et al,. [55] as:

$$\frac{\bar{x}}{x_{ref}} \times 100 = relative\ spike\ recovery\ (\%) \quad (11)$$

This equation was used to calculate the relative spike recovery (%) because none of the samples had analyte response above the LOD before spiking and was therefore determined to have no concentration of the analytes. In this context "$\bar{x}$" was the mean of all spiked samples and "x_{ref}" was the theoretical concentration of the spiked samples.

The relative spike recovery (%) for tacrolimus, sirolimus, everolimus and cyclosporin A are described in Table 4-3.

4.4.3 Precision – repeatability

The repeatability of this method is presented as the % CV of concentration (ng/mL) between 10 aliquots of TDM QC Low (99999941), TDM QC Medium (99999942) and TDM QC High (99999943) analysed simultaneously. The % CV was calculated with eq. 10, and the results are described in Table 4-3.

$$\left(\frac{SD}{\bar{x}}\right) \times 100 = CV\ (\%) \qquad (12)$$

4.4.4 Precision - reproducibility

The reproducibility of this method is presented as the % CV of concentration (ng/mL) between 23 aliquots of TDM QC (99999506), TDM QC Low (99999941), TDM QC Medium (99999942), and TDM QC High (99999943) analysed over an extended timescale. The % CV was calculated with eq. 10, and the results are described in Table 4-3.

The results are presented with two values for each quality control. The first values represent the % CV for the quality controls that had been quantified with different calibration standards. The second values represent the % CV for the quality control aliquots that were quantified with the same calibration standard (TDM Lys-WB-PBS STD#0-7).

These values are very different from each other. There are several explanations as to why this could happen. There are always uncertainties when preparing different lots of calibration standards, even is the protocol is carefully followed. In the case of these reproducibility experiments, the different calibration standards had slightly different preparation procedures. This would cause more uncertainty, especially because the composition of each calibration standard was different.

This large variation could also partially be explained by the stability of the calibration standards. Changes in linearity was observed in aliquots of calibration standards that had been refrozen after use and then reused the next day. This was particularly apparent in the calibration standards TDM NBCS STD#0-7. Two sets of this calibration standard were included in the same run. One set was freshly thawed directly from -80 °C, while the other set had been analysed once before and stored in temperatures of -20 °C. There was no difference observed in the curve for tacrolimus, but the slope appeared to decrease

significantly in sirolimus and everolimus. A similar effect was observed in cyclosporin A, but it was not as severe.

Figure 4-22 depicts a calibration curve for sirolimus where the values for both sets were included. The arrows mark the points belonging to the calibration standard set that had been stored at -20 °C. There seems to be a significant, systematic decrease in the response. Calibration with this old calibration standard would have given false high concentrations.

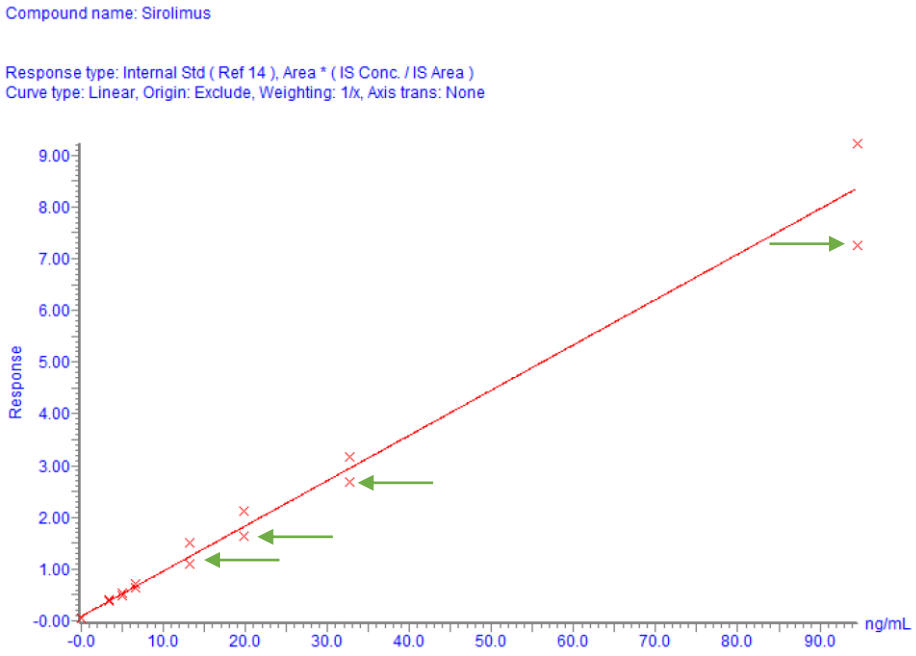


Figure 4-22 Calibration curve of sirolimus in TDM NBCS STD#0-7. The values of two sets included to show decreasing slope. Analyte response on the y-axis and theoretical concentration (ng/mL) on the x-axis. Arrows mark points belonging to the set of calibration standards stored at -20 °C.

A study published in the scientific journal Chromatographia in 2020 ([59]) reported a loss of analyte when PP vials containing a solution of sirolimus in ACN were evaporated until dry and then re-dissolved. The conclusion in the article was that there was an irreversible adsorption of sirolimus in the PP vails. This suggests there could be a loss of sirolimus in the calibration standards and quality controls discussed in this thesis due to adsorption to the PP vials. As everolimus is structurally similar to sirolimus, it is possible that the same could happen to everolimus.

4.5 Method comparison with established immunoassay method

The results from this experiment was compared with the results from analysis of the 100 samples with the immunoassay-based analytical instrument Architect iSR2000 from Abbott. A Bland-Altman plot was made for each analyte with the relative difference (%) on the y-axis and the mean difference on the x-axis. A linear regression plot was also made for both analytes. These plots can be found in Appendix 3 – 4.

The samples containing tacrolimus had an estimated mean relative difference of -28 % (95 % CI [-31 %, -26 %]), 95 % lower limit of agreement (LoA) of -50 % (95 % CI [-54 %, -45 %]), and 95 % lower LoA of -7 % (95 % CI [-12 %, -2 %]).

The samples containing cyclosporin A had an estimated mean relative difference of -20 % (95 % CI [-31 %, -11 %]), 95 % lower limit of agreement (LoA) of -76 % (95 % CI [-93 %, -59 %]), and 95 % lower LoA of 35 % (95 % CI [18 %, 51 %]).

The linear regression and Bland-Altman plot for tacrolimus showed there was a constant bias with dispersion between the two methods and that the results from the new LC-MS/MS was significantly lower than the immunoassay method. This could have been caused by a difference in the calibration of the two different methods. It is also possible that the constant bias was due to cross-reactivity in the immunoassay with substances endogenous to the sample matrix. The linear regression and Bland-Altman plot for cyclosporin A showed however that there was a proportional bias between the two methods. The SD increased in the higher concentrations. This was suspected to have been caused by cross-reactivity with cyclosporin A metabolites in the immunoassay. This will be explained in greater detail below.

Two new MRM transitions were added to the MS method later in the experiments. These new MRM transitions were cyclosporin A $[M + Na]^+$ *m/z* 1225.1 > 377.1, and cyclosporin A D_4 $[M + Na]^+$ *m/z* 1228.6 > 377.1. When comparing the chromatograms for these new MRM transitions and the chromatograms for the old MRM transitions, an obvious difference could be seen.

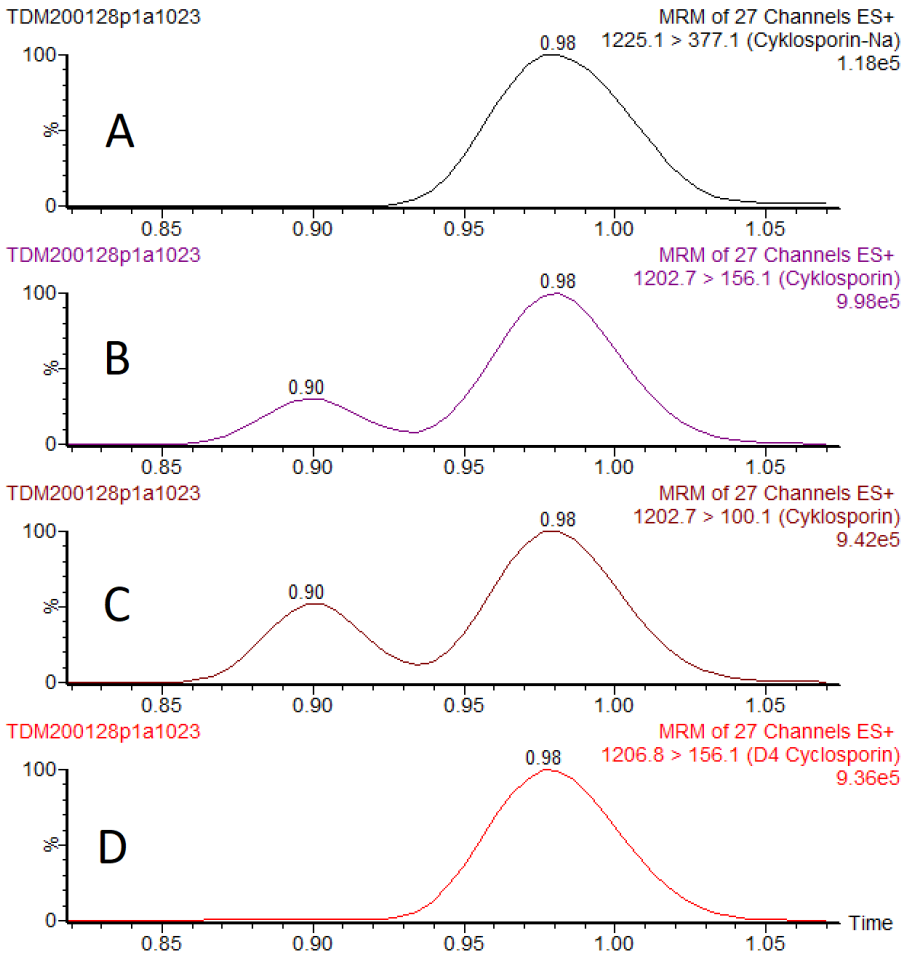


Figure 4-23 Chromatogram of cyclosporin A with different MRM transitions from a patient sample. Chromatogram A is a molecular ion with a Na^+ adduct, while B and C are H^+ adducts with two different fragments. Chromatogram D is the Na^+ of the ISTD.

As is shown in Figure 4-23, the H^+ adducts (chromatogram B and C) have a distinct peak at around 0.9 min. These peaks were not present in the ISTD MRM transition (chromatogram D), nor in the Na^+ adduct MRM transition. This indicated that the peak pertained to something endogenous in the sample. These peaks were only present in patient samples and not in spiked samples (like quality controls and calibration standards). Moreover, the intensity of these peaks seemed to be lower in samples with low concentrations of cyclosporin A and increased in samples with higher concentrations. The tR was around 0.9 min for all samples as well. These observations indicated there was some substance in the patient samples with the same molecular weight, but a different tR. It is possible these peaks belonged to metabolites of cyclosporin A with an additional functional group which affected the tR but was detected as the analyte itself in the MS/MS due to in-source fragmentation.

Immunoassays are often vulnerable to cross-reaction with metabolites, and if the peaks found in the LC-MS/MS at a tR around 0.9 min really pertained to metabolites, this observation could be a very good explanation as to why there was a proportional difference between the immunoassay method and the LC-MS/MS.

4.6 Method comparison with Oslo University Hospital, Department of Pharmacology

The sample preparation and analysis method discussed in this thesis was compared to an existing sample preparation and analysis method used in Oslo University Hospital, Department of Pharmacology. The methods were compared by making an Bland-Altman plot for each analyte, with the relative difference (%) (y-axis) against the mean difference (x-axis). A linear regression plot was also made for each analyte. These plots can be found in Appendix 5 – 8.

The samples containing tacrolimus had an estimated mean relative difference of 10 % (95 % CI [7 %, 13 %]), 95 % lower limit of agreement (LoA) of -5 % (95 % CI [-9 %, 0 %]), and 95 % lower LoA of 25 % (95 % CI [20 %, 30 %]). The correlation was 0.988.

The samples containing had an estimated mean relative difference of -12 % (95 % CI [-16 %, -8%]), 95 % lower LoA of -33 % (95 % CI [-41 %, -26 %]), and 95 % upper LoA of 10 % (95 % CI [3 %, 17 %]). The r^2 was calculated to be 0.892.

The samples containing everolimus had an estimated mean relative difference of -17 % [95 % CI [-22 %, -12 %]), 95 % lower LoA of -41 % [95 % CI [-49 %, -33 %]), and 95 % upper LoA of 7 % (95 % CI [-1 %, 15 %]). The r^2 was calculated to be 0.9679.

The samples containing cyclosporin A had an estimated mean relative difference of 2 % [95 % CI [-5%, 10 %]), 95 % lower LoA of -36 % (95 % CI [-50 %, -23 %]), and 95 % upper LoA of 41 % (95 % CI [27 %, 54 %]). The r^2 was calculated to be 0.9674.

The Bland-Altman plots showed that there was a good correlation between the new and existing methods for tacrolimus and cyclosporin A. Cyclosporin A had a low mean relative difference (%) but a larger LoA interval. The mean relative difference (%) for tacrolimus was a bit higher, but the LoA interval was narrow. Both analytes were measured to have a little

higher concentration (ng/mL) in the new method in relative to the existing method. This difference could be caused by something pertaining to the instrument itself, but it is also possible that it is due to differences in calibration. The method in OUS was calibrated with a different commercial calibration standard than the method discussed in this thesis.

The correlation was not good in sirolimus and everolimus. The new method measured lower concentrations in both, and the mean relative difference (%) in everolimus was quite large. The 95 % LoA for everolimus was also quite large.

The poor correlation between the new method and the existing method was not surprising. Experiences from earlier experiments showed that there was larger SD in samples with sirolimus and everolimus compared to tacrolimus and cyclosporin A. It was difficult to determine whether this was due to the sample preparation itself, or if the variations were caused by degradation of the analytes themselves. The samples were not fresh and could have been vulnerable to degradation due to the shipping and storage before analysis.

5 CONCLUSION

The purpose for this thesis was to find the best combination for extracting immunosuppressants from whole blood with the use of salting-out assisted liquid-liquid extraction (SALLE), and to develop a method for analysing these samples on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for the purpose of therapeutic drug monitoring.

The best sample preparation method was determined to be SALLE with NaCl (5 M, 100 µL) and 12.5 % (v/v) methanol in acetonitrile. This combination gave clean and clear separations of the organic and aqueous phases. The extraction of the analytes was also reliable, with great extraction yield and low ion suppression.

The LC-MS/MS analysis method had satisfying performance characteristics with low limit of detection and limit of quantification, a wide working range, good calibration linearity, and adequate recovery, trueness, and precision, especially in repeatability. These performance characteristics gave the laboratory grounds to finish the method validation for the purpose of implementing the method into the routine analysis.

The method comparisons with both an immunoassay-based method from Stavanger University Hospital and a LC-MS/MS method from Oslo University Hospital gave interesting results. Such experiments are performed with the purpose of comparing the performance between a new method and established methods. Differences between new and established methods are expected, especially when based on different analytical methods. Such differences were also apparent in these experiments. In addition to comparing the performance, these experiments also gave interesting indications about the possible storage instability of sirolimus and everolimus, as well as a potential presence of metabolites in patient samples with cyclosporin A.

gave interesting indications about the performance of the new method, but also about the possible storage instability of sirolimus and everolimus, and a presence of potential interfering metabolites in samples with cyclosporin A.

6 REFERENCES

[1] X. C. Li and A. M. Jevnikar, *Transplant immunology*, Chichester, England: Wiley Blackwell : American Society of Transplantation, 2016.

[2] C. Enderby and C. A. Keller, "An overview of immunosuppression in solid organ transplantation," *The American Journal of Managed Care,* vol. 21, 1, pp. 12-23, 31 January 2015. [Online]. Available: https://www.ajmc.com/journals/supplement/2015/ace022_jan15_organtransplant_ce/ace022_jan15_enderby.

[3] A. Corthay, "A Three-cell Model for Activation of Naïve T Helper Cells," *Scandinavian Journal of Immunology,* vol. 64, no. 2, pp. 93-96, 07 July 2006, doi: 10.1111/j.1365-3083.2006.01782.x.

[4] S. Goral, "The three-signal hypothesis of lymphocyte activation/targets for immunosuppression," *Dialysis & Transplantation,* vol. 40, no. 1, pp. 14-16, 2011, doi: 10.1002/dat.20527.

[5] V. R. Dharnidharka, "43 - Pediatric Renal Transplantation," in *Chronic Kidney Disease, Dialysis, and Transplantation (Fourth Edition)*, J. Himmelfarb and T. A. Ikizler Eds. Philadelphia: Elsevier, 2019, pp. 661-675.e7.

[6] M. R. Ganji and B. Broumand, "Acute cellular rejection," (in eng), *Iran J Kidney Dis,* vol. 1, no. 2, pp. 54-6, Oct 2007.

[7] P. Halloran, "Immunosuppressive Drugs for Kidney Transplantation," *The New England Journal of Medicine,* vol. 351, no. 26, pp. 2715-2729, 23 December 2004. [Online]. Available: https://www.nejm.org/doi/pdf/10.1056/NEJMra033540?articleTools=true.

[8] T. Kino *et al.*, "FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics," (in eng), *J Antibiot (Tokyo),* vol. 40, no. 9, pp. 1249-55, Sep 1987, doi: 10.7164/antibiotics.40.1249.

[9] W. E. Fitzsimmons, "Tacrolimus," ed. Oxford, UK: Oxford, UK: Wiley-Blackwell, 2010, pp. 224-240.

[10] G. Wiederrecht, E. Lam, S. Hung, M. Martin, and N. Sigal, "The mechanism of action of FK-506 and cyclosporin A," (in eng), *Ann N Y Acad Sci,* vol. 696, pp. 9-19, Nov 30 1993, doi: 10.1111/j.1749-6632.1993.tb17137.x.

[11] J. R. Azzi, M. H. Sayegh, and S. G. Mallat, "Calcineurin inhibitors: 40 years later, can't live without," (in eng), *J Immunol,* vol. 191, no. 12, pp. 5785-91, Dec 15 2013, doi: 10.4049/jimmunol.1390055.

[12] H. Tanaka *et al.*, "Structure of FK506, a novel immunosuppressant isolated from Streptomyces," *Journal of the American Chemical Society,* vol. 109, no. 16, pp. 5031-5033, 1987/08/01 1987, doi: 10.1021/ja00250a050.

[13] PubChem. Tacrolimus, CID=445643 [Online] Available: https://pubchem.ncbi.nlm.nih.gov/compound/Tacrolimus

[14] C. Vézina, A. Kudelski, and S. N. Sehgal, "Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle," (in eng), *J Antibiot (Tokyo),* vol. 28, no. 10, pp. 721-6, Oct 1975, doi: 10.7164/antibiotics.28.721.

[15] S. N. Sehgal, "Sirolimus: its discovery, biological properties, and mechanism of action," (in eng), *Transplant Proc,* vol. 35, no. 3 Suppl, pp. 7s-14s, May 2003, doi: 10.1016/s0041-1345(03)00211-2.

[16] R. Sedrani, S. Cottens, J. Kallen, and W. Schuler, "Chemical modification of rapamycin: the discovery of SDZ RAD," *Transplantation Proceedings,* vol. 30, no. 5, pp. 2192-2194, 1998/08/01/ 1998, doi: https://doi.org/10.1016/S0041-1345(98)00587-9.

[17] J. C. Hong and B. D. Kahan, "SIROLIMUS-INDUCED THROMBOCYTOPENIA AND LEUKOPENIA IN RENAL TRANSPLANT RECIPIENTS: RISK FACTORS, INCIDENCE, PROGRESSION, AND MANAGEMENT12," *Transplantation,* vol. 69, no. 10, 2000. [Online]. Available: https://journals.lww.com/transplantjournal/Fulltext/2000/05270/SIROLIMUS_INDUCED_THROMBOCYTOPENIA_AND_LEUKOPENIA.19.aspx.

[18] PubChem. Sirolimus, CID=5284616 [Online] Available: https://pubchem.ncbi.nlm.nih.gov/compound/5284616

[19] S. N. Sehgal, H. Baker, and C. Vézina, "Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization," (in eng), *J Antibiot (Tokyo),* vol. 28, no. 10, pp. 727-32, Oct 1975, doi: 10.7164/antibiotics.28.727.

[20] PubChem. Everolimus, CID=6442177 [Online] Available: https://pubchem.ncbi.nlm.nih.gov/compound/6442177

[21] H. F. Stähelin, "The history of cyclosporin a (SandimmuneR) revisited: Another point of view," *Experientia,* vol. 52, no. 1, pp. 5-13, 1996, doi: 10.1007/BF01922409.

[22] J. F. Borel, C. Feurer, H. U. Gubler, and H. Stähelin, "Biological effects of cyclosporin A: a new antilymphocytic agent," (in eng), *Agents Actions,* vol. 6, no. 4, pp. 468-75, Jul 1976, doi: 10.1007/bf01973261.

[23] PubChem. Cyclosporin A, CID=5284373 [Online] Available: https://pubchem.ncbi.nlm.nih.gov/compound/5284373

[24] J.-S. Kang and M.-H. Lee, "Overview of therapeutic drug monitoring," *Korean J Intern Med,* vol. 24, no. 1, pp. 1-10, 2009, doi: 10.3904/kjim.2009.24.1.1.

[25] A. Johnston and D. W. Holt, "Immunosuppressant drugs - the role of therapeutic drug monitoring: TDM of immunosuppressant drugs," *British Journal of Clinical Pharmacology,* vol. 52, no. S1, pp. 61-73, 2001, doi: 10.1111/j.1365-2125.2001.00365.x.

[26] N. Mohammadpour, S. Elyasi, N. Vahdati, A. H. Mohammadpour, and J. Shamsara, "A review on therapeutic drug monitoring of immunosuppressant drugs," (in eng), *Iran J Basic Med Sci,* vol. 14, no. 6, pp. 485-98, Nov 2011.

[27] J. E. Adaway and B. G. Keevil, "Therapeutic drug monitoring and LC–MS/MS," vol. 883-884, pp. 33-49, 2012, doi: 10.1016/j.jchromb.2011.09.041.

[28] M. Winkler, B. Ringe, J. Baumann, M. Loss, K. Wonigeit, and R. Pichlmayr, "Plasma vs whole blood for therapeutic drug monitoring of patients receiving FK 506 for immunosuppression," (in eng), *Clin Chem,* vol. 40, no. 12, pp. 2247-53, Dec 1994.

[29] T. G. Rosano, "Effect of hematocrit on cyclosporine (cyclosporin A) in whole blood and plasma of renal-transplant patients," (in eng), *Clin Chem,* vol. 31, no. 3, pp. 410-2, Mar 1985.

[30] R. Yatscoff, D. LeGatt, R. Keenan, and P. Chackowsky, "Blood distribution of rapamycin," (in eng), *Transplantation,* vol. 56, no. 5, pp. 1202-6, Nov 1993, doi: 10.1097/00007890-199311000-00029.

[31] T. Koal, M. Deters, B. Casetta, and V. Kaever, "Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry," *J Chromatogr B Analyt Technol Biomed Life Sci,* vol. 805, no. 2, pp. 215-22, Jun 15 2004, doi: 10.1016/j.jchromb.2004.02.040.

[32] S. Mei *et al.*, "Simultaneous determination of cyclosporine and tacrolimus in human whole blood by ultra-high performance liquid chromatography tandem mass spectrometry and comparison with a chemiluminescence microparticle immunoassay," *J Chromatogr B Analyt Technol Biomed Life Sci,* vol. 1087-1088, pp. 36-42, Jun 15 2018, doi: 10.1016/j.jchromb.2018.04.028.

[33] M. Karapirli *et al.*, "Simultaneous determination of cyclosporine A, tacrolimus, sirolimus, and everolimus in whole-blood samples by LC-MS/MS," *ScientificWorldJournal,* vol. 2012, p. 571201, 2012, doi: 10.1100/2012/571201.

[34] R. A. Koster, E. C. Dijkers, and D. R. Uges, "Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood," *Therapeutic drug monitoring,* vol. 31, no. 1, pp. 116-125, 2009.
[35] C. Seger, K. Tentschert, W. Stöggl, A. Griesmacher, and S. L. Ramsay, "A rapid HPLC-MS/MS method for the simultaneous quantification of cyclosporine A, tacrolimus, sirolimus and everolimus in human blood samples," *Nature Protocols,* vol. 4, no. 4, p. 526, 2009. [Online]. Available: https://www.nature.com/articles/nprot.2009.25.
[36] Z. S. Gong *et al.*, "A high-throughput LC-MS/MS method for the quantification of four immunosu- ppressants drugs in whole blood," *Clin Chim Acta,* vol. 498, pp. 21-26, Nov 2019, doi: 10.1016/j.cca.2019.07.026.
[37] B. C. Sallustio, B. D. Noll, and R. G. Morris, "Comparison of blood sirolimus, tacrolimus and everolimus concentrations measured by LC-MS/MS, HPLC-UV and immunoassay methods," *Clinical biochemistry,* vol. 44, no. 2-3, pp. 231-236, 2011. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S0009912010004649.
[38] S. J. Gee, B. D. Hammock, and J. M. Van Emon, "Section 1 - Introduction," in *Environmental Immunochemical Analysis Detection of Pesticides and Other Chemicals*, S. J. Gee, B. D. Hammock, and J. M. Van Emon Eds. Westwood, NJ: William Andrew Publishing, 1996, pp. 1-6.
[39] J. Tate and G. Ward, "Interferences in immunoassay," (in eng), *Clin Biochem Rev,* vol. 25, no. 2, pp. 105-120, 2004. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1904417/.
[40] Z. G. Fang *et al.*, "Analysis of cyclosporine A and its metabolites in rat urine and feces by liquid chromatography-tandem mass spectrometry," *J Chromatogr B Analyt Technol Biomed Life Sci,* vol. 878, no. 15-16, pp. 1153-62, May 1 2010, doi: 10.1016/j.jchromb.2010.03.032.
[41] T. Lea, *Immunologi og immunologiske teknikker*, 3. utg. ed. Bergen: Fagbokforl., 2006.
[42] B. K. De, E. Jimenez, S. De, J. C. Sawyer, and G. A. McMillin, "Analytical performance characteristics of the Abbott Architect i2000 Tacrolimus assay; comparisons with liquid chromatography-tandem mass spectrometry (LC-MS/MS) and Abbott IMx methods," *Clinica Chimica Acta,* vol. 410, no. 1, pp. 25-30, 2009/12/08/ 2009, doi: https://doi.org/10.1016/j.cca.2009.09.009.
[43] D. A. Skoog, *Principles of instrumental analysis*, 7th edition. ed. Boston, Mass: Cengage Learning, 2017.
[44] R. E. Ardrey, *Liquid chromatography - mass spectrometry : an introduction* (Analytical techniques in the sciences). Chichester: Wiley, 2003.
[45] A. Dahlin, "ESI positive mode," ed: Flickr, 2015.
[46] D. L. Pavia, *Introduction to spectroscopy*, 5th ed. ed. Stamford, Conn: Cengage Learning, 2015.
[47] J. Stone, "Chapter 3 - Sample preparation techniques for mass spectrometry in the clinical laboratory," in *Mass Spectrometry for the Clinical Laboratory*, H. Nair and W. Clarke Eds. San Diego: Academic Press, 2017, pp. 37-62.
[48] W. Tszyrsznic *et al.*, "Two rapid ultra performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) methods with common sample pretreatment for therapeutic drug monitoring of immunosuppressants compared to immunoassay," *J Chromatogr B Analyt Technol Biomed Life Sci,* vol. 928, pp. 9-15, Jun 1 2013, doi: 10.1016/j.jchromb.2013.03.014.
[49] B. G. Keevil, S. J. McCann, D. P. Cooper, and M. R. Morris, "Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography-tandem mass spectrometry," *Ann Clin Biochem,* vol. 39, no. Pt 5, pp. 487-92, Sep 2002, doi: 10.1258/000456302320314502.
[50] A. Pohanka, S. Rosenborg, J. D. Lindh, and O. Beck, "Experiences from using LC-MS/MS for analysis of immunosuppressive drugs in a TDM service," *Clin Biochem,* vol. 49, no. 13-14, pp. 1024-31, Sep 2016, doi: 10.1016/j.clinbiochem.2016.06.013.

[51] M. Deters, G. Kirchner, K. Resch, and V. Kaever, "Simultaneous quantification of sirolimus, everolimus, tacrolimus and cyclosporine by liquid chromatography-mass spectrometry (LC-MS)," *Clin Chem Lab Med,* vol. 40, no. 3, pp. 285-92, Mar 2002, doi: 10.1515/CCLM.2002.045.

[52] N. J. K. Simpson, *Solid-phase extraction : principles, techniques, and applications*. New York: Marcel Dekker, 2000.

[53] Y. Su, S. Xia, R. Wang, and L. Xiao, "13 - Phytohormonal quantification based on biological principles," in *Hormone Metabolism and Signaling in Plants*, J. Li, C. Li, and S. M. Smith Eds.: Academic Press, 2017, pp. 431-470.

[54] J. Zhang, H. Wu, E. Kim, and T. A. El-Shourbagy, "Salting-out assisted liquid/liquid extraction with acetonitrile: a new high throughput sample preparation technique for good laboratory practice bioanalysis using liquid chromatography–mass spectrometry," *Biomed Chromatogr,* vol. 23, no. 4, pp. 419-425, 2009, doi: 10.1002/bmc.1135.

[55] B. Magnusson, U. Örnemark, Ed. *Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics*, 2nd ed. 2014.

[56] M. J. Bogusz, E. A. Enazi, H. Hassan, J. Abdel-Jawaad, J. A. Ruwaily, and M. A. Tufail, "Simultaneous LC–MS–MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure," *Journal of Chromatography B,* vol. 850, no. 1, pp. 471-480, 2007/05/01/ 2007, doi: https://doi.org/10.1016/j.jchromb.2006.12.048.

[57] A. Kruve, K. Kaupmees, J. Liigand, M. Oss, and I. Leito, "Sodium adduct formation efficiency in ESI source," *Journal of Mass Spectrometry,* vol. 48, no. 6, pp. 695-702, 2013, doi: 10.1002/jms.3218.

[58] R. A. Koster, J.-W. C. Alffenaar, B. Greijdanus, and D. R. Uges, "Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery," *Talanta,* vol. 115, pp. 47-54, 2013. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S0039914013003317.

[59] A. D. Rogachev, D. V. Trebushat, A. N. Kudryashov, and A. G. Pokrovsky, "Study of Sirolimus Adsorption and Preparation of Its Samples in Methanol, Acetonitrile and Their Mixtures with Water for HPLC–MS/MS Analysis," *Chromatographia,* vol. 83, no. 2, pp. 299-304, 2020/02/01 2020, doi: 10.1007/s10337-019-03835-5.

APPENDICES

Appendix 1: Case documents from Regional Ethics Committee (REK vest)

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK vest	Jessica Svärd	55978497	06.02.2020	80982
			Deres referanse:	

Cato Brede

80982 Ny metode for måling av immunosuppressiva

Forskningsansvarlig: Helse Stavanger HF - Stavanger universitetssjukehus

Søker: Cato Brede

Søkers beskrivelse av formål:

Prosjektet tar sikte på å utvikle og validere en ny og forbedret metode for bestemmelse av immunosuppressiva i blodprøver ved Stavanger Universitetssykehus. Den nye metoden er basert på væskekromatografi koblet med tandem massespektrometri (LC-MS/MS) og vil anvende en ny og forbedret ekstraksjonsmetode for blodprøvene. For å validere den nye metoden må det analyseres 100-500 av et tilfeldig utvalg pasientprøver (EDTA fullblod) fra pasienter som inntar medikamentene tacrolimus, sirolimus, everolimus og/eller cyclosporin. Dette må være prøver hvor det allerede foreligger et prøvesvar, enten fra Stavanger Universitetssykehus eller fra Rikshospitalet. Målsetningen er å undersøke samsvar med de eksisterende analysemetodene.

REKs vurdering

Deltakere
Prosjektet skal inkludere 500 pasienter som inntar medikamentene tacrolimus, sirolimus, everolimus og/eller cyclosporin og som får sine prøver analysert ved Stavanger Universitetssjukehus eller Rikshospitalet.

Deltakere identifiseres ved å plukke ut blodprøver hvor det er bestilt analyse av immunosuppressiva.

Alle skriftlige henvendelser om saken må sendes via REK-portalen
Du finner informasjon om REK på våre hjemmesider rekportalen.no

Tidligere registrerte opplysninger
Opplysningene hentes fra Laboratoriets datasystem (LIMS). Analysesvar for medikamentene tacrolimus, sirolimus, everolimus og cyclosporin.

Humant biologisk materiale
Fullblod som er innhentet for diagnostikk skal benyttes. Prøvemateriale anonymiseres før analyse. Materiale destrueres etter analyse.

Oppbevaring av data og koblingsliste
Det oppgis at det ikke er nødvendig med kobling til navn på pasient. Prøvenummeret er koblet til navn en kort tid før det blir gjenbrukt i laboratoriet. Prosjektet planlegger å opprette en liste som kun inneholder prøvenummer, medikament og måleresultatene.

Det søkes om fritak fra kravet om å innhente samtykke
For at REK skal kunne innvilge fritak fra samtykke må kravene etter helseforskningsloven §§ 28 (humant biologisk materiale) og 35 (helseopplysninger) være oppfylt: 1) det må være vanskelig å innhente samtykke, 2) velferden og integriteten til deltakerne må være ivaretatt, og 3) interessen for samfunnet må være vesentlig stor. For humant biologisk materiale må pasientene i tillegg være informert om at biologisk materiale tatt for diagnostikk kan bli brukt i forskning og at de har hatt anledning til å reservere seg mot dette jf. *hfl* §28.

Komiteen vurderer at kriteriene for å innvilge fritak fra samtykkekravet er oppfylt for prosjektet og innvilger dermed dispensasjon fra taushetsplikten for å få utlevert de nevnte opplysningene fra pasientjournal og humant biologisk materiale innhentet for diagnostisk formål, jf. helseforskningsloven §§ 28 og 35.

Prosjektslutt og håndtering av data etter prosjektslutt
Prosjektslutt er 30.06.2020. Data er anonymiserte under prosjektperioden.

Vedtak

Godkjent

REK vest har gjort en helhetlig forskningsetisk vurdering av alle prosjektets sider. Prosjektet godkjennes med hjemmel i helseforskningsloven § 10.

Med vennlig hilsen,

Marit Grønning
Prof. Dr. med.

Komitéleder, REK vest

Jessica Svärd
rådgiver, REK vest

Sluttmelding
Søker skal sende sluttmelding til REK vest på eget skjema senest seks måneder etter godkjenningsperioden er utløpt, jf. hfl. § 12.

Søknad om å foreta vesentlige endringer
Dersom man ønsker å foreta vesentlige endringer i forhold til formål, metode, tidsløp eller organisering, skal søknad sendes til den regionale komiteen for medisinsk og helsefaglig forskningsetikk som har gitt forhåndsgodkjenning. Søknaden skal beskrive hvilke endringer som ønskes foretatt og begrunnelsen for disse, jf. hfl. § 11.

Appendix 2: Calculated mass-to-charge ratio (*m/z*) of predicted molecular ions

Analyte	Formula	M	$M + H^+$ (m/z)	$M + 2\ H^+$ (m/z)	$M + NH_4^+$ (m/z)	$M + Na^+$ (m/z)	$M + Na^+ + H^+$ (m/z)	$M + 2\ Na^+$ (m/z)
Tacrolimus	$C_{44}H_{69}NO_{12}$	803.48	804.49	402.75	821.52	826.47		
Sirolimus	$C_{51}H_{79}NO_{13}$	913.56	914.56	457.79	931.59	936.54		
Everolimus	$C_{53}H_{83}NO_{14}$	957.58	958.59	479.80	975.62	980.57		
Cyclosporin A	$C_{62}H_{111}N_{11}O_{12}$	1201.84	1202.85	601.93	1219.88	1224.83	612.92	623.91
Tacrolimus (13C, D4)	$C_{43}{}^{13}CH_{67}D_2NO_{12}$	806.50	807.51	404.26	824.53	829.49		
Sirolimus (D3)	$C_{51}H_{76}D_3NO_{13}$	916.57	917.58	459.29	934.61	939.56		
Everolimus (D4)	$C_{53}H_{79}D_4NO_{14}$	961.61	962.61	481.81	979.64	984.60		
Cyclosporin A (D4)	$C_{62}H_{107}D_4N_{11}O_{12}$	1205.87	1206.87	603.94	1223.90	1228.86	614.93	625.92

Appendix 3: Bland-Altman plot comparing new LC-MS/MS method to immunoassay-based method, tacrolimus

Method comparison: Immunoasay: tacrolimus, LC-MS/MS: tacrolimus

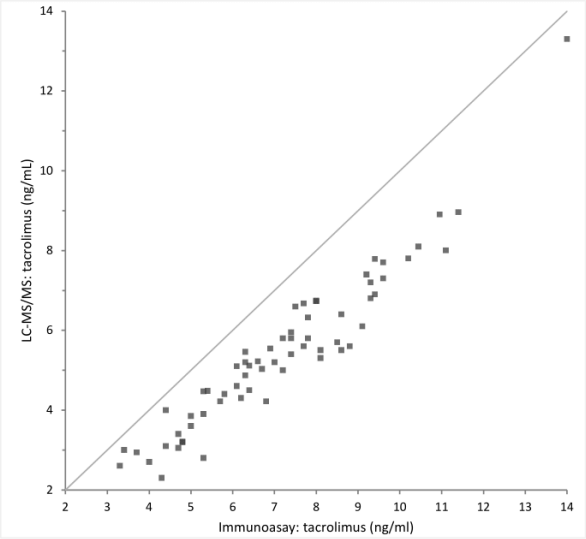
Analyse-it v5.30

Tacrolimus A4:D88

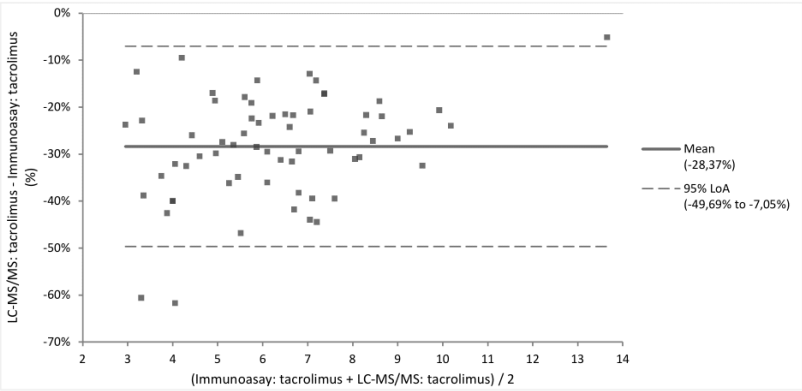
Filter: No filter

Last updated 20 juni 2020 at 13:42 by Kvamsøe, Marianne Meling

Descriptives



Difference plot



N	64

	Minimum	Maximum
Immunoasay: tacrolimus	3.3000	14.0000
LC-MS/MS: tacrolimus	2.3000	13.3000
(Immunoasay: tacrolimus + LC-MS/MS: tacrolimus) / 2	2.9500	13.6500

Fit Differences

Parameter	Estimate	95% CI	SE
Mean difference	-28.37 %	-31.083% to -25.649%	1.360%
95% Lower LoA	-49.69 %	-54.353% to -45.017%	2.336%
95% Upper LoA	-7.05 %	-11.714% to -2.378%	2.336%

SD	10.88 %

Appendix 4: Bland-Altman plot comparing new LC-MS/MS method to immunoassay-based method, cyclosporin A

Method comparison: Immunoassay: cyclosporin A, LC-MS/MS: cyclosporin A

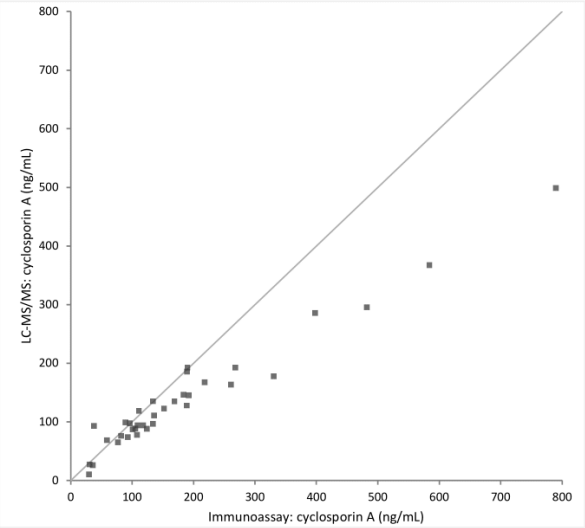
Analyse-it v5.30

Cyclosporin A1:D82

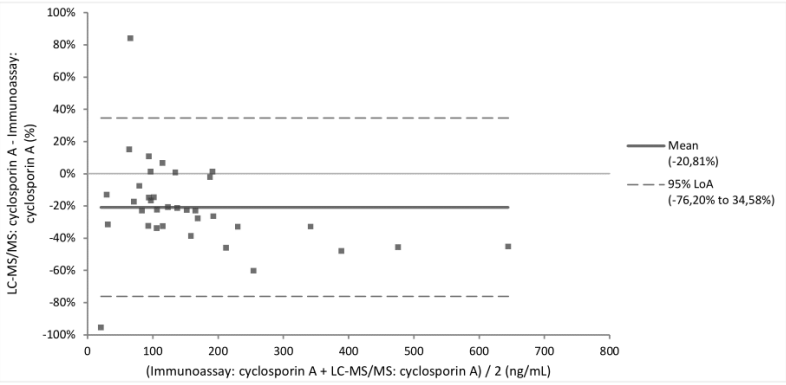
Filter: No filter

Last updated 20 juni 2020 at 13:42 by Kvamsøe, Marianne Meling

Descriptives



Difference plot



N	35

	Minimum	Maximum
Immunoassay: cyclosporin A	30.000	790.000
LC-MS/MS: cyclosporin A	10.600	498.800
(Immunoassay: cyclosporin A + LC-MS/MS: cyclosporin A) / 2	20.300	644.400

Correlation - r	0.977

Fit Differences

Parameter	Estimate	95% CI	SE
Mean difference	-20.81 %	-30.518% to -11.101%	4.777%
95% Lower LoA	-76.20 %	-92.954% to -59.451%	8.243%
95% Upper LoA	34.58 %	17.833% to 51.336%	8.243%

SD	28.26 %

Appendix 5: Bland-Altman plot comparing new method with established method from Oslo University Hospital, tacrolimus

Method comparison: OUS (tacrolimus (ng/mL)), SUS (tacrolimus (ng/mL))

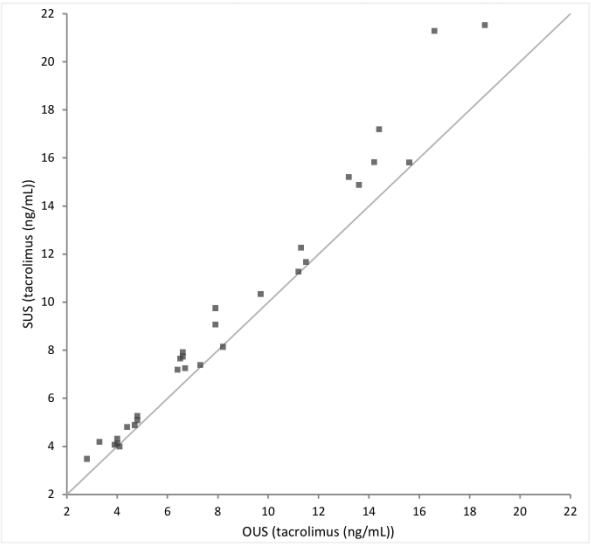
Analyse-it v5.30

Tacrolimus A3:C33

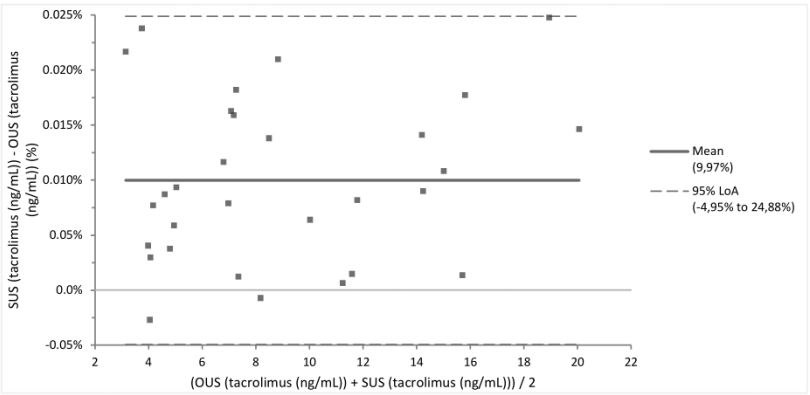
Filter: No filter

Last updated 20 juni 2020 at 13:16 by Kvamsøe, Marianne Meling

Descriptives



Difference plot



N	30

	Minimum	Maximum
OUS (tacrolimus (ng/mL))	2.8	18.6
SUS (tacrolimus (ng/mL))	3.48	21.53
(OUS (tacrolimus (ng/mL)) + SUS (tacrolimus (ng/mL))) / 2	3.14	20.065

Correlation - r	0.988

Fit Differences

Parameter	Estimate	95% CI	SE
Mean difference	9.97 %	7.125% to 12.809%	1.390%
95% Lower LoA	-4.95 %	-9.862% to -0.038%	2.402%
95% Upper LoA	24.88 %	19.973% to 29.796%	2.402%

SD	7.61 %

Appendix 6: Bland-Altman plot comparing new method with established method from Oslo University Hospital, sirolimus

Method comparison: OUS (sirolimus (ng/mL)), SUS (sirolimus (ng/mL))

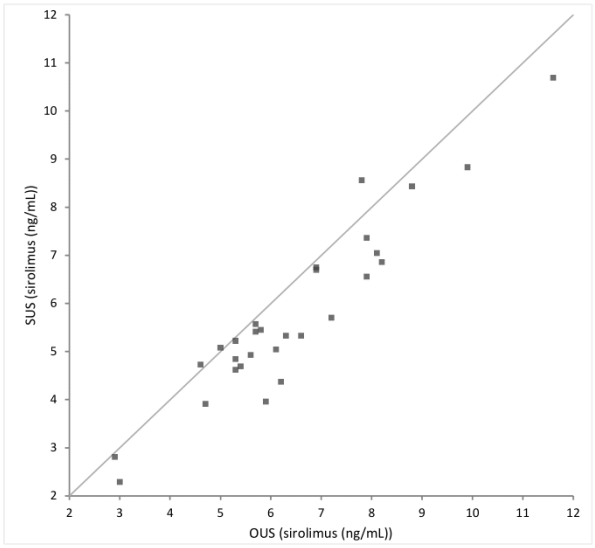
Analyse-it v5.30

Sirolimus A1:C30

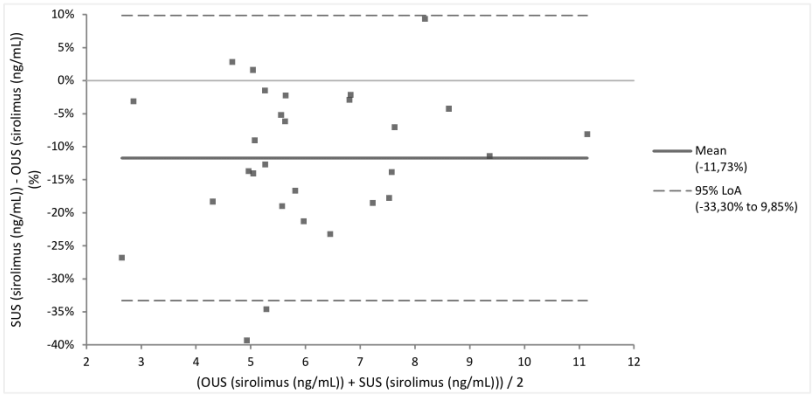
Filter: No filter

Last updated 20 juni 2020 at 13:03 by Kvamsøe, Marianne Meling

Descriptives



Difference plot



N	29

	Minimum	Maximum
OUS (sirolimus (ng/mL))	2.9	11.6
SUS (sirolimus (ng/mL))	2.29	10.69
(OUS (sirolimus (ng/mL)) + SUS (sirolimus (ng/mL))) / 2	2.645	11.145

Correlation - r	0.944

Fit Differences

Parameter	Estimate	95% CI	SE
Mean difference	-11.73 %	-15.914% to -7.539%	2.044%
95% Lower LoA	-33.30 %	-40.544% to -26.064%	3.535%
95% Upper LoA	9.85 %	2.611% to 17.092%	3.535%

SD	11.01 %

Appendix 7: Bland-Altman plot comparing new method with established method from Oslo University Hospital, everolimus

Method comparison: OUS (everolimus (ng/mL)), SUS (everolimus (ng/mL))

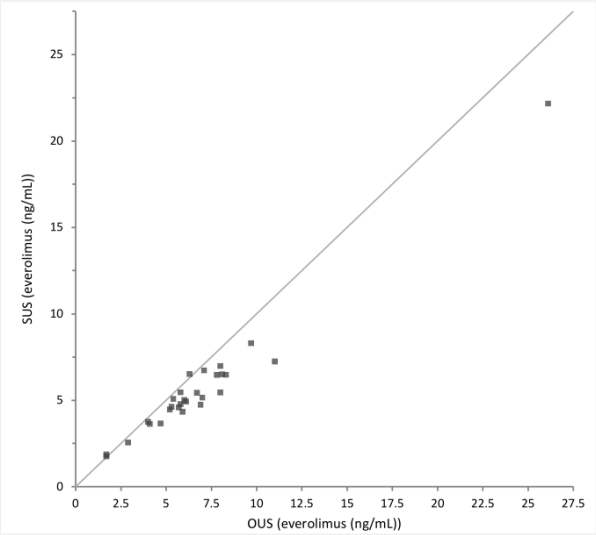
Analyse-it v5.30

Everolimus A1:C30

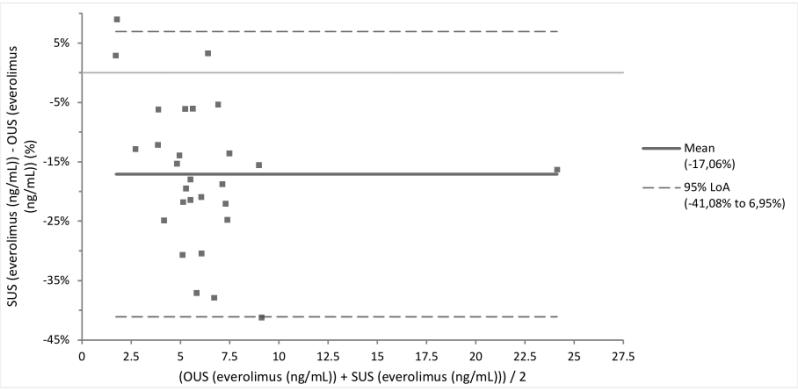
Filter: No filter

Last updated 20 juni 2020 at 13:03 by Kvamsøe, Marianne Meling

Descriptives



Difference plot



N	28

	Minimum	Maximum
OUS (everolimus (ng/mL))	1.70	26.10
SUS (everolimus (ng/mL))	1.75	22.16
(OUS (everolimus (ng/mL)) + SUS (everolimus (ng/mL))) / 2	1.73	24.13

Correlation - r	0.984

Fit Differences

Parameter	Estimate	95% CI	SE
Mean difference	-17.06 %	-21.817% to -12.313%	2.316%
95% Lower LoA	-41.08 %	-49.305% to -32.865%	4.006%
95% Upper LoA	6.95 %	-1.265% to 15.175%	4.006%

SD	12.26 %

Appendix 8: Bland-Altman plot comparing new method with established method from Oslo University Hospital, cyclosporin A

Method comparison: OUS (cyclosporin A (ng/mL)), SUS (cyclosporin A (ng/mL))

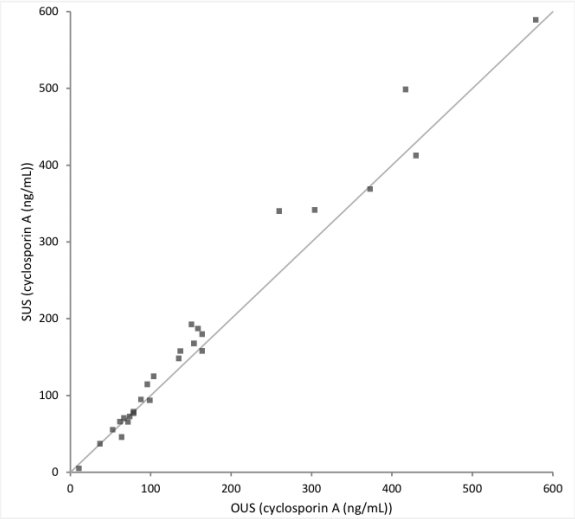
Analyse-it v5.30

Cyclosporin A1:C28

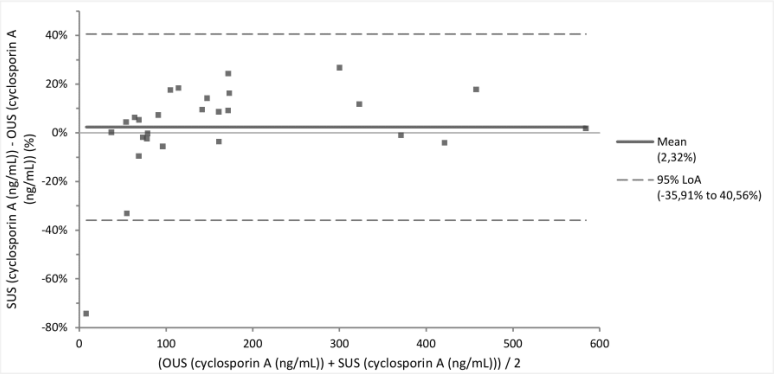
Filter: No filter

Last updated 20 juni 2020 at 13:03 by Kvamsøe, Marianne Meling

Descriptives



Difference plot



N	27

	Minimum	Maximum
OUS (cyclosporin A (ng/mL))	11	579
SUS (cyclosporin A (ng/mL))	5.04	589.4
(OUS (cyclosporin A (ng/mL)) + SUS (cyclosporin A (ng/mL))) / 2	8.02	584.2

Correlation - r	0.988

Fit Differences

Parameter	Estimate	95% CI	SE
Mean difference	2.32 %	-5.394% to 10.042%	3.755%
95% Lower LoA	-35.91 %	-49.270% to -22.559%	6.497%
95% Upper LoA	40.56 %	27.207% to 53.918%	6.497%

SD	19.51 %