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Solution Speciation and Antimicrobial Properties of Silver(I) Nitrate Complexes with Pyridine and Quinoline type Ligands

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Abstract

Due to the emergence of multi-drug resistant strains of bacteria (MDRS) the current treatment methods of superficial wounds, burns and ulcers are becoming less and less effective (Socialstyrelsen, 2010). Hence there is a need for innovative biocides capable of withstanding the onslaught of microorganisms which have been rendered more or less impervious to existing antibiotics (World Health Organization, 2010).

In this thesis an attempt to further understand and document the chemical properties of six silver-coordination complexes which, in a previous study, have indicated biocidal effects comparable to more conventional antibiotics (Abu-Youssef *et al.*, 2007 and Massoud, 2011). Even though silver has been utilized in medicine in various forms throughout history there is little evidence of bacterial resistance development (Alt *et al*, 2004 and Adams, 2000). The hope is therefore that such compounds/complexes as the ones being scrutinized here can even the odds in the field of wound healing and burn management, where the issue of ineffective treatments have been particularly severe (Williamson, 2005).

The intention was to utilize two analysis techniques; proton nuclear magnetic resonance (^{1}H-NMR) titration to obtain data for determination of equilibrium constants for the complexation reactions and electrospray ionization mass spectrometry (ESI-MS) to study chemical behaviour under *in vivo*-mimicking conditions. Synthesis and analysis of complexation of the complexes and tests to study biocidal effect were also carried out on a smaller scale.

X-ray powder diffraction performed on newly synthesized [Ag(5-nitroquinoline)$_2$], as well as previously performed XRPD-analysis by Shimo al Massoud (2011), confirmed that the suggested synthesis method for the complexes here studied is functional.

^{1}H-NMR trials were able to detect chemical shifts which could be utilized to predict complexation behaviour, to some degree, for silver(I) and ligands 5-nitroquinoline, isopropylnicotinate and isonicotinamide. ^{1}H-NMR data was also utilized to obtain equilibrium constants, K_{eq}, for the formation of four of the complexes; $K_{[Ag(5\text{-nitroquinoline})2]} = 15.84$, $K_{[Ag(isopropylnicotinate)2]} = 2.51*10^4$ $K_{[Ag(isonicotinamide)2]} = 1.58*10^4$ and $K_{[Ag(2\text{-aminopyridine})2]} = 1.99*10^6$.

The ESI-MS experiments were largely unsuccessful, however, one spectrum was obtained and the data gave a notion of the chemical behaviour of [Ag(2-aminopyridine)$_2$] in solution. Unfortunately no data could be obtained from the trials with bovine serum albumin in solution with either of the complexes.

The diffusion-tests (tests for biocidal effect) were performed on too small a scale to be statistically significant; however, they could determine with some certainty that ligands 5-nitroquinoline and ethyl nicotinate does not have inherent antibacterial properties.

List of abbreviations

BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
K_{eq}	Equilibrium Constant
ESI-MS	Electrospray Ionization Mass Spectrometry
HRI	Healthcare Related Infection
MDRS	Multi-Drug Resistant Strain
MeOH	Methanol
NMR	Nuclear Magnetic Resonance
XRPD	X-ray Powder Diffraction

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1. Introduction

In an article published in 2005 the journalist Julie Williamson reported that 10% of all patients in North American hospitals are suffering from superficial wounds and pressure ulcers (Williamson, 2005). Since traumatic or thermal damage to the skin causes a breach in the body's primary defence-barrier against microorganisms, the risk for infections in these patients increase dramatically. The healing rate of an infected wound is reduced and causes the patient more pain and difficulty and if not properly treated, or if untreatable, the infection may lead to sepsis (Robson, 1997). Hospitalized patients suffering from infected wounds are estimated to represent more than 40% of total healthcare costs and have a ten times higher mortality rate than other patients (Socialstyrelsen, 2010).

Hence, there is both a substantial need and market for more effective treatments of slow-healing and infected wounds and burns (Williamson, 2005). Novel solutions to the issue are however necessary since the currently used antimicrobial agents are no longer sufficient to prevent certain infections from propagating and spreading due to resistance development (Socialstyrelsen, 2010).

A possible approach may be to further develop antimicrobial agents already proven to have a biocidal effect but that have not yet been used to the extent that bacterial resistance has had the chance to develop. An example of such an agent is silver, which has been utilized in medicine in various forms at for at least six millennia (Wesley, 2009). Silver is known to be antimicrobial but there is still some speculation on the exact mechanism (Rentz, 2012).

However, many of the existing forms of medicinal silver have drawbacks that, to some extent, discourage from usage. The compounds are relatively unstable and may easily lose silver ions to the surrounding tissue when applied to a wound. Once in contact with bodily fluids the ions are prone to start forming stable complexes with anions, such as chloride, and precipitate (Choi *et al*, 2008). This does not only lead to the inactivation of silver as an antibacterial agent but may also have adverse effects to the tissue in question (Drake and Hazelwood, 2005).

If silver is to be utilized in medicine it will therefore need to be studied further and more apposite and stable compounds have to be developed. This thesis will focus on six molecules that have shown potential as suitable ligands to silver for the purpose of improving antibacterial properties as well as reducing the risk for precipitation.

1.1. Aim

The overall objective for the project is to thoroughly study six pyridine and quinoline type ligands in complex with silver ions in order to more fully be able to evaluate the possibility to utilize them as antibacterial agents. The intention is both to study the complexes capabilities as antimicrobial agents as well as try to determine chemical properties related to macromolecular binding and complex formation.

The ligands are intended for complexation with Ag(I) cations in a molar ratio of one ion to two ligands or 1:2 Ag(I)/ligand ratio. ^{1}H-NMR titration will be utilized to study chemical equilibrium properties of the complex formation and, to try to determine equilibrium constants for the complexes.

Since the *in vivo*-environment is constituted of a complex combination of various substances the possibility of making an exact evaluation of silver/ligand behaviour once applied to the wound is limited. Solutions containing bovine serum albumin (BSA) will be used to mimic *in-vivo* conditions and the effect of the silver complexes interaction with such media will be sought to be

determined. By utilizing a mass spectrometer combined with an electrospray ionization ion source (ESI-MS) it may be possible to study such interactions *in vitro* and define their possible influence on the complexes efficiency.

2. Background

2.1. Historical utilization of silver-compounds

There exists evidence that suggests that silver has been utilized to improve wellbeing as far back as 4000 B.C. if not even longer (Wesley, 2009). Since the existence of microorganisms was not discovered until 1675 A.D. the benign properties of silver have been observed but they have in most cases been considered supernatural or unfathomable (Wesley, 2009). Though the exact effects of silver on microorganisms are still, to some extent, unknown the continued use of the element in the field of medicine 6000 years later is a testament to its qualities.

Silver was one of the first metals to be discovered and used since it occurs naturally in unalloyed form and silver relics such as jewellery, weapons and vessels from several ancient civilizations have been excavated. The "purifying" properties of the metal might have been noticed as it was utilized as a container for various liquids and foodstuffs (Wesley, 2009). As a biocide, silver would, to some extent, have been able to prevent proliferation of microorganisms thereby allowing for better storage capabilities.

The belief that serving food on silver plates and drinks in silver goblets would cleanse the foodstuffs of evil is thusly not completely incorrect. It could be as simple as plates made from silver were more easily washed and kept clean than plates made from wood or tin. However, a more scientific explanation is that the daily utilization of silverware caused a small but continual ingestion of the metal to those that could afford such luxury. A small amount of silver in the circulatory system probably resulted in a slightly increased resistance to bacterial infection giving the upper classes an increased chance of survival during times of disease (Wesley, 2009). It might also have brought about the belief of the nobilities "blue blood" since a high enough concentration of silver in the blood system causes a condition known as argyria. Symptoms involve a blue-grey discolouration of the skin and bodily fluids (Drake and Hazelwood, 2005).

The medicinal use of inorganic silver salts has been described by several historical profiles such as Hippocrates and Herodotus around 400 B.C. (Adams, 2000). The compositions of these salts are of course hard to determine but according to translations of Hippocrates's texts the compounds were utilized in much the same way and for the same purposes as they are today (Adams, 2000).

2.2. Bacterial resistance development

On the 20th of August 2010 the World Health Organisation released a report in which the global community was alerted that the threat of bacterial resistance to antibiotics had to be addressed even more seriously than previously (World Health Organization, 2010). One of the reasons for this alarm was the discovery of a powerful bacterial antibiotics resistance gene in an Indian strain of *Enterobacteriaceae* (Kumarasamy *et al*, 2010). Due to the bacterial ability to pass on genetic material by means of horizontal gene transfer, not only to its own strain but to other susceptible bacteria in its vicinity, this could allow infectious microorganisms to quickly develop resistance to almost all currently used antibiotics (Kumarasamy *et al*, 2010).

The problem with microbial resistance is not a new phenomenon; hospitals around the world have battled hard treated infections for a long time (Barrett *et al*, 1999). The discovery in India is however alarming since a rapid spread of almost untreatable bacteria could be the consequence.

Statistics gathered 2010 on behalf of the Swedish national board of health and welfare indicate that around 10% of all patients in western hospitals are affected by healthcare associated

infections (HRI) (Smittskyddsinstitutet, 2010). This is to be expected since it is hard to keep infections from spreading in such an environment even with good clinical practises. However, more and more of these contagions are caused by multi-drug resistant strains (MDRS) of microorganisms which require more aggressive treatment methods than previously encountered strains (Socialstyrelsen, 2010).

Studies have shown that 1 of every 100 patients spend more than 50 days in hospitals or other medical facilities. It is estimated that over 90% of these patients are suffering from HRIs and that about 25% will not recover (Socialstyrelsen, 2010). The number of patients with chronic infections can be expected to rise along with increasing mortality because of the inability to appropriately treat infections caused by MDRS.

The main reason for the rapid rise of bacteria capable of withstanding one or several types of antibiotics is the unconsidered utilization of biocidal substances (Barrett *et al*, 1999). It is therefore crucial not only to develop new antibiotics but also to restrict their usage in order to avoid further resistance development (Barrett *et al*, 1999).

2.3. Silver in medicine

As mentioned in the introduction to this paper; silver substances could be part of the solution to circumvent the problem with bacterial resistance, at least for some time. However, there is a necessity to improve the current treatments and to gain further understanding of the mechanisms behind the biocidal effect of silver.

Evidence suggests that silver ions prevent bacterial cell wall-activity by binding to certain membrane proteins and thereby obstruct enzymatic activity (Choi *et al*, 2008). Since bacterial energy producing complexes mostly are situated in the membrane and not inside the cell, as is the case of mammalian cell mitochondria, this could explain why non-eukaryotic microorganisms are sensitive to silver influence (Alt *et al*, 2004). It could also explain why mammalian cells suffer only minor adverse effects from silver exposure at the same concentrations (Alt *et al*, 2004). The main concern in mammals is the potential build-up of silver compounds in tissues due to over-exposure. Various recommendations concerning exposure-limitations to silver and silver complexes exist. It is generally considered that a concentration in the body of 0.01 mg/m^3 and over can lead to a chronic blue-grey discolouration of the skin (argyria) or the eyes (argyrosis) (Drake and Hazelwood, 2005). High levels of soluble forms of silver in the body can also lead to damages to the liver, kidneys and other vital organs whilst metallic silver seems to cause only minor health problems (Drake and Hazelwood, 2005).

The aforementioned adverse effects of exposure to certain types of silver compounds must be taken into account when developing an effective antimicrobial system for wound treatment. One of the main concerns is that the environment *in vivo* will allow the silver to react with naturally occurring elements of the bloodstream and precipitate without contributing any beneficial effects or even cause an unnecessary increase of the silver-substance in the body.

However, by combining silver with molecules designed to both enhance potency against bacteria as well as decrease the risk of random chemical reactions in wound fluids, an effective and less-toxic compound may be synthesised. By applying such compounds, for example to medical adhesives it could be possible to ascertain that the complex reaches the intended target without losing too much potency. Several factors must be taken into account, such as leaching from the adhesive to the intended area and perfusion into the tissues. This project, however, focused mainly on the complexes themselves and their antimicrobial effect and did only to a lesser extent study material diffusion properties.

2.4. Ligands

Two groups, or types, of ligands are included in this study; nitroquinolines and nicotinates. They have been included in this study due to various interesting properties such as an ability to increase complex stability or inherent antibacterial effect. It must be explained that this thesis focuses on the overall antibacterial properties of the functional complexes. Consequently, a complex containing a ligand that is in itself antibacterial or a complex that is more stable *in vivo* due to its ligands may be considered equally effective biocides.

2.4.1. Quinolines

One of the two quinoline-type ligands here studied, 5-nitroquinoline, have in hydroxylated form (8-hydroxy-5-nitroquinoline) in previous studies shown potential as antineoplastic properties and have also been utilized to treat minor infections (Jiang *et al*, 2011). There has been some concern regarding the carcinogenicity of 8-nitroquinoline but a study on hamsters in 1985 found no conclusive evidence suggesting that 8-nitroquinoline increases the risk for tumour formation (Takahashi *et* al. 1978 and Furukawa *et al*. 1985). Both quinolines are utilized as precursors to the metal-dependent antibiotic "streptonigrin" which is produced by the actinobacterium *Streptomyces flocculus* (Kimber *et al.* 2000).

In figure 1 a depiction of the chemical structure of both ligands as well as the two complexes in which they are coordinated around a silver ion (Ag^+).

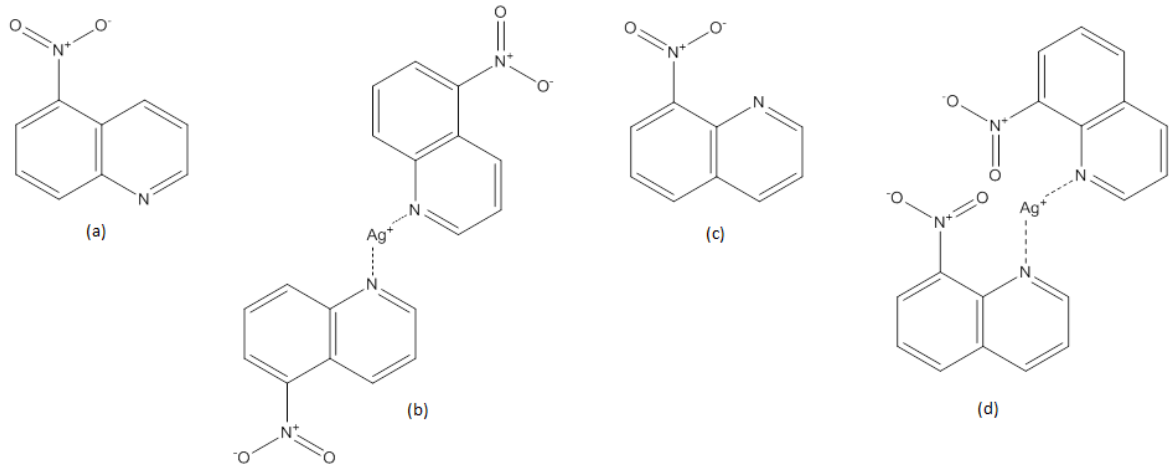

Figure 1: Depiction of the quinoline-type ligands, (a) 5-nitroquinoline single ligand, (b) [Ag(5-nitroquinoline)$_2$]-complex, (c) 8-nitroquinoline single ligand and (d) [Ag(8-nitroquinoline)$_2$]-complex

2.4.2. Pyridines

Four different pyridine-type ligands are included in the study (see figure 2). The nicotinates (isopropylnicotinate, isonicotinamide and ethyl nicotinate) are closely related to the vitamin B group member nicotine amide and have been studied for various purposes in medicine or as agrochemicals (Báthori *et al.*, 2011).

In an article published in 2007 the researchers discuss and test the possibility of utilizing the nicotinates as ligands, coordinated around silver, to produce novel biocides for the purpose of wound treatment (Abu-Youssef *et al*., 2007).

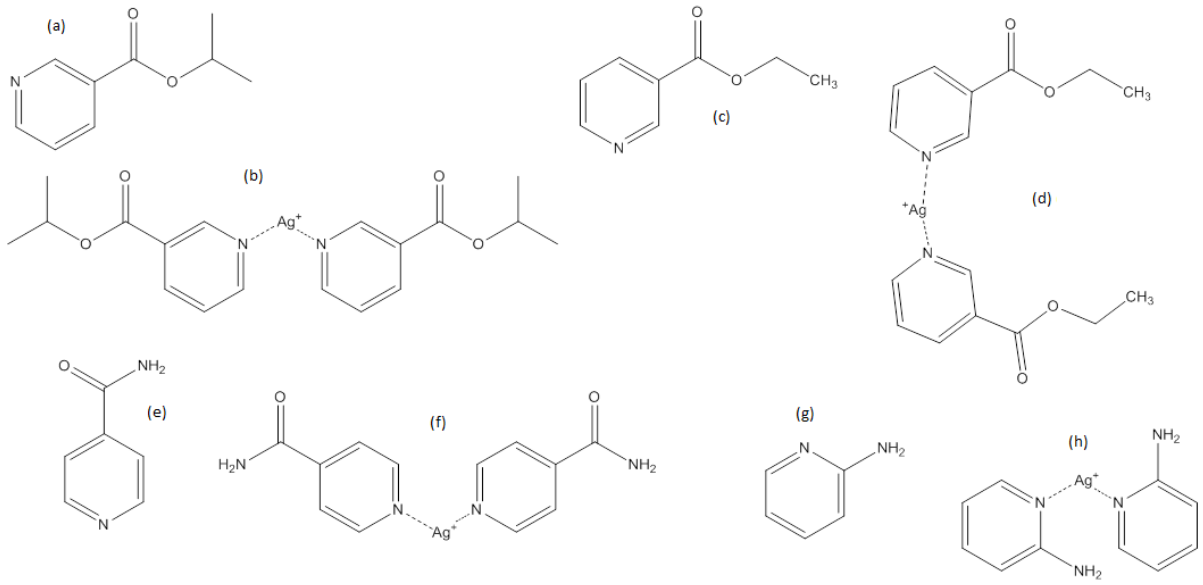

Figure 2: *Depiction of the pyridine-type ligands, (a) isopropyl nicotinate single ligand, (b) [Ag(isopropyl nicotinate)$_2$]-complex, (c) ethyl nicotinate single ligand and (d) [Ag(ethyl nicotinate)$_2$]-complex, (e) Isonicotinamide single ligand, (f) [Ag(isonicotinamide)$_2$]-complex, (g) 2-aminopyridine single ligand and (h) [Ag(2-aminopyridine)$_2$]-complex*

3. Methods and Instrumentation

Primarily a literature study was performed to evaluate the current level of silver complex development and to gain understanding of the intended analysis techniques. The literature study continued throughout the project but focus shifted from analysis to result interpretation and comparison to other similar studies.

Speciation analysis by mass spectrometry and ^{1}H-NMR were used to quantify the complexes present in solution. Proton nuclear magnetic resonance, or ^{1}H-NMR, titration was performed to determine equilibrium constants for each of the complexes.

Electrospray ionization mass spectrometry (ESI-MS) tests were carried out to study complex behaviour in a solution resembling that of wound fluids. Analysing the complexes in a medium resembling that of *in vivo* conditions was necessary in order to study the functionality of the complexes when allowed to interact with ions, proteins and other macromolecules.

Antibacterial activity was studied utilizing diffusion-tests. Trials were performed on the gram-negative strains; *Proteus mirabilis* and *Pseudomonas aeruginosa*, and the gram-positive; *Staphylococcus aureus* and *Streptococcus pyogenes*.

3.1. List of chemicals

Ligands 5- and 8-nitroquinoline, 2-aminopyridine, isonicotinamide and ethyl nicotinate were obtained from Sigma-Aldrich as well as silver nitrate and bovine serum albumin (BSA), as analytical reagent grade and they were utilized as received except for BSA. The bovine serum albumin from Sigma-Aldrich contains a small amount of mineral salts which had to be removed in order to effectively perform ESI-MS analysis. ^{1}H-NMR solvents; DMSO-d6 and D_2O were also obtained from Sigma-Aldrich and utilized in the same condition as they were received. The ligand isopropyl nicotinate was acquired from Chemos GmbH and utilized as received.

3.2. Synthesis of complexes

Due to the fact that silver cations (Ag(I)) are sensitive to both light and reducing agents, which reduces them to stable metallic silver (Ag(0)), delicate synthesis procedures must be utilized. Slow evaporation from solution and filtration drying are two synthesis techniques that can be considered non-detrimental to silver/ligand-complexation if proper precautions are taken. However, evaporation at room temperature is time consuming and filtration drying can allow for retention of the dissolving agents. Hence, analysis by x-ray diffraction must be performed to evaluate the synthesis results.

Both techniques are simple and whether slow evaporation or filtration is utilized depends only on the solution obtained from mixing the solution components. If the result of mixing is a clear liquid evaporation follows and if there is a precipitation filtration is possible to save time.

3.2.1. Synthesis procedure

Primarily $AgNO_3$ is dissolved in water and the organic ligands are dissolved in ethanol so that a silver/ligand-molar ratio of 1:2 is obtained (data included in appendix I). The solutions are combined and the mixture is allowed to settle at room temperature until crystals are formed. In the case of solution $AgNO_3$/5-nitroquinoline the mixture precipitates and can therefore be vacuum-filtered which reduces the amount of time needed.

In this study only complex [Ag(5-nitroquinoline)$_2$] was synthesized and analysed since the remaining complexes were in stock after a previous study.

3.3. Analysis techniques

Bellow follows a short summation of the techniques and their utilization in this project.

- ^{1}H-NMR – To study silver/ligand-behaviour and complexation.
- XRPD – To control and describe crystallised complexes after synthesis.
- ESI-MS – For speciation and to study the complexes in convoluted solutions.

3.3.1. Proton Nuclear Magnetic Resonance

Probably being one of the most commonly used technique for analysing the content of organic solutions, ^{1}H-NMR utilizes magnetic spin properties of the hydrogen atom core to create a spectrum allowing for substance recognition (Derome, 1987). H-MNR is particularly well suited for the study of most organic compounds because of their abundance of hydrogen atoms (in the future referred to as protons or ^{1}H) (Silverstein *et al*, 1991).

^{1}H has two magnetic spin states, up or down, depending on its alignment to an external magnetic field (Derome, 1987). If aligned along the field the spin state is considered to be in its lower energy state or “up”, and vice versa. Several short bursts or “pulses” of electromagnetic radiation is emitted, exciting ^{1}H to a higher energy state (Derome, 1987). When the pulses have subsided, the protons will return to their less excited state in a process known as population relaxation which sends back a photon that can be detected (Derome, 1987). Other atoms in the molecule are not detected, as such, by this particular type of NMR but they influence the protons by means of shielding from the EM-pulses. Hence it is possible to draw conclusions of the proton’s surroundings by studying the so called "chemical shifts" which arises due to the various shielding properties of connecting atoms (Derome, 1987).

Proton nuclear magnetic resonance test were performed with a Varian 500-MHz spectrometer, using a 5-mm probe operating at 500 MHz, all measurements were performed at room temperature. The technique will be utilized to study complexation behaviour since it is possible

to draw conclusions on ligand coupling from studying shift-changes whilst titrating. By adding a small amount of ligand solution into a sample tube containing a fixed amount of Ag(I) and observing the produced spectrum it is possible to deduce both suitable silver/ligand concentrations and how the ligands are bound to the cation.

In order to minimize interference from the solute both the ligands and the silver nitrate will be solved in deuterated dimethyl sulfoxide (DMSO-d6) or deuterated water (D_2O or heavy water). Since deuterium, or ^2H, has both a proton and a neutron its spin properties differ from that of ^1H, and excitation/relaxation of the solute will therefore not affect the spectrum analysis adversely.

3.3.1.1. Equilibrium constant calculations

To be able to evaluate the reactions taking place during the complexes formation it is necessary to determine the stepwise equilibrium constant, K_{eq}, for each such reaction. The ^1H-NMR titration data were collected for this purpose since it is possible to determine equilibrium for a reaction by studying chemical shifts.

K_{eq} is most easily explained as the concentration or activity ratio between atomic/molecular species (Martell and Motekaitis, 1992). There are several ways to calculate the equilibrium and a number of methods to obtain the data. The general formula for determination of K_{eq}, from which the others are derived, is:

$$aA + bB \rightleftharpoons cC + dD \tag{1}$$

$$K_{eq} = \frac{\alpha_C^c \times \alpha_D^d}{\alpha_A^a \times \alpha_B^b} \tag{2}$$

Where α is the ratio of the activities of the reaction products, capital letters are the ingoing species and lower case are their stoichiometric coefficients.

However, according to Martell and Motekaitis, the determination of activities for species in solution is considered a both complicated and time-consuming procedure. A simpler and less laborious equation instead utilizes concentration of chemical species in a similar manner. Since concentrations and activities can be considered correspondent in ionic solutions in the presence of a non-reacting electrolyte at high concentration, this simplification is possible under such conditions (Martell and Motekaitis, 1992 and Fielding, 2000). The corresponding formula becomes:

$$K_{eq} = \frac{[C]^c \times [D]^d}{[A]^a \times [B]^b} \tag{3}$$

In which the capital letters in brackets symbolize species concentration and the lower-case letters are stoichiometric coefficients.

It is considered more practical to separate the large number of various forms of equilibriums (more than 800 different equilibrium constant denominations defined in 1992). In the case of coordination compounds in solution where metal ions (Lewis acids) are combined with ligands (Lewis bases) the cumulative equilibrium constant, β_2, is determined (Martell and Motekaitis, 1992).

In this study the hope is to be able to find the β_2 for several complexation reactions between silver anions and ligands. A previous study have conferred that the complexes here scrutinized

are most effective as biocides when the silver/ligand-ratio is 1:2, two ligands to each silver (Massoud, 2011). Establishing which reactant concentrations are most favourable to such complexations is therefore necessary. The stepwise and cumulative equilibrium constants equations corresponding to the general equation for this type of complexation is:

$$ML_{n-1} + L \rightleftharpoons ML_n \qquad K_n = \frac{[ML_n]}{([ML_{n-1}][L])} \tag{4}$$

$$M + nL \rightleftharpoons ML_n \qquad \beta_n = \frac{[ML_n]}{([M][L]^n)} \tag{5}$$

Where M is the silver anion and L corresponds to the ligand studied. The relationship between the two constants is therefore:

$$\beta_n = \prod_1^n K_n \qquad \text{Example: } \beta_2 = K_1 K_2 \tag{6}$$

For a metal, double ligand system (n = 2), equation (5) gives:

$$\beta_2 = \frac{[ML]}{([M] \times [L])} \times \frac{[ML_2]}{([ML] \times [L])} \tag{7}$$

Since the concentration of complex in solution is unknown the equation must be further adapted. It is possible to determine the complex/ligand-concentration by utilizing the chemical shifts obtained from ^{1}H-NMR titration data on that particular mixture (Suchidat *et al*. 1972). The relationship between observed and real chemical shifts is:

$$\delta_{obs} = \alpha_{fr}\delta_{fr} + \sum \alpha_{comp}\delta_{comp} \tag{8}$$

Where δ_{obs} is the observed chemical shift, α_{fr} is the fraction of free ligand, δ_{fr} is the chemical shift for free ligand, α_{comp} is the fraction of ligand in complex and δ_{comp} is the chemical shift for ligand in complex (Billo, 2001).

In a solution containing silver and ligands there is a rapid exchange between molecules in complex and unbound molecules/anions (Suchidat *et al*., 1972). The NMR spectrum obtained from each titration therefore displays peaks which are the weight average of the ligand's protons in free form and in complex (Fielding, 2000)). As the concentration of reactants increase so does the chance of them forming complexes at any given time, thus the chemical shift will alter accordingly. The NMR data obtained will therefore need, along with the observed chemical shift (δ^L_{obs}), to include; chemical shift for free reactant, in this case the ligand, (δ^L_{fr}) and chemical shift (or estimated chemical shift) for the reactant in complex (δ^L_{comp}).

The span of the chemical shifts is crudely displayed by figure 3.

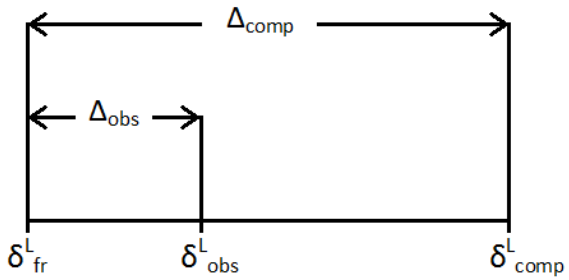

Figure 3: *The implication of five units necessary for calculating K_{eq} from ^{1}H-NMR data.*

When studying a complexation reaction including only two constituents (a 1:1 system) it is possible to utilize the following relationships:

$$K_1 = \frac{[ML]}{([M] \times [L])} = \frac{[ML]}{([M]_0 - [ML]) \times ([L]_0 - [ML])} \tag{9}$$

and

$$\delta^L_{obs} = \delta^L_{fr} \frac{[L]}{[L] \times [ML]} + \delta^L_{comp} \frac{[ML]}{[L] \times [ML]} \tag{10}$$

and

$$\Delta_{obs} = \delta^L_{fr} - \delta^L_{obs} \tag{11}$$

$$\Delta_{comp} = \delta^L_{fr} - \delta^L_{comp} \tag{12}$$

to derive a formula from which the stepwise equilibrium constant can be calculated:

$$\frac{1}{\beta_1} = [L]_0 \left(\frac{\Delta_{comp}}{\Delta_{obs}} - 1 \right) - [M]_0 \left(1 - \frac{\Delta_{obs}}{\Delta_{comp}} \right) \tag{13}$$

Where $[L]_0$ and $[M]_0$ are the total concentrations of ligands and metal ions, both free and in complex (Suchidat *et al*., 1972).

In the case of a 1:1 complex the stepwise and cumulative equilibrium constants are the same (see equation (6)) and can be determined utilizing equation (13) with the ^{1}H-NMR data obtained for Δ_{obs} and Δ_{comp} (Suchidat *et al*., 1972 and Fielding, 2000).

Determining the cumulative equilibrium constant for a 1:2 (or higher) metal/ligand-complexes is, however more complicated. For the purpose of determining the equilibrium constants for larger complexes, involving more than two components, an analytical software known as "HypNMR2008" is utilized.

A thorough explanation of the procedures of the program has been described by Frassineti *et al*.; however, a simplified description of the program follows below (Frassineti *et al*., 1995 and

Frassineti *et al*., 2003). Basically HypNMR2008 performs a four step regime to calculate β from the obtained data:

1. Data input: specification of stoichiometric coefficients for assumed species in solution studied with NMR, estimations of β (if possible), specifications of contributions from each species to the overall chemical shifts and input of raw data such as concentrations and chemical shifts.

2. Linear lest squares calculations utilized to determine the individual contribution to the overall chemical shifts from each proton.

3. Minimization of the sum of squares residual utilizing Gauss-Newton-Marquardt iterative refinement process.

4. Statistical analysis and presentation of results

By utilizing HypNMR2008 it is possible to simultaneously determine several equilibrium constants for a certain system (Frassineti *et al*., 1995 and Frassineti *et al*., 2003).

3.3.2. X-ray Powder Diffraction

A powerful tool for the study of crystalline substances the x-ray powder diffraction technique can be utilized to determine purity of a sample or to quantify the contents of a mixture of compounds. The technique utilizes an x-ray source which propels high intensity photons towards the sample and a detector that identifies the scattered photons and produces a diffraction pattern. Once a pattern is obtained it is necessary to compare it to a known standard in order to identify the crystals.

The instrument used in this particular study was a Siemens SMART D5000 powder diffractometer with a cupper-anode that utilizes a germanium crystal monochromator to produce $K_{\alpha 1}$-radiation (Cu-Kα with λ = 1.5405Å).

The crystals are studied by placing them in a small indentation on a plate and putting them in a sample holder which rotates during the measurement procedure. The x-ray tube (containing the x-ray source) and the detector are situated opposite to one-another and continually, slowly increase their angle (“scattering angle”) to the horizontal axis and the sample plate.

By rotating the sample the effects of orientation preference of the crystals can be avoided and the angular variation of the photon source and the detector allows for greater diffraction data collection (Liss *et al*, 2003).

After detection the peak intensities are plotted against the diffraction angle and since the peaks are characteristic for each crystal they can be compared to the standard and the sample identified (Liss *et al*, 2003). The peak intensity can be utilized to quantify the various components in the sample if it is a mixture but for this thesis the main utilization will be to simply identify the crystals to control synthesis results.

3.3.3. Electrospray Ionization Mass Spectrometry

ESI-MS spectra were collected by a Bruker Daltonics APEX Qe Fourier transform ion cyclotron resonance mass spectrometer equipped with a 12 T superconducting magnet and an Apollo II ion funnel-based ESI source.

The electrospray ionization (ESI) was developed for use in mass spectrometry in order to produce ions in a non-interfering manner. It works particularly well for analysis of larger molecules such as proteins since the technique does not utilize electron bombardment, which may cause deterioration in molecular structures (Fenn *et al*, 1989). Simplified the ESI instead functions, as the name indicates, by letting droplets of analyte in solution wander over an electric field, letting the solvent evaporate continuously (Kebarle and Verkerk, 2009).

More accurately the solution containing the analyte is forced through a capillary and into a positively charged nozzle, which allows for removal of negative charges (mostly ions from the solvent) from the forming tip-cone. Charge separation leads to the formation of an electric field between the nozzle and the opposite wall. If the field is sufficiently strong the tip-cone will emit a jet of solution droplets, covered with positively charged ions, which will travel downfield towards the negatively charged electrode (see figure 1). Due to in-flight solvent evaporation the concentration of positive ions on the droplets will increase causing repulsion forces to act over the droplet surface (Kebarle and Verkerk, 2009). When the build-up of positive charges has reached a critical level the droplet surface tension is no longer enough to hold it together and it "explodes", turning into several progeny droplets in an event known as Coulomb fission (Kebarle and Verkerk, 2009). The progression of Coulomb fission over the field continues as the progeny droplets undergo the same reaction until gas-phase ions is all that remains (Kebarle and Verkerk, 2009).

Once the solvent has evaporated the ions are led into a mass spectrometer for analysis. In this study a comparison between solutions containing only the complex, solutions containing the complexes and chloride ions and, solutions containing BSA and complex will be made in order to determine binding and complexation behaviour. Since the ESI does not affect the macro-molecules in the solution the silver complexes interactions with, for instance, proteins such as albumin and other common blood constituents can potentially be detected.

3.3.3.1. ESI-MS function

Electrospray ionization mass spectroscopy allows for detection of mass to charge (m/z) ratios (x-axis) and relative intensity (y-axis) in gas-phase molecules (see figure 4). The m/z-ratio is a measurement of molecular weight divided by number of charges deposited on the molecule (Domon and Aebersold, 2003). Depending on whether the MS is set on positive or negative ion mode the charge detected can be either a proton (H^+) or an electron (e^-), in this study only positive ion mode was utilized. The relative intensity is a percentile of the tallest peak in the current spectrum, that is, the tallest peak is considered 100 % and all other peaks are fractions of its intensity (Domon and Aebersold, 2003).

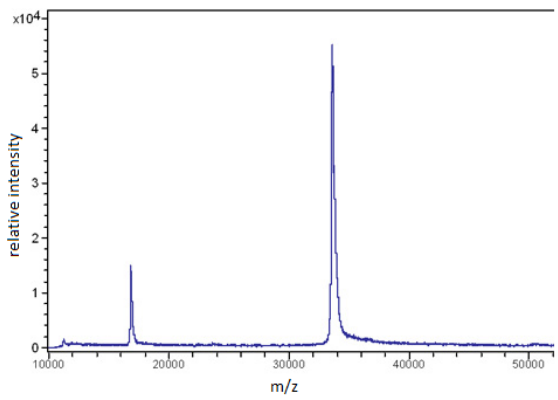

Figure 4: *An example of ESI-MS spectra depicting two unknown peaks*

As previously mentioned, the m/z ratio is molecular weight divided by the number of charges that a particular molecule is carrying. Positive charges are generally correlated to the amount of sites that can be protonated at low pH. This means that small molecules, such as the ligands here studied, mostly have only one or two protons connected to them whilst proteins and other macromolecules are able to carry larger numbers. Hence, even though BSA-proteins can be several orders of magnitude larger than the silver/ligand-complexes they also carry more charge and thus their m/z-ratios are not too far apart. Furthermore, before analysis the BSA are also digested in order to simplify detection, this abridge the possibility to distinguish between BSA that does or does not interact with the complexes (Domon and Aebersold, 2003).

It is important to explain that small molecules such as the complexes will produce a spectrum consisting of one or a few peaks corresponding to their m/z-ratio while the peptides will produce a large spectrum of peaks with a very distinctive wave-like appearance (Domon and Aebersold, 2003).

3.3.3.2. ESI-MS procedure

In order to be able to test whether or not the complexes interact in a certain manner in a mixed solution containing, for instance, chlorine or BSA-peptides a table has to be made, listing all possible (or at least most probable) combinations of Ag(I), ligands and other ions or macromolecules (see an example in table 2). The ESI-MS detects mass to charge ratios (m/z) along the x-axis with a four decimal precision. Due to complexation or other interaction, the peaks shifts slightly and the table therefore has to contain a meticulous list of the expected m/z-ratios in order to allow for a rapid evaluation of the results (Lomeli et al. 2010).

Table 1: *An example of an entry of the possible outcomes from a test with silver nitrate ($AgNO_3$) in solution with chloride (Cl^-) ions. Listed are the expected chemical compositions of included elements, silver to ligand ratio, m/z-ratios (both theoretical and experimentally found) as well as molarity for the components.*

Expected chemical formula from ESI-MS in solution	Expected ratio Ag:L	Mass (m/z)		M [g/mol]
		Found	Calculated	
$[Ag(5\text{-nitroquinoline})_2]^+$	1:2		454,99	456,18
$[Ag(5\text{-nitroquinoline})_2]^+(HNO_3)$	1:2		516,98	518,19
$[Ag(5\text{-nitroquinoline})]^+$	1:2		280,95	282,02
$[(5\text{-nitroquinolinium})]^+$	0:1		174,04	174,16
$[Ag(8\text{-nitroquinoline})_2]^+$	1:2		454,99	456,18
$[Ag(8\text{-nitroquinoline})_2]^+(HNO_3)$	1:2		516,98	518,19
$[Ag(8\text{-nitroquinoline})]^+$	1:2		280,95	282,02
$[(8\text{-nitroquinolinium})]^+$	0:1		174,04	174,16

The calculated values for m/z-ratios and molar mass (M) differ slightly since the m/z-ratios take into account only one of the two main isotopes of silver, whilst molar mass is a mean value of both. If peaks are found outside the span between the two calculated values for m/z and M it is possible to assume that the peak does not correspond completely to the molecule in the chart. Slight variations can be caused by measurement errors, however, a general guideline is that a deviation of ± 0,01 from the span between m/z and M is considered acceptable, above/below

that and the peak corresponds to another compound. In figure 5 are depicted two ESI-MS spectra, one measured and one theoretically determined.

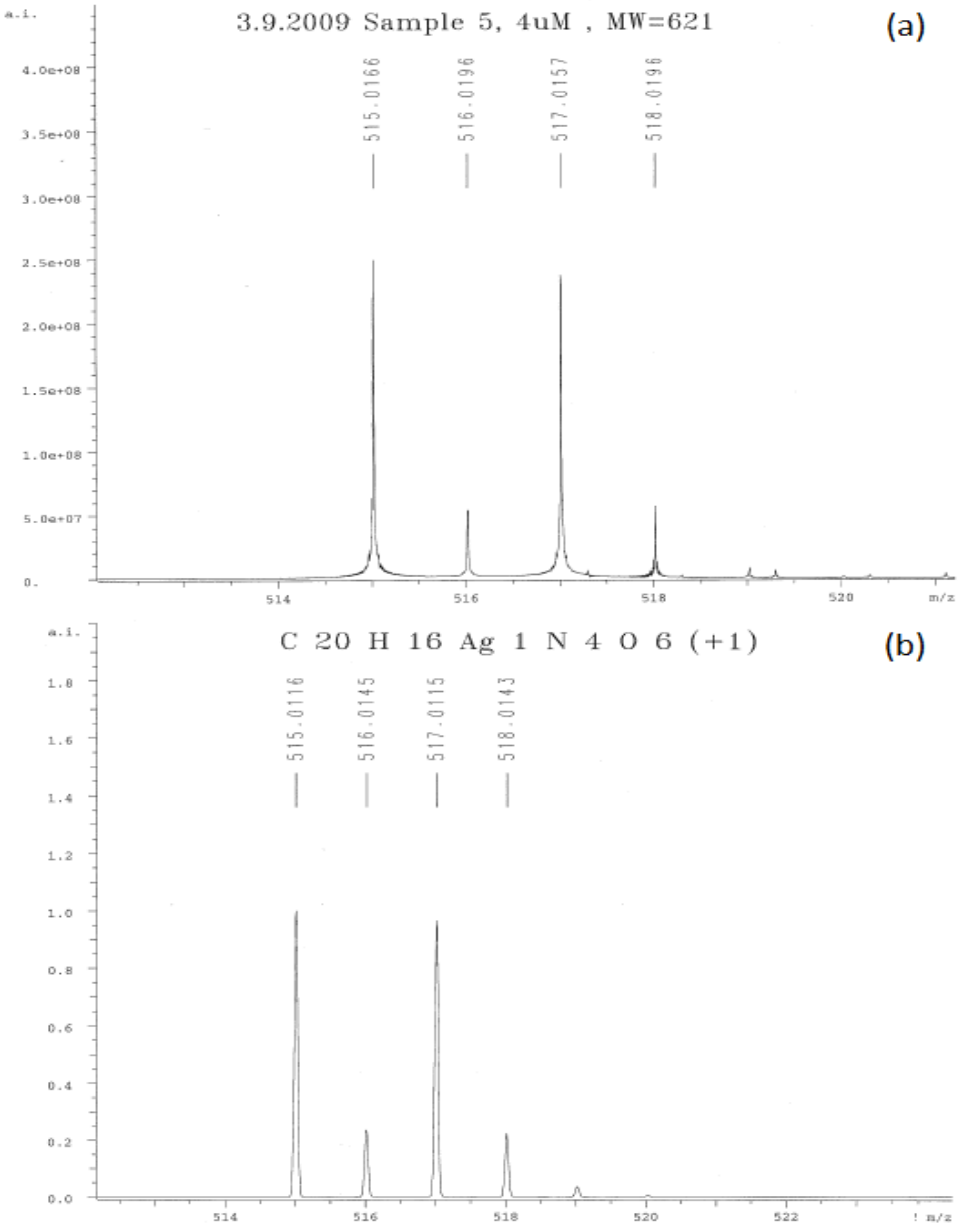

Figure 5: *ESI-MS spectra for [Ag(6-MeO-8-quinoline)$_2$], the measured spectrum (a) is compared to the theoretical (b)*

The alignments of the spectra in figure 5 are sufficient to be able to determine that the measured spectrum correspond to the studied molecule.

For each trial where interaction is to be tested it is necessary to first acquire a standard for the particular components included in that trial. Hence, if a trial to study the behaviour of one of the silver complexes with BSA, primarily m/z-ratios must be determined individually and secondly in combination.

The interactions are multifaceted and there is an almost infinite number of possible ways for the molecules to adhere or influence each other. Hence, the data obtained must be thoroughly scrutinized in order to be able to decide with some certainty what might have occurred. Most probably the time available for this project is insufficient to be able to describe silver/ligand

interaction with proteins in detail. However, if the molecules bond in some way the ESI-MS spectra will detect it.

It is possible to assume that if the BSA m/z-ratio shifts, when present in solution along with silver-complex, there is an interaction between the two. Depending on the size of the shift-change it is conceivable to deduce if the complex interacts as a whole or if a certain element is responsible. If the complexes are degraded and if for instance a ligand is lost so that the rest of the complex can adhere to the BSA, this will be possible to detect.

Even if the method provides quantifiable data only under special conditions, not present in this study, the data may be able to give an indication on whether or not the silver complexes are interacting with elements of wound fluids and of the circulatory system. The knowledge can then be utilized to advice on further research on the subject.

3.4. Diffusion tests

The diffusion tests were conducted in order to study the effect of the ligands mixed in solution with silver, not to study the complexes themselves. The tests were performed by treating punches made from a suitable material, in this case bacterial cellulose, with the silver/ligand-mixtures solved in DMSO. The punches were placed on an agar plate and incubated along with the tested bacterial strain. Since the punches leached out solution into their vicinity and into the agar gel, in a manner similar to that of a silver containing medical adhesive into a wound, the tests were intended to give a notion of the effect of the ligands on silvers ability to inhibit bacterial growth.

Primarily bacteria were seeded onto an agar plate, utilizing Mc Farland standards for turbidity measurements and dilution to obtain a bacterial concentration of 2*10^8 cells/ml. A Saveen & Werner NanoDrop ND-1000 was utilized for absorbance measurements, ensuring the correct cell concentration in each sample. Once seeded the bacteria were incubated for 24 hours at 37°C and atmospheric pressure.

Silver/ligand-mixtures were prepared by observing data acquired in a parallel study that was performed in conjunction with this thesis and in which antibacterial properties of the silver/ligand-complexes were thoroughly tested (Lindberg *et al*. 2012). In the parallel study, among other things, was tested the complexes antibacterial effectiveness compared to some silver-containing medical adhesives on the market (containing $AgNO_3$ or Ag_2SO_4). The results obtained by Lindberg *et al*. included what concentration of the complexes in solution was needed to cause the same amount of bacterial growth inhibition as those products.

The Ag(I):Ligand-ratios utilized in this study were decided from diffusion tests concentrations obtained by Lindberg *et al*. for pure complex in DMSO-solution. Diffusion test-values for the two complexes and bacteria tested in this thesis are included in table 4 below.

Table 4: *Diffusion-values for the two complexes [Ag(5-nitroquinoline)$_2$] and [Ag(ethyl nicotinate)$_2$] against the two bacteria Staphylococcus Aureus and Pseudomonas Aeruginosa obtained by Linberg et al. in 2012.*

Conc. (µg/ml)	[Ag(5-nitroquinoline)$_2$]	[Ag(ethyl nicotinate)$_2$]
Staphylococcus Aureus	32	4,1
Pseudomonas Aeruginosa	8,2	4,1

The relative amount of silver in complex with each ligand needed to inhibit bacterial growth comparable to silver-containing medical adhesives was transferred to this trial and utilized in the silver/ligand-mixtures. The tests were performed with a fixed concentration of silver, decided individually for each ligand/bacterial strain and four silver/ligand-ratios; 1:3, 1:2, 1:1 and 1:0,5 were utilized. A control was included, containing $AgNO_3$ with the same Ag(I) concentration as the samples, as well as a blank-sample containing only ligand at a silver/ligand-ratio of 0:2. For precise sample preparation see appendix III.

The punches were prepared by putting them into 1,5 ml Eppendorf-tubes, adding 10 µl of the silver/ligand-mixtures and incubating them at room temperature for at least eight hours.

The punches were then applied to the agar plates, on which the bacteria had been allowed to propagate for 24 hours, and the agar plates were then again allowed to incubate for 24 hours at 37°C and atmospheric pressure.

After incubation the plates were removed and the diameter of the inhibition zones formed around the samples was measured utilizing a slide calliper.

4. Results and Discussion

This master thesis was conducted with the intention to study complexes of silver in combination with six different ligands which have indicated biocidal capabilities. The thesis project is a continuation of a doctor of philosophy dissertation previously undertaken by PhD Shimo Al Massoud and most work described in this thesis is based on her efforts (Massoud, 2011). Hence, some of the results here presented were obtained by replicating and slightly altering her tests or by continuing on paths suggested in her dissertation.

The two main parts of interest in the results section of this thesis do differ from previous work performed at this institution however. The use of ^{1}H-NMR titration to obtain equilibrium constants for the formation of the complexes and the utilization of ESI-MS to study the influence of wound fluids on the complexes have not been performed up until now.

4.1. Complex synthesis

[Ag(8-nitroquinoline)$_2$], [Ag(isonicotineamide)$_2$], [Ag(2-aminopyridine)$_2$], [Ag(ethyl isonicotinate)$_2$] and [Ag(isopropyl nicotinate)$_2$] were available at the start of the project and only [Ag(5-nitroquinoline)$_2$] had to be synthesized.

Primarily both silver nitrate ($AgNO_3$) and the organic ligands (L) were dissolved in water and ethanol respectively and, depending on sought after constitution of the complexes, molar concentrations were decided. A molar ratio of two ligands to every silver, or Ag:L ratio 1:2, was intended for all complexes.

Thereafter the solutions were mixed together, if the result turned into a clear liquid, evaporation synthesis was utilized to form crystals. However, if the mixed solutions precipitated it was possible to assume that the complexation had already occurred and filtration could then be used instead of evaporation since it was less time consuming.

4.1.1. XRPD analysis

X-ray powder diffraction analysis on the synthesized [Ag(5-nitroquinoline)$_2$]-complexes was performed to evaluate purity. The spectrum obtained was compared to an existing spectrum obtained by x-ray single crystal diffraction in a previous study (see figure 6)(Massoud *et al*. 2011). The two spectra align almost exactly and the synthesis can therefore be considered successful.

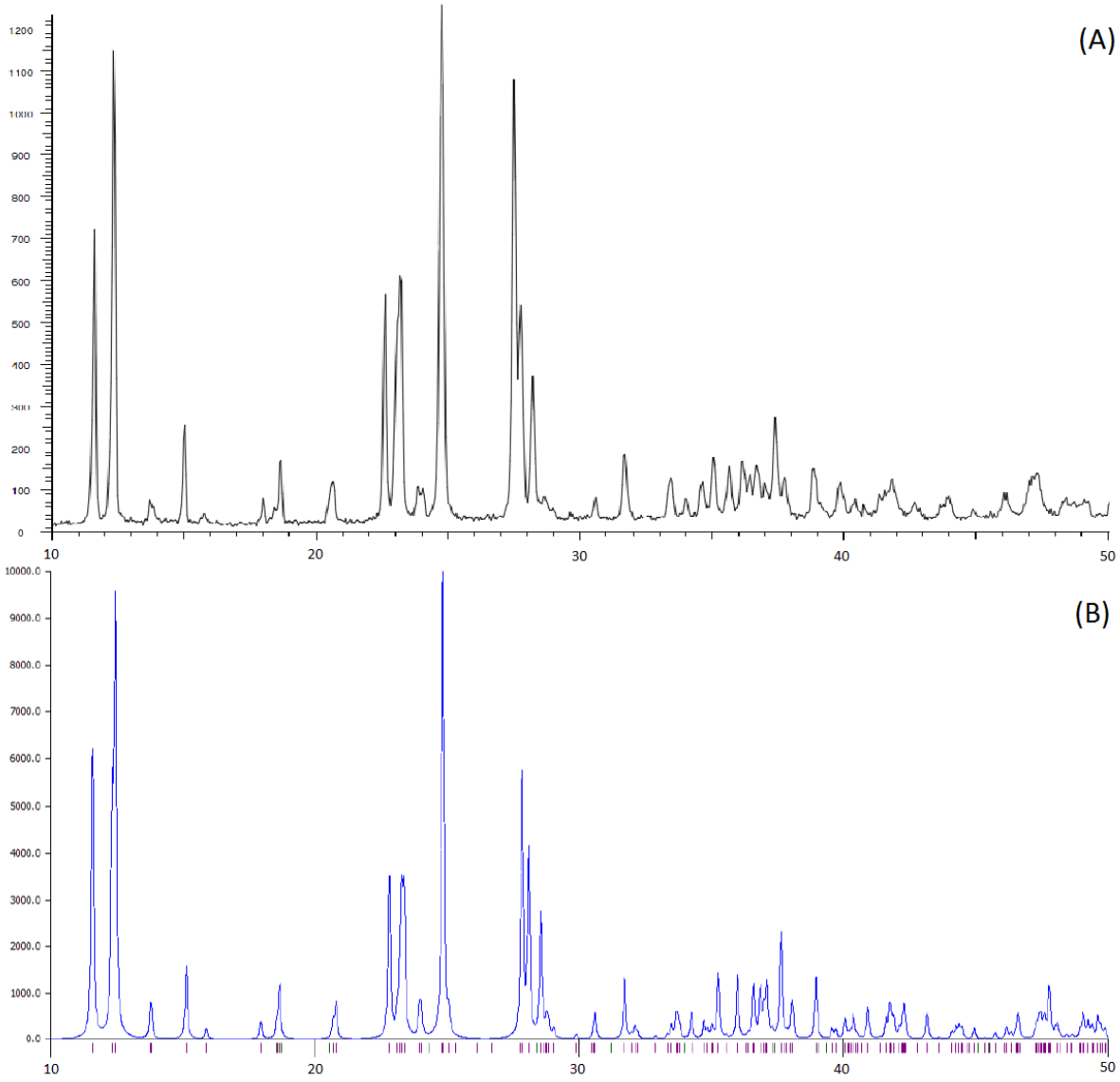

Figure 6: *Combined spectra of the (A) synthesized [Ag(5-nitroquinoline)$_2$] and (B) the standard obtained from Cambridge Structural Database.*

The previously synthesized complexes had undergone the same analysis and their purity had been established utilizing the same technique. Since storage conditions for those complexes were sufficient the [Ag(8-nitroquinoline)$_2$], [Ag(isonicotinamide)$_2$], [Ag(2-aminopyridine)$_2$], [Ag(ethyl isonicotinate)$_2$] and [Ag(isopropyl nicotinate)$_2$] complexes were considered still functional.

4.2. ^{1}H-NMR data and equilibrium constant determination

The ^{1}H-NMR was primarily conducted to study the complexation reaction in solution between silver ions and ligands. It was also concluded that the ^{1}H-NMR -data could be utilized to estimate the K_{eq} (β) of the reactions.

4.2.1. ^{1}H-NMR -titration

By plotting the measured chemical shift, δ_n, against [Ag^+]/[L]-molar equivalence for each of a series of successive additions of a solution of $AgNO_3$ solved in DMSO-d_6 it was possible to draw

some conclusions on complexation behaviour for four of the silver/ligand mixtures. Below is included the graphs and result-interpretation of Isonicotinamide, isopropylnicotinate as well as 5- and 8-nitroquinoline.

For titration data see appendix II.

4.2.1.1. Isopropyl nicotinate

Six peaks were detected for pure ligand at δ = 9,4905; 9,2253; 8,6860; 7,9785; 5,5860 and 3,7340 ppm which corresponds to the identified seven ^{1}H on the molecule (identical shifts recorded for the two peaks labelled "6"). The increase of $[Ag^{+}]$ in the solution caused a small but noticeable change in chemical shifts as figure 7 depicts. The graph gives a suitable overlook of the peaks in relation to one-another even though there are only slight variations in chemical shifts.

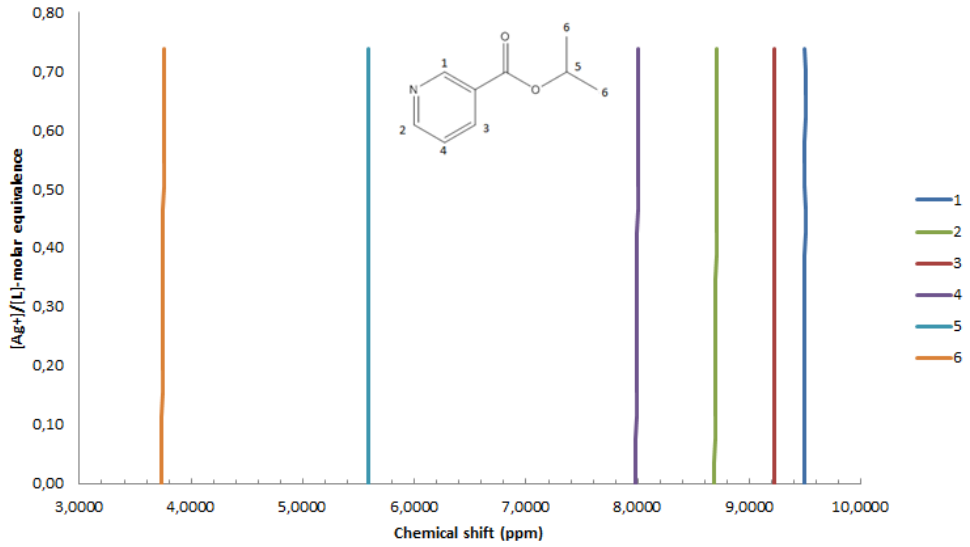

Figure 7: *Graph depicting ^{1}H-NMR titration of isopropylnicotinate with $AgNO_3$, molar equivalence has been plotted against the chemical shift recorded after each titration*

For all peaks a slight inflection commences at $[Ag^{+}]/[L]$ = 0,10 and continues throughout the graph. The shift change in some of the peaks may be so small that they are barely perceptible in this view and peaks 3 and 5 are almost completely stationary after the inflection point. Figure 8 allows for a better overview of the chemical shift change.

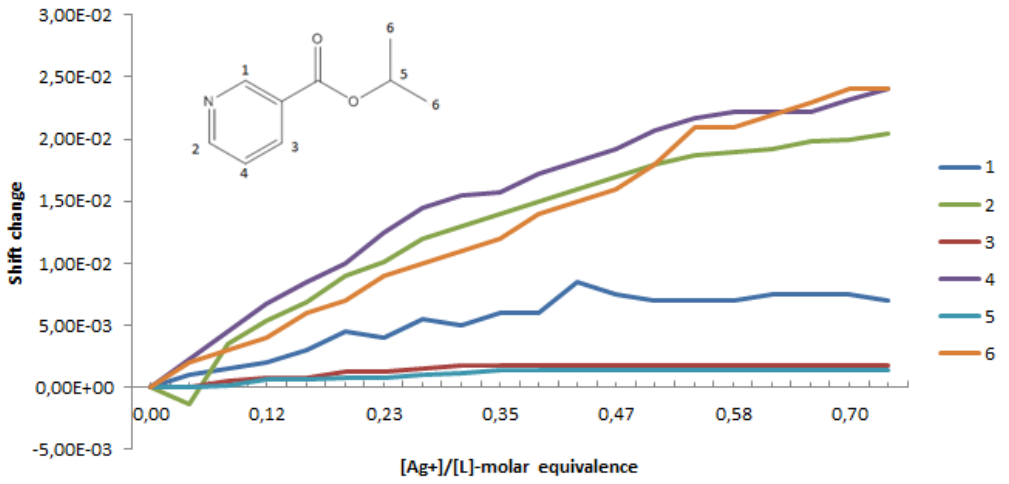

Figure 8: *Graph depicting ^{1}H-NMR titration of isopropylnicotinate with $AgNO_3$, chemical shift change ($\Delta\delta_n$) from the start of the experiment is plotted against $[Ag^+]/[L]$-molar equivalence*

Proton 1 presents a concave curve with a temporary increase at $[Ag^+]/[L] = 0{,}43$ of $\Delta\delta_1 = 0{,}0025$ ppm and proton 2 displays a total $\Delta\delta_2$ of 0,0205 ppm and. Protons 3 and 5 seem to be almost unaffected by the reaction while protons 4 and 6 (double protons) continually increase their chemical shift summing up an identic shift change of $\Delta\delta_4 = \Delta\delta_6 = 0{,}024$. In summation the overall alteration of chemical shifts was downfield, indicating a decreased amount of shielding.

The formation of a bond between the nitrogen on the ligands benzene group and the positive silver ion would decrease shielding in the area around protons 1 and 2 due to decreased electron density around that area.

The largest shift changes occurred in protons 4 and 6, one explanation could be that they interacted with free Ag^+ in their surroundings. Since these protons are protruding from the molecule, decreasing possible steric hindrance, they could be susceptible to such interaction.

4.2.1.2. Isonicotinamide

Two strong peaks were detected at δ = 9,1300 and 8,1790 ppm for the pure isonicotinamide sample. The peaks correspond to four protons on the molecule, two protons corresponding to each peak because of the benzene rings symmetrical proton distribution. The increased presence of silver (I) ions mainly caused a downfield shift in peak 1 corresponding to protons nearest the nitrogen (see figure 9) but also brought on a subtle increase of the shift of peak 2.

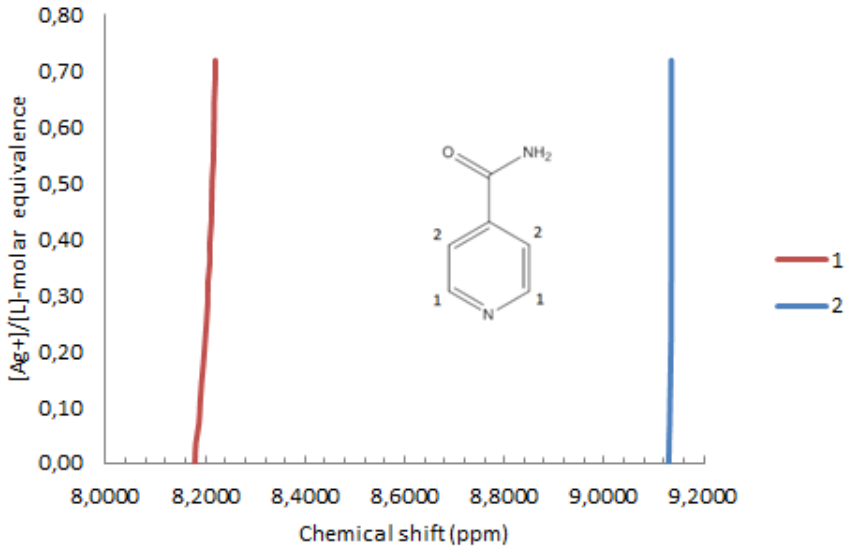

Figure 9: *Graph depicting ^{1}H-NMR titration of isonicotinamide with $AgNO_3$, molar equivalence has been plotted against the chemical shift recorded after each titration*

A distinct and continuous shift increase of peak 1 can be seen from $[Ag^+]/[L] \geq 0$. Peak 2 shift change is less divergent and is easier to analyse in figure 10, where the $\Delta\delta_n$ is plotted against molar equivalence.

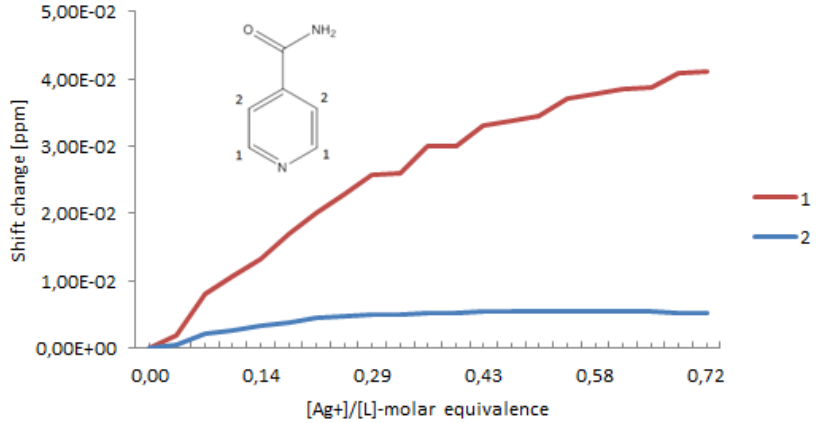

Figure 10: *Graph depicting ^{1}H-NMR titration of isonicotinamide with $AgNO_3$, chemical shift change ($\Delta\delta_n$) from the start of the experiment is plotted against $[Ag^+]/[L]$-molar equivalence*

Peak 2 shows a concave curve, increasing primarily, peaking at $\Delta\delta_2 \approx 0{,}0055$ (corresponding $\delta_2 = 9{,}1355$ ppm) somewhere in the $0{,}43 \leq ([Ag^+]/[L]) \leq 0{,}65$ – span and lastly declining slightly. The interpretation of this behaviour could be that free Ag^+ interferes with nearby electrons, causing a slight decrease in shielding. Since there may be steric hindrance due to the proximity of the amide-group the protons are not as susceptible as the 4 and 6 protons observed in isopropylnicotinate.

The total chemical shift change of peak 1, $\Delta\delta_1$, amounted to 0,041 ppm. As with the corresponding protons of isopropylnicotinate (peaks 1 and 2) this may have corresponded to a bond formation between nitrogen and Ag^+. Since the chemical shift describes a mean value of the detected proton-content in solution an increase would indicate that a larger and larger amount of this or comparable incidents were occurring. Hence, if the chemical shift changes of peak 1 indicated silver-nitrogen coordination the concentration of complexes, involving at least one silver ion and one ligand, was increasing.

4.2.1.3. 5-nitroquinoline

The 5-nitroquinoline experiment was somewhat of a pilot test in the study and solely five titrations were performed in comparison to twenty for the subsequent trials. The [$AgNO_3$] however was higher, thus the [Ag^+]/[L]-ratio lies in the same span as in succeeding tests. Molar equivalence/chemical shift-graph for the experimental data acquired in this experiment can be found in Appendix I (A.1.1).

Five peaks were detected for the pure ligand at δ = 9,082; 8,838; 8,440; 7,950 and 7,814 ppm. The addition of $AgNO_3$ caused a downfield shift of all peaks, indicating a decreased shielding over the entire proton-population. Studying the $\Delta\delta_n/([Ag^+]/[L])$-graph (figure 11) shows that the shift-change is relatively uniform with the exception that at [Ag^+]/[L] = 0,42 peak 4 surges by 0,01 ppm and then drops after the next titration. This is most probably a measurement error since the other peaks show little indication of a similar behaviour.

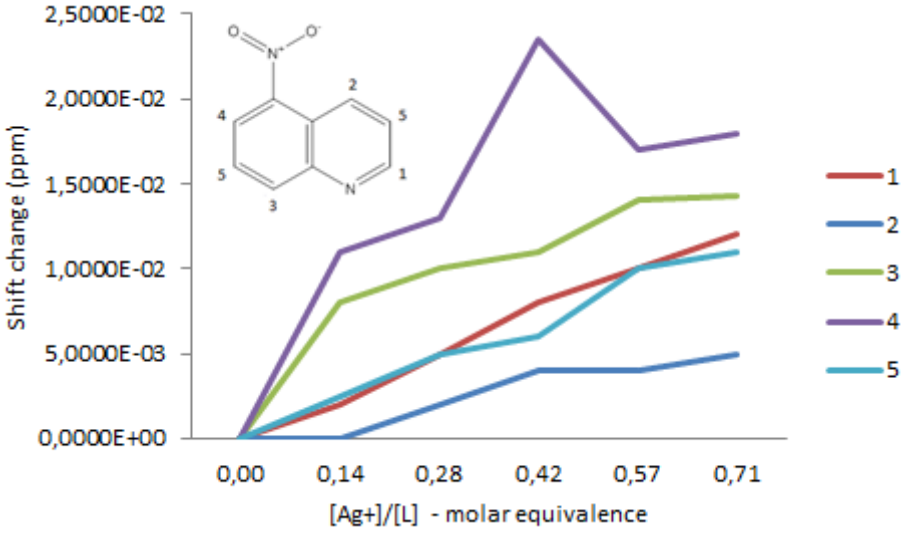

Figure 11: *Graph depicting ^{1}H-NMR titration of 5-nitroquinoline with $AgNO_3$, chemical shift change ($\Delta\delta_n$) from the start of the experiment is plotted against [Ag^+]/[L]-molar equivalence*

Drawing conclusions from these particular results were complicated because of the uniform deviation of the peaks. Peak 1 behaved in such a manner as to indicate silver-nitrogen coordination; however, since all peaks acted in a similar way, it was not possible with the sparse amount of data, to determine if that was the case and if so to what extent. As with both previously displayed ^{1}H-NMR data sets, there may be interference from free Ag^+ present that could have caused the de-shielding as well as the sought after coordination.

4.2.1.4. 8-nitroquinoline

Six distinguishable peaks were recorded for 8-nitrquinoline ligand in solution; δ = 9,04375; 9,59325; 8,3040; 8,2725; 7,7740 and 7,7470 ppm. This trial varied from the others because of the higher concentration of $AgNO_3$ that was added to the ligand solution. The $\Delta\delta_n/([Ag^+]/[L])$-graph is displayed in figure 12 and the molar equivalence/chemical shift-graph for the experimental data can be found in Appendix I (A.1.2).

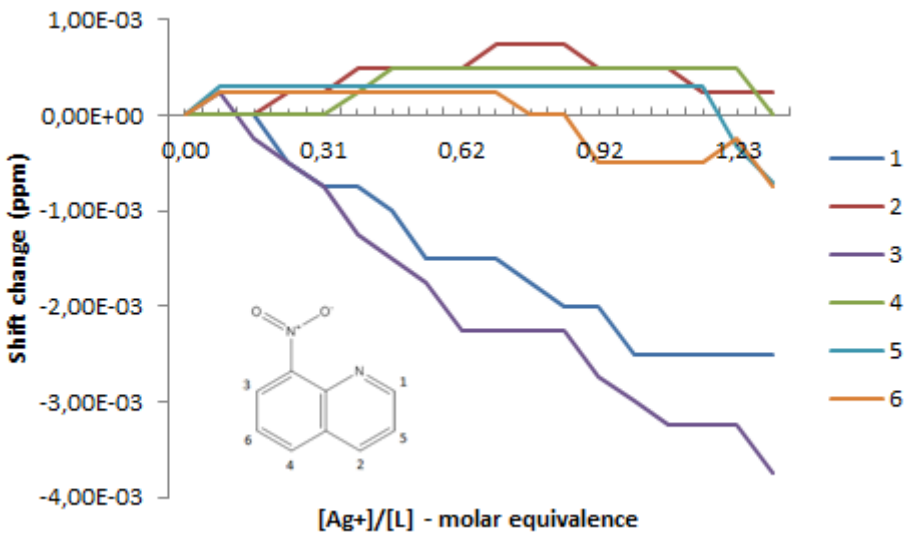

Figure 12: *Graph depicting ^{1}H-NMR titration of 8-nitroquinoline with $AgNO_3$, chemical shift change ($\Delta\delta_n$) from the start of the experiment is plotted against $[Ag^+]/[L]$-molar equivalence*

Peaks 1 and 3 were continuously shifted up-field, indicating an increased amount of shielding whilst the other peaks (2, 4, 5 and 6) shifted slightly down-field until $[Ag^+]/[L] \approx 0{,}70$, where peak 6 dropped to a $\Delta\delta_6 = 0$ and thereafter shifted downfield to a final $\Delta\delta_6$ of -0,00075 ppm. Peaks 2, 4 and 5 all displayed more or less concave curves; peak 2 terminated in a net shift change of 0,00025 ppm ($\Delta\delta_2$ = 0,00025 ppm), peak 4 showed no net change ($\Delta\delta_4 = 0$) and peak 5 ended in a net shift change of -0,0007 ppm ($\Delta\delta_5$ = -0,0007 ppm). The behaviour of peaks 2, 4, 5 and 6 is hard to determine but the primary down-field shift-change might be contributed to interaction with Ag^+ in the solution. The later up-field shift could have been caused by NO_3^- or interaction with other nearby ligands.

The relatively large up-field shift changes of peaks 1 and 3, $\Delta\delta_1$ = -0,0025 and $\Delta\delta_3$ = -0,00375 respectively, would probably have been caused by increased presence of shielding from unknown source. The proximity of the nitro-group to a ligand coordinated around the same Ag^+ would be able to have caused the up-field shift changes indicating the formation of either a 1:2 or higher ligand containing silver/ligand-complex. However, the molecular structure of the molecule insinuates the possibility of large steric hindrance. It must also be noted that the shifts are one order of magnitude smaller than those detected in the remaining titration experiments. Since the recorded NMR-shifts are a mean value of the detected peaks over a time-span it might be possible that small amounts of Ag(I)/ligand interactions were formed for brief periods of time. The increase of [Ag(I)] would increase the probability of such interactions occurring, thusly presenting larger over-all shift-changes.

A comparison between the [Ag(5-nitroquinoline)$_2$] and [Ag(8-nitroquinoline)$_2$] complexes crystal structures (see figure 13) were performed in order to find supporting evidence for the hypothesis that there was little or no complexation during the titration of 8-nitroquinoline with $AgNO_3$.

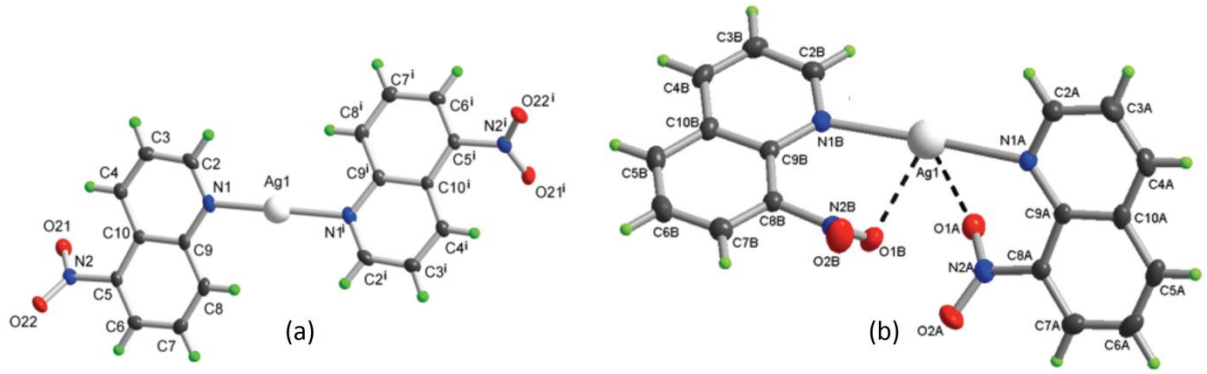

Figure 13: *The two crystal structures of (a) [Ag(5-nitroquinoline)$_2$] and (b) [Ag(8-nitroquinoline)$_2$]*

From the crystal structures it was possible to determine that the bond lengths N-Ag and Ag-N in the [Ag(5-nitroquinoline)$_2$] were both 2.142(2) Å and that the corresponding bonds in [Ag(8-nitroquinoline)$_2$] were 2.303(2) Å and 2.330(2) Å (Massoud *et al*. 2011). The difference in bond lengths indicate that the [Ag(8-nitroquinoline)$_2$] complex is less stable and that the formation of such complexes in solution therefore less likely.

4.2.2. Determination of equilibrium constants

The software "HypNMR2008" was utilized to calculate K_{eq} for the complexes tested in the ^{1}H-NMR study. The program was set to calculate K_{eq} (log β_2) only for complexes with a molar ratio (Ag:L) of 1:2, for each titration trial even though several different complexes might have been present in the solution simultaneously. However, since the relative concentration of ligand was excessive in comparison to silver ions in the first titrations it is reasonable to estimate that the large majority of compounds detected in in the ^{1}H-NMR were [Ag(L)$_2$]-complexes and free ligands.

In figure 14 is included the graphs produced by HypNMR2008 in which the calculated and measured data points from the K_{eq} computations are plotted and compared to one another.

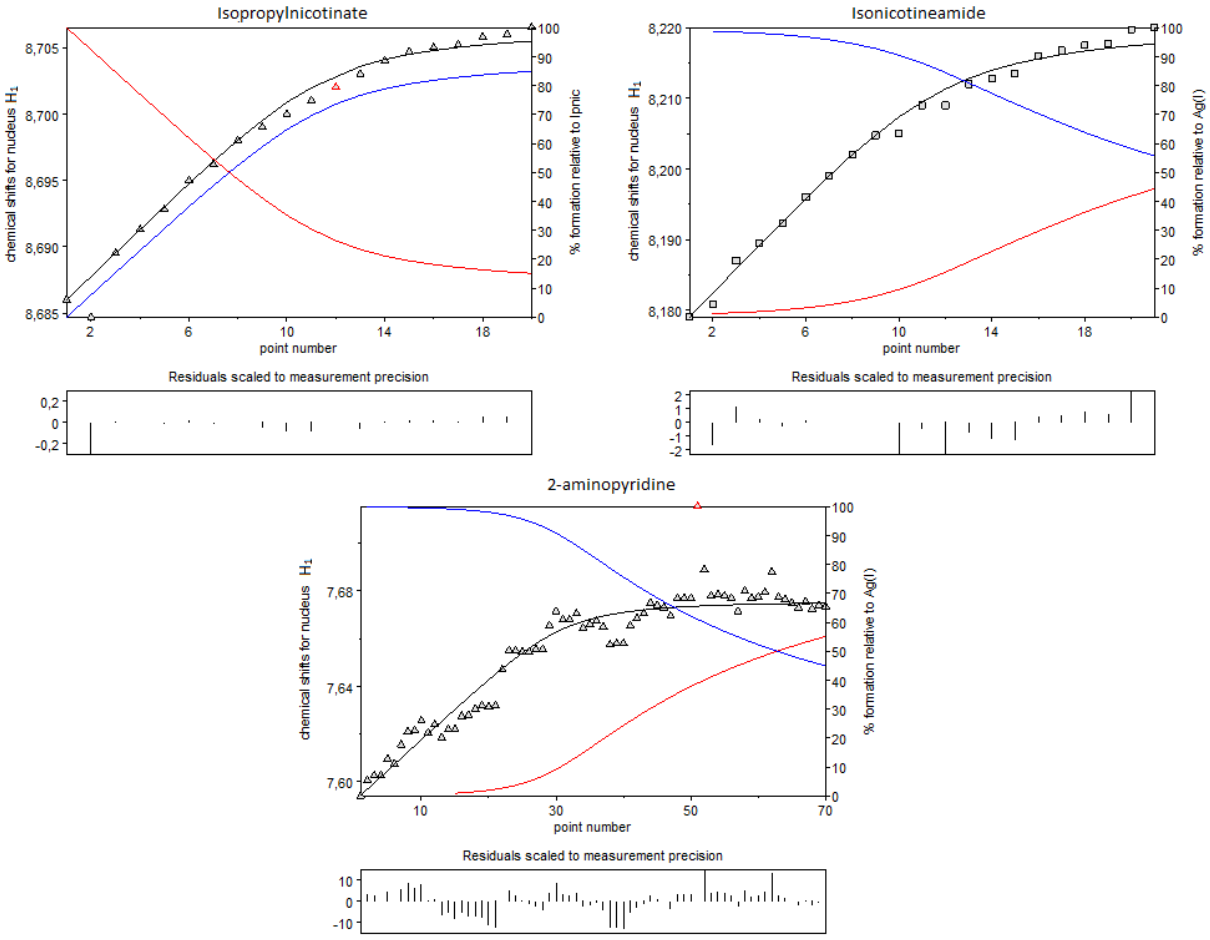

Figure 14: *Graphs representing the chemical shift-change of proton 1 (H_1) in four ligands due to complexation with silver. Presumed depicted are the shift-changes caused by the formation of [Ag(isopropylnicotinate)$_2$], [Ag(isonicotinamide)$_2$] and [Ag(aminopyridine)$_2$] respectively.*

β_2 was acquired for formation of complexes; [Ag(5-nitroquinoline)$_2$], [Ag(isopropylnicotinate)$_2$], [Ag(isonicotinamide)$_2$] and [Ag(2-aminopyridine)$_2$] and data is included in table 2. An equilibrium constant could not be determined for the formation of [Ag(8-nitroquinoline)$_2$], which furthers the hypothesis that, due to various factors, the complex does not form or if so, forms in too small amounts to be sufficiently detected by ^{1}H-NMR.

Table 2: *From HypNMR2008 estimated log β_2-values for the formation of the complexes.*

Complex	log β_2	Solvent
[Ag(5-nitroquinoline)$_2$]	1.2	DMSO-d_6
[Ag(8-nitroquinoline)$_2$]	x	DMSO-d_6
[Ag(isopropyl nicotinate)$_2$]	4.4	DMSO-d_6
[Ag(isonicotinamide)$_2$]	4.2	DMSO-d_6
[Ag(2-aminopyridine)$_2$]	6.3	D_2O

The log β_2 for the formation of [Ag(5-nitroquinoline)$_2$] was determined utilizing data from only five titration and the accuracy was therefore low.

No reference values for log β_2 for the particular complexes studied in this thesis could be found. Values determined for the pyridine-type ligands were instead compared to log β_2 for [Ag(pyridine)$_2$] in two different media (DMSO-d_6 and D_2O); log β_2(DMSO-d_6) = 1.96 and log β_2(D_2O) = 4.26. The structural similarities of ligands isopropyl nicotinate, isonicotinamide and 2-aminopyridine and pyridine made such estimation, to some extent, acceptable. There were no similarly utilizable data for comparison for [Ag(5-nitroquinoline)$_2$].

The lack of proper references is unfortunate; however, this may be one of the first trials in which these particular complexes have been studied for the purpose of determining the equilibrium constant.

4.3. ESI-MS

The electrospray ionization mass spectrometer was intended for the study of the possible interactions between silver/ligand-complexes and molecules typically present in wound fluids. The sensitivity of this particular device would have allowed for the study of variations in chemical peak shift brought on by minute alterations in molecular bonding which would in turn allow for determination of the hypothetical molecular interaction.

The biocidal effectiveness of the complexes is strongly dependent on their behaviour in the presence of biomolecules, minerals and other compounds present in wound fluids and in the circulatory system. It was therefore important to this project to try to study the complexes in a solution resembling such an environment.

One of the main concerns when using complexes containing silver is, as have been mentioned previously in this report, that silver ions have a strong affinity for halides such as chlorine which is common in bodily fluids. The effect of the complexes “loosing” ligands on behalf of chlorine would most probably have the effect of rendering them less efficient or even completely inert. However, there was also a possibility that the complexes could bind or in other manners interact with proteins or other macromolecules and the implications of such cogency are hard to overlook.

The ESI-MS has the ability to analyse dilute solutions with high precision which is the strength of the technique. In the case of this study, however, the sensitivity of the apparatus ruled out the possibility of detecting the studied molecules. Most likely due to inadequate cleaning procedure there were residues from previous experiments left in the ionization chamber. Although there were only small amounts of the disruptive elements present it was enough to make analysis impossible. Unfortunately this was not detected until after the time made available for the tests had run out.

4.3.1. ESI-MS experiments

Below is a description of the experiments performed with the ESI-MS. As mentioned before, investigates were mostly unsuccessful in the sense that only one of a number of trials produced a readable spectrum. Hence, the list is cut short since there was little purpose in continuing testing as almost every experiment turned out a spectrum that was unreadable.

Primarily the two most common silver-containing substances currently in medicinal use, silver nitrate ($AgNO_3$) and silver sulphate (Ag_2SO_4), were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 16 µM and studied. The read-outs were littered with unknown peaks and the experiments were interrupted. At this point it was believed that the solvent could have caused the emergence of the unknown peaks by washout of leftovers from previous experiments and thusly the solvent was switched to methanol. The alteration of solvent had no discernable effect which led to the belief that a concentration of 16 µM was too high and that it caused interference by formation of adducts. A new mixture of $AgNO_3$ in methanol at 4 µM was tested and still no positive outcome.

By only ejecting solvent (DMSO, MeOH and NH_4OAc) into the capillary it was discovered that there in fact were traces of pollutants and the capillary was switched. New trials were performed but still there were numbers of unknown peaks present. Three of the silver complexes were also studied; $[Ag(5\text{-nqu})_2]$, $[Ag(8\text{-nqu})_2]$ and $[Ag(ipnic)_2]$ in solution with DMSO, MeOH and NH_4OAc, at 4 µM but without improvement regarding the interfering peaks.

Considering the possibility that the interference was located to a specific span of the spectrum, hypothetically at relatively low m/z-ratios (100-1000 m/z) there was a prospect that higher weight molecules could be effectively studied. Therefore a test with digested BSA, which may be found in an m/z span of 2000-4000 in the spectrum, was performed. The sought after patterns were distinguishable but there still were unknown peaks disturbing analysis. Hence, none of the trials performed could be considered successful and this part of the project was therefore inconclusive.

One useable spectrum was obtained however and the results are presented in figure 15 below. The spectrum was obtained after a thorough abstersion of the apparatus several weeks after the original trial-period was over.

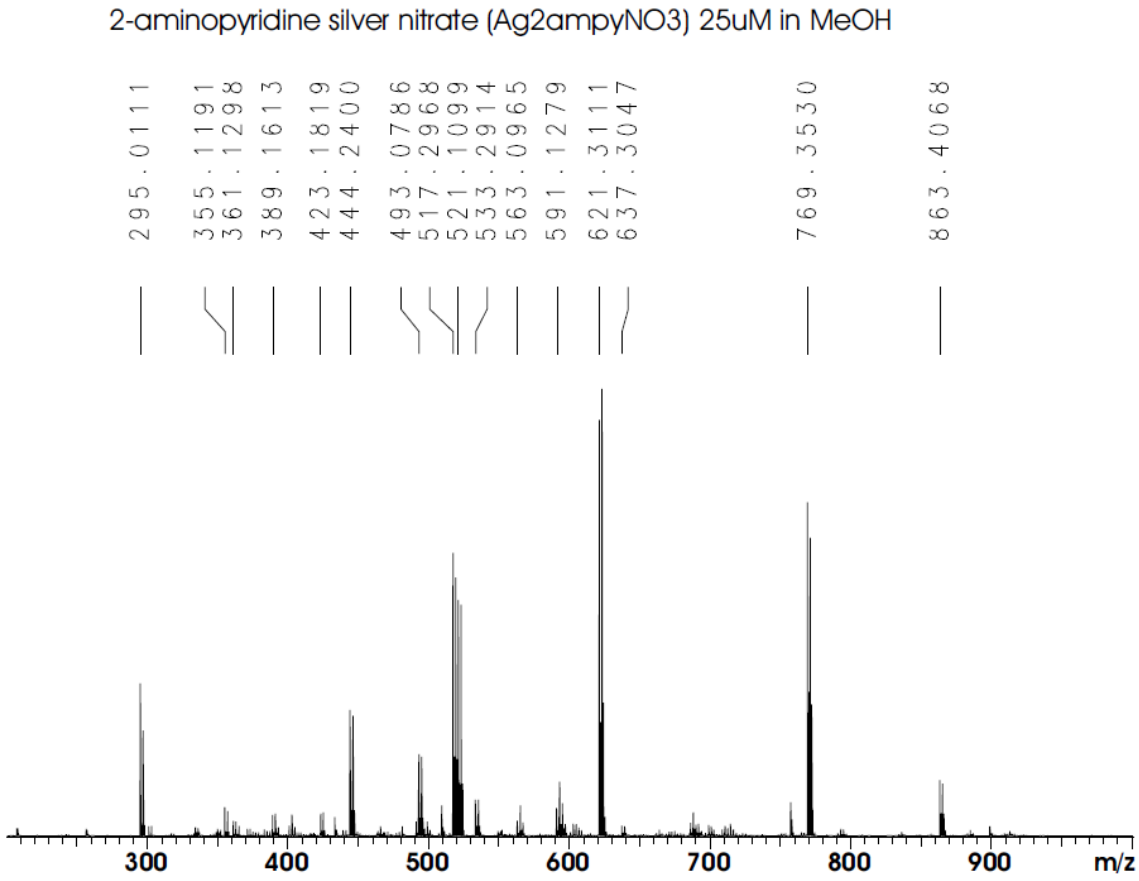

Figure 15: *The ESI-MS spectrum of pure $[Ag(2\text{-aminopyridine})_2]$-complex dissolved in methanol with a concentration of 25 µM*

There are two predominant isotopes of silver; 51,839 % of bulk mass is constituted of ^{107}Ag and 48,161 % of ^{109}Ag. This gives silver a characteristic double-peak (the first slightly more intense than the second) in an MS-spectrum which simplifies detection. In figure 15, a number of such peaks are visible along with several unidentified peaks. The calculated m/z-ratio for $[Ag(2\text{-aminopyridine})_2]^+$ is 295,0113 and a deviation of ±0,5 is acceptable.

The spectrum indicates that the $[Ag(2\text{-aminopyridine})_2]^+$-complex is present in a relatively high concentration in the solution. It also indicates that several other, larger, silver-containing complexes had formed. In table 3 a list of the most probable sources of those spectra is presented and the molecular structure of the complexes with the five most intense peaks are depicted in figure 16.

Table 3: *A listing of the most probable complexes detected by the ESI-MS for a solution of 25µM $[Ag(2\text{-aminopyridine})_2]$ in methanol. The theoretical m/z-ratios were calculated by ChemDraw Ultra 12.0.*

Mass to charge ratio (m/z)			Contains or coordinates with solvent and/or solutes
Theoretical	Found	Probable complex	
295,0113	295,0111	$[Ag(2\text{-aminopyridine})_2]^+$	
354,9895	355,1191	$[Ag(2\text{-aminopyridine})_2(HNO_3)]^+$	x
361,0998	361,1298	$[Ag_2(2\text{-aminopyridine})_2]^{2+}(MeOH)_2$	x
389,0644	389,1613	$[Ag(2\text{-aminopyridine})_3]^+$	
423,0682	423,1819	$[Ag_3(2\text{-aminopyridine})_5]^{3+}(MeOH)$	x
444,1985	444,2400	$[Ag_3(2\text{-aminopyridine})_4(NO_3)]^{2+}(MeOH)_4$	x
493,1500	493,0786	$[Ag_6(2\text{-aminopyridine})_6(NO_3)_3]^{3+}(MeOH)_3$	x
517,2701	517,2968	$[Ag_6(2\text{-aminopyridine})_6(NO_3)_3]^{3+}(MeOH)_5$	x
521,4613	521,1099	$[Ag_3(2\text{-aminopyridine})_6(NO3)]^{2+}(MeOH)_3$	x
533,8403	533,2914	$[Ag_5(2\text{-aminopyridine})_3(NO_3)_3]^{2+}(MeOH)_2$	x
591,0300	591,1279	$[Ag_2(2\text{-aminopyridine})_2]^+$	
621,4910	621,3111	$[Ag_4(2\text{-aminopyridine})_6(NO_3)_2]^{2+}(MeOH)_4$	x
769,3300	769,3530	$[Ag(2\text{-aminopyridine})_5]^+(MeOH)_6$	x
863,3800	863,4098	$[Ag(2\text{-aminopyridine})_6]^+(MeOH)_6$	x

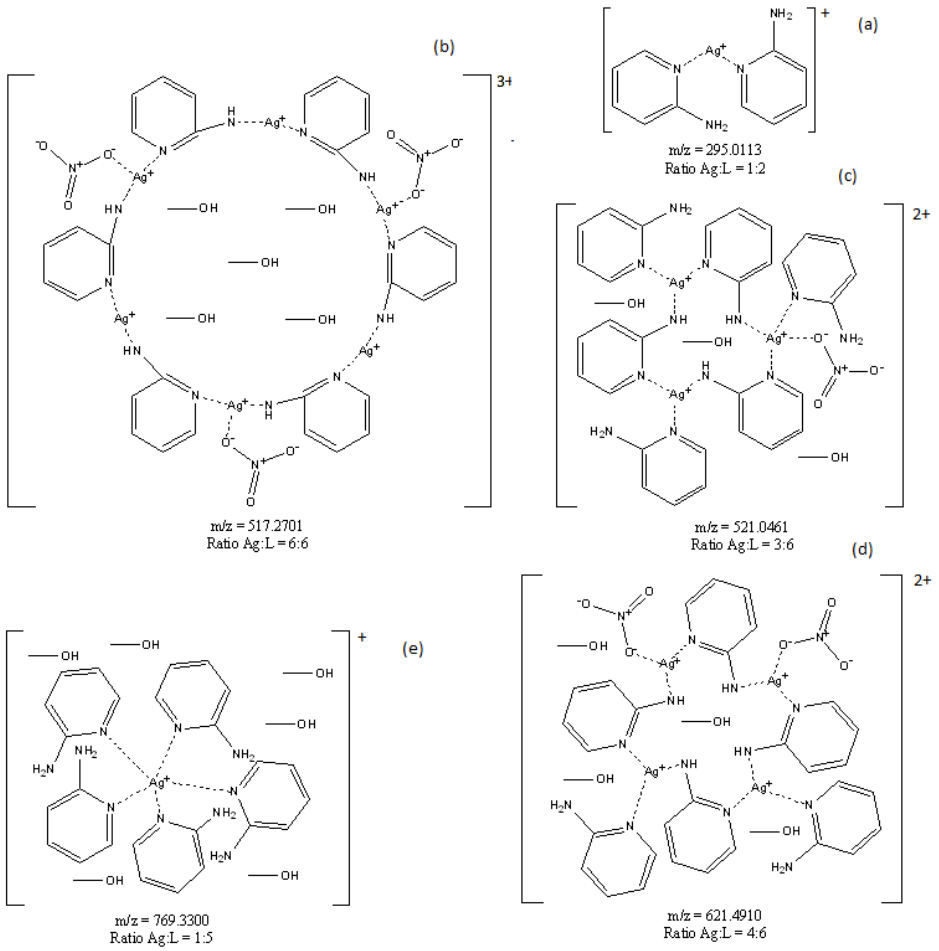

Figure 16: *The complexes that may have caused the peaks detected in the ESI-MS spectrum depicted in figure 15. (a) shows the sought after complex* $[Ag(2\text{-aminopyridine})_2]^+$*, (b)* $[Ag_6(2\text{-aminopyridine})_6(NO_3)_3]^{3+}(MeOH)_5$*, (c)* $[Ag_3(2\text{-aminopyridine})_6(NO3)]^{2+}(MeOH)_3$*, (d)* $[Ag_4(2\text{-aminopyridine})_6(NO_3)_2]^{2+}(MeOH)_4$ *and (e)* $[Ag\ (2\text{-aminopyridine})_5]^+(MeOH)_6$

It must be noted that the complexes displayed in figure 16 have been estimated solely from their m/z-ratios. The solutions tested by the ESI-MS were not analysed by any other equipment and there was therefore no certainty that the complexes depicted here were ones corresponding to the detected peaks.

4.4. Diffusion tests

The trials were performed with silver/ligand-mixtures in solution of DMSO and with four Ag(I):Ligand-ratios: 1:0,5; 1:1, 1:2 and 1:3. The reason for not utilizing complexes was partly because a parallel study in this institution was already conducting such tests, however, the main motive was to study the antimicrobial effect of the ligands themselves compared to silver (Lindberg *et al.*, 2012).

This was a small scale trial performed at two separate occasions, on two ligands and two bacterial strains. Bacteria *Staphylococcus Aureus* (gram positive) and *Pseudomonas Aeruginosa* (gram negative) were tested against ligands 5-nitroquinoline and ethyl nicotinate, adding up to a total of eight tests.

Since these tests can be directly associated with the wound-healing abilities of medical adhesives, the amount of silver in each test was decide by a comparison to common silver-containing products on the market. For a certain amount of $AgNO_3$ a corresponding inhibition zone for a particular bacterium was expected. The silver/ligand-mixture's composition for each

bacterial strain was therefore decided individually in order to be comparable to diffusion-tests performed with only $AgNO_3$.

In none of the trials, described below, were the blank samples able to show any biocidal effect and have therefore been left-out from the figures.

4.4.1. Results *Staphylococcus Aureus*

In order to expect the same effect from [Ag(5-nitroquinoline)$_2$] and [Ag(ethyl nicotinate)$_2$] as from existing products containing only $AgNO_3$ on *S. Aureus*, a silver concentration of 32 µg/ml and 4,1 µg/ml respectively was utilized. The data were obtained at two separate occasions and the results have been normalized against $AgNO_3$ to simplify result interpretation.

The results are presented in figure 17 as a normalized diagram displaying the measured diameter of the inhibition zones resulting from that particular treatment.

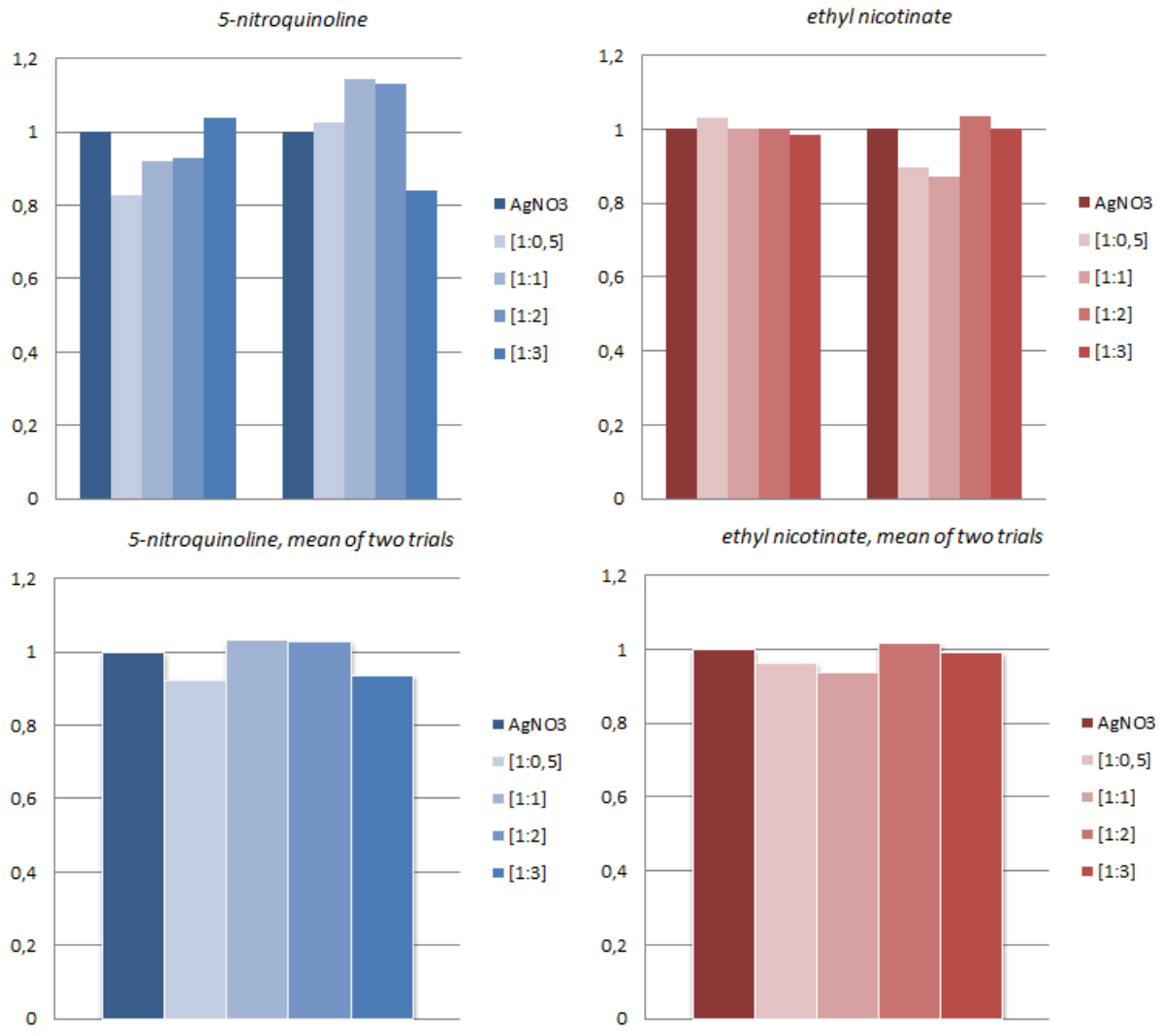

Figure 17: *The two diagrams on-top depicts the normalized inhibition zones for S. Aureus resulting from treatment with $AgNO_3$ or silver/ligand-mixtures at four different Ag:L-ratios. Below are two graphs of the mean normalized inhibition zones for the trials.*

The 5-nitroquinoline-trial displayed irregular inhibition on *S. Aureus*. All samples (except blank) contained the same amount of silver which in this case could indicate that the ligand have both promoting and demoting effects on Ag(I) biocidal properties. Hence, from the graph it is not possible to draw any particular conclusions on what effect the treatment may have. The blank

sample caused no measurable inhibition and it may therefore be possible to assume that the antibacterial effect stems from silver/ligand-complex or from silver only and what effects the ligand has is yet undecided.

Treatment with ethyl nicotinate was more consistent even though samples [1:1] and [1:0,5] differ somewhat between the two trials. The graph indicates that the presence of ligand has a neutral or possibly a slightly positive effect on the biocidal properties of Ag(I). Neither in this trial the blank sample indicated any antibacterial effect of the ligand itself.

From the mean inhibition graphs it is possible to see that the sum effect of the ligands over the two separate trials is basically non-existing or even slightly detrimental compared to the biocidal properties of silver compared to the effect of $AgNO_3$.

4.4.2. Results *Pseudomonas Aeruginosa*

For these trials silver concentrations of 8,1 µg/ml for tests with 5-nitroquinoline and 4,1 µg/ml for ethyl nicotinate were utilized. For unknown reason the first trial was a failure, neither control ($AgNO_3$) nor did the samples have any inhibiting effect on the bacterium. The data were therefore obtained only from the second trial. The results have been normalized against $AgNO_3$ to simplify result interpretation.

The results are presented in figure 18 as a diagram displaying the measured and normalized diameter of the inhibition zones resulting from that particular treatment.

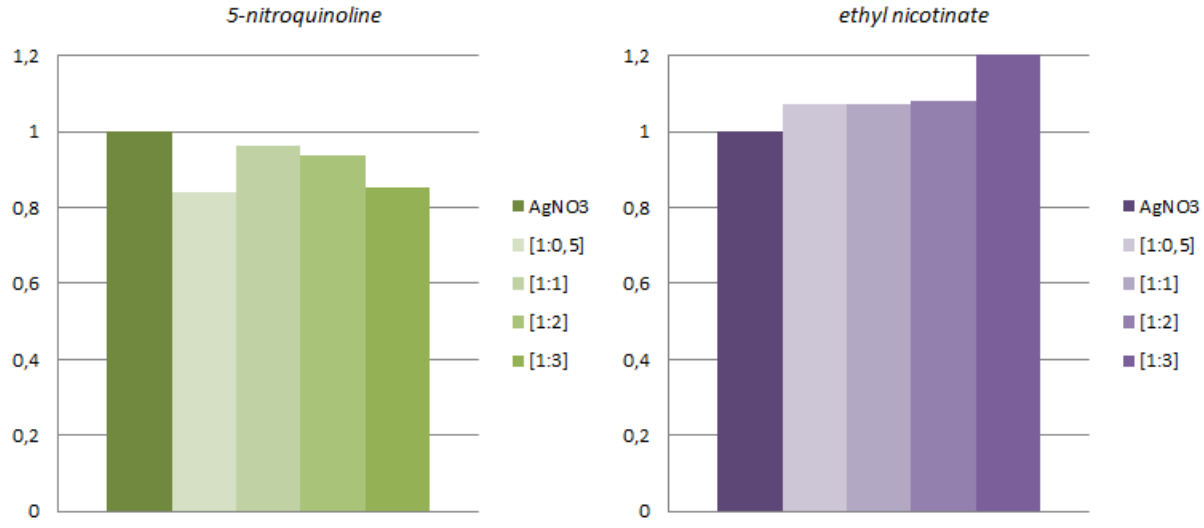

Figure 18: *The two diagrams depicting normalized inhibition zones for P. Aeruginosa resulting from treatment with $AgNO_3$ or silver/ligand-mixtures at four different Ag:L-ratios.*

The fact that only two of the four samples were successful makes any statistically relevant determination of possible effects of the ligands presence impossible, especially as the *S. Aureus* results displayed significant variations between the two trials.

Treatment with silver and 5-nitroquinoline mixture seems to be less efficient than treatment with $AgNO_3$ in this trial and the blank sample indicates that the ligand has, on its own, no more effect on *P. Aeruginosa* than it did on *S. Aureus*.

The ethyl nicotinate does; however, seem to have a positive influence on biocidal effect of silver as the increase in inhibition seems to correlate with ligand concentration. The blank sample, without silver, is ineffectual as was the case also in the other tests.

4.5. Concentrations of complex in silver/ligand-diffusion mixtures

The samples prepared for diffusion tests in this thesis contained uncomplexed amounts of silver and ligands directly corresponding to the amount of 1:2-complexed silver and ligand utilized in diffusion studies performed by Linberg *et al*. in 2012.

By utilizing the calculated values for log β_2 (section 4.2.2.) the actual amounts of complex formed in the silver/ligand-mixtures could be determined (see table 4). The log β_2 value for formation of [Ag(5-nitroquinoline)$_2$] was not utilized since the results were not satisfyingly accurate. Ethyl nicotinate was not tested utilizing ^{1}H-NMR titration and hence no equilibrium constant was determined for the formation of [Ag(ethyl nicotinate)$_2$]. However, ethyl nicotinate and isopropyl nicotinate are structurally closely related and hence it was estimated that their log β_2-values were similar. Hence, the log β_2-value for isopropyl nicotinate was utilized to study the actual amount of [Ag(5-nitroquinoline)$_2$] in the performed diffusion tests.

Table 4: *Concentrations of complex formed during diffusion tests with $AgNO_3$ in mixture with ethyl nicotinate. The column label "free Ag(I)" includes all silver that was not in a 1:2 silver/ligand-complex with ethyl nicotinate.*

Diffusion tests

[conc. in mol/l]	K (from log β_2) = 2,511*10^4				
Bacteria	Reactant		Products		
Staphylococcus Aureus/Pseudomonas Aeruginosa	Ag(I)	ethyl nicotinate	Ag(I) in [Ag(ethyl nicotinate)$_2$]-complex in solution	free ethyl nicotinate	free Ag(I)
AgNO3	3,8E-05	0	0	0	3,8E-05
blank	0	7,6E-05	0	4,8E-05	0
1:3	3,8E-05	1,14E-04	1,239E-8	1,139E-04	3,798E-5
1:2	3,8E-05	7,6E-05	5,508E-9	7,598E-5	3,799E-5
1:1	3,8E-05	3,8E-05	1,377E-9	1,377E-9	3,799E-5
1:0,5	3,8E-05	1,9E-05	3,442E-10	1,899E-5	3,799E-5

Due to low initial concentrations of reactants the product concentration, silver ions in complex, is correspondingly low despite the large value of the equilibrium constant.

In Lindberg *et al.*'s study, tests showed that the minimum inhibitory concentration (MIC) of Ag(I)-ions in [Ag(ethyl nicotinate)$_2$]-complex against the same bacteria were 16 µgAg(I)/ml (1,48*10^{-4} molAg(I)/l) for *S. Aureus* and 8 µgAg(I)/ml (7,42*10^{-5} molAg(I)/l) for *P. Aeruginosa*. This gives that the MICs of silver in [Ag(ethyl nicotinate)$_2$] against *S. Aureus* and *P. Aeruginosa* are 3,235*10^{-7} and 1,617*10^{-7} molAg(I)/l. Both concentrations are two powers of magnitude higher than the 1:2 Ag(I)/ethyl nicotinate-mixture utilized in the diffusion tests performed in this thesis. However, the concentration of "free Ag(I)" (all silver that was not in a 1:2 silver/ligand-complex with ethyl nicotinate) in the diffusion trials is still higher.

Hence, it is uncertain whether the effect of the complex is possible to detect since its influence might be overshadowed by the combined effect of free Ag(I)-ions, other silver/ligand-complexes, $AgNO_3$ or other species possibly present in the solution.

In figure 19 is displayed a graph in which the calculated concentrations of products [Ag(5-nitroquinoline)$_2$] and free Ag(I) have been plotted against the theoretical concentrations of reactants in mixture; Ag(I) and ethyl nicotinate. The same value for K as in table 4 was utilised.

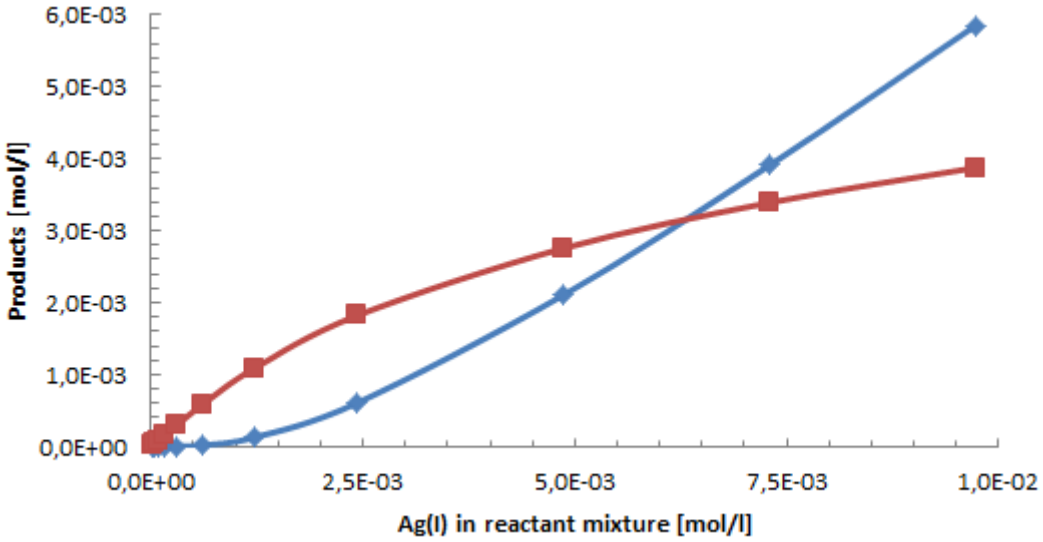

Figure 19: *Graph displaying the calculated concentrations of [Ag(5-nitroquinoline)$_2$] (blue line) and free Ag(I) (red line) as a response to an increased amount of reactants in mixture. For easier visualisation lines have been included between data points.*

The graph visualises at which point reaction product [Ag(5-nitroquinoline)$_2$] overtakes free Ag(I) as the main species in a mixture initially containing only Ag(I) and ethyl nicotinate. It is important to stress that figure 19 does not compare the antibacterial effectiveness of the two species, only their concentrations. From the plot it is however possible to ascertain when it is safe to assume that the antibacterial effect of [Ag(5-nitroquinoline)$_2$] is no longer overshadowed by other silver-containing species in solution.

Research has shown that the minimum inhibitory concentration of [Ag(5-nitroquinoline)$_2$] is lower than the standard $AgNO_3$ (Linberg *et al*. 2012). Which means that even though there is only a small amount of complex present it can have a detectable impact on bacterial growth. Hence, the graph in figure 19 gives a notion of from where the main antibacterial effect stems.

5. Conclusion

In this thesis the chemical behaviour of six quinoline and pyridine-type ligands was studied and even though there is much research still to be conducted it is possible to draw some conclusions from the data collected here.

5.1. The experiments and sources of error

The synthesis of [Ag(5-nitroquinoline)$_2$] was studied utilizing x-ray powder diffraction and standard comparison confirmed correct complexation. Seeing as how the remaining complexes were synthesised utilizing the same method in a previous study and, they also were determined utilisable, the synthesis method can be confirmed as functional.

^{1}H-NMR titration was successfully utilized and the obtained data was able to depict a, probably, correct pattern of proton behaviour for the four ligands tested. From the protons chemical shifts it was possible to draw conclusions on the complexation to some extent or, in the case of 8-nitroquinoline, the inability to form such complexes.

In conjunction with the HypNMR2008 software, the obtained data was able to give reasonable estimations of the equilibrium constants for several of the complexes. The experimental setup

can thusly be deemed correct but there is, as always, room for improvement. Including pH measurements in the titration experiments will allow for more precise determination of K_{eq}. Since the pKa of the ligands tend to change as they coordinate in a complex, pH can be utilized in conjunction with the shift-change data.

The ESI-MS experiments performed were largely unsuccessful in the sense that little data could be obtained; however, in theory the method is functional. The reasons the experiments failed can probably be contributed to lack of experience as well as unfortunate timing. The one successful experiment was able to show the potency of the technique and the data shed light on what actually happens to the complexes as they are passing through the mass spectrometer.

The diffusion tests performed in this thesis must be considered more or less as a pilot study. It is of interest to determine the antibacterial effect of the silver/ligand-mixture, however, more tests are needed to give statistical significance to the results obtained. What could be concluded with some certainty was that the two ligands tested did not have any inherent biocidal effect and thusly that the antibacterial properties stem from complexes formed in the solution mixtures or from silver(I) alone.

A possible reason for the inability to determine the biocidal properties of the silver/ligand-mixtures is that the silver concentrations utilized were too high and that the effects of possible silver/ligand-complexes in the studied solutions were indiscernible from the effects of the silver ions.

5.2. Recommendations for future research

- Include pH-meter in the ^{1}H-NMR trials since the data can be utilized in conjunction with the chemical shifts to improve accuracy when determining equilibrium constants. From the current pH at each titration it may also be possible to explain certain phenomena occurring in the titration mixture.
- Include further analysis methods to determine log β, such as potentiometry or spectrophotometry.
- Further ESI-MS trials. The experiments conducted during the course of this thesis were unsuccessful most likely due to outside sources or insufficient abstersion of the machinery, however, the method is most likely sound and should be possible to utilize for this purpose.
- No clear pattern emerged from neither of the diffusion tests, which were to be expected since the growth and general behaviour of bacteria is inherently rather erratic and may be affected by slight variations in their vicinity. In order to fully determine the antibacterial properties of the ligands it is therefore necessary to perform more trials to complement the ones performed here. It may also be prudent to include other tests, such as minimum inhibitory concentration (MIC) or time-kill assays.

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Appendix I – Complex synthesis data

A.1. Weights and volumes for synthesis of [Ag(5-nitroquinoline)$_2$]

Primary solutions:

0,340 g $AgNO_3$ (0,002 moles) solved in 20 ml distilled water

0,700 g 5-nitroquinoline (0,004 moles) solved in 20 ml of ethanol

Result:

0,8307 g [Ag(5-nitroquinoline)$_2$] (0,001821 moles) - correct complex synthesized confirmed by XRPD)

Yeild:

91,05 %

Appendix II – H-NMR titration data

A.2.1. Titration of 5-nitroquinoline with $AgNO_3$

H-NMR titration of 5 x 0,1 ml AgNO3 in DMSO-d6 to a 0,75 ml solution of 5-nitroquinoline (5-nqu)in DMSO-d6

M(AgNO3):	**169,87**	g/mol	Vol. titrated in each step:	**0,1**	ml
m(AgNO3):	**0,0062**	g	Moles added / titration:		
n(AgNO3):	**3,65E-05**	mol	**4,87E-06**	mol	
ci(AgNO3):	**4,87E-05**	mol/ml			
M(5-nqu):	**174,16**	g/mol			
m(5-nqu):	**0,006**	g			
n(5-nqu):	**3,45E-05**	mol			
ci(5-nqu):	**4,59E-05**	mol/ml			

Titration #	Tot. am. of Ag(I) [mol]	Mol eq. Ag(I) to 5-nqu	Tot. vol. of DMSO-d6 [ml]	conc. Ag(I) [mol/ml]	conc. 5-nqu [mol/ml]
0	0,0000E+00	0,00	0,75	0	4,59E-05
1	4,8665E-06	0,14	0,85	5,72525E-06	4,05E-05
2	9,7329E-06	0,28	0,95	1,02452E-05	3,63E-05
3	1,4599E-05	0,42	1,05	1,39042E-05	3,28E-05
4	1,9466E-05	0,57	1,15	1,69268E-05	3,00E-05
5	2,4332E-05	0,71	1,25	1,94659E-05	2,76E-05

A.2.2. Titration of 8-nitroquinoline with $AgNO_3$

H-NMR titration of 17 x 0,0375 ml AgNO3 in DMSO-d6 to a 0,75 ml solution of 8-nitroquinoline (8-nqu) in DMSO-d6

M(AgNO3):	**169,87**	g/mol	Vol. titrated in each step:	0,0375	ml
m(AgNO3):	**0,01**	g	Moles added / titration:	2,94375E-06	moles
n(AgNO3):	**5,89E-05**	mol			
c*i*(AgNO3):	**7,85E-05**	mol/ml			
M(8-nitroquinoline):	**174,16**	g/mol			
m(8-nitroquinoline):	**0,005**	g			
n(8-nitroquinoline):	**2,87E-05**	mol			
c*i*(8-nitroquinoline):	**3,83E-05**	mol/ml			

Titration #	Tot. am. of Ag(I) [mol]	Mol eq. Ag(I) to 8NOq	Tot. am. of DMSO-d6 [ml]	conc. Ag(I) [mol/ml]	conc. 8NOq [mol/ml]
1	0	0,00	0,75	0	5,10386E-05
2	2,944E-06	0,08	0,7875	3,738E-06	4,86082E-05
3	5,888E-06	0,15	0,825	7,136E-06	4,63988E-05
4	8,831E-06	0,23	0,8625	1,024E-05	4,43814E-05
5	1,178E-05	0,31	0,9	1,308E-05	4,25322E-05
6	1,472E-05	0,38	0,9375	1,570E-05	4,08309E-05
7	1,766E-05	0,46	0,975	1,812E-05	3,92605E-05
8	2,061E-05	0,54	1,0125	2,035E-05	3,78064E-05
9	2,355E-05	0,62	1,05	2,243E-05	3,64562E-05
10	2,649E-05	0,69	1,0875	2,436E-05	3,51991E-05
11	2,944E-05	0,77	1,125	2,617E-05	3,40258E-05
12	3,238E-05	0,85	1,1625	2,785E-05	3,29282E-05
13	3,533E-05	0,92	1,2	2,944E-05	3,18991E-05
14	3,827E-05	1,00	1,2375	3,092E-05	3,09325E-05
15	4,121E-05	1,08	1,275	3,232E-05	3,00227E-05
16	4,416E-05	1,15	1,3125	3,364E-05	2,91649E-05
17	4,710E-05	1,23	1,35	3,489E-05	2,83548E-05
18	5,004E-05	1,31	1,3875	3,607E-05	2,75885E-05

A.2.3. Titration of isopropyl nicotinate with $AgNO_3$

H-NMR titration of 17 x 0,05 ml AgNO3 in DMSO-d6 to a 0,75 ml solution of isopropyl nicotinate (ipnic) in DMSO-d6

M(AgNO3):	**169,87**	g/mol	Vol. titrated in each step:	**0,03**	ml
m(AgNO3):	**0,01**	g	Moles added / titration:	**2,35E-06**	mol
n(AgNO3):	**5,89E-05**	mol			
ci(AgNO3):	**7,85E-05**	mol/ml			
M(ipnic):	**165,19**	g/mol			
m(ipnic):	**0,01**	g			
n(ipnic):	**6,05E-05**	mol			
ci(ipnic):	**8,07E-05**	mol/ml			

Titration #	Tot. am. of Ag(I) [mol]	Mol eq. Ag(I) to ipnic	Tot. vol. of DMSO-d6 [ml]	conc. Ag(I) [mol/ml]	conc. ipnic [mol/ml]
0	0,0000E+00	0,00	0,75	0	8,07E-05
1	2,3547E-06	0,04	0,78	3,0189E-06	7,76E-05
2	4,7095E-06	0,08	0,81	5,81418E-06	7,47E-05
3	7,0642E-06	0,12	0,84	8,40979E-06	7,21E-05
4	9,4190E-06	0,16	0,87	1,08264E-05	6,96E-05
5	1,1774E-05	0,19	0,9	1,30819E-05	6,73E-05
6	1,4128E-05	0,23	0,93	1,51919E-05	6,51E-05
7	1,6483E-05	0,27	0,96	1,717E-05	6,31E-05
8	1,8838E-05	0,31	0,99	1,90282E-05	6,11E-05
9	2,1193E-05	0,35	1,02	2,07771E-05	5,93E-05
10	2,3547E-05	0,39	1,05	2,24261E-05	5,77E-05
11	2,5902E-05	0,43	1,08	2,39835E-05	5,61E-05
12	2,8257E-05	0,47	1,11	2,54567E-05	5,45E-05
13	3,0612E-05	0,51	1,14	2,68523E-05	5,31E-05
14	3,2966E-05	0,54	1,17	2,81764E-05	5,17E-05
15	3,5321E-05	0,58	1,2	2,94343E-05	5,04E-05
16	3,7676E-05	0,62	1,23	3,06308E-05	4,92E-05
17	4,0031E-05	0,66	1,26	3,17703E-05	4,80E-05
18	4,2385E-05	0,70	1,29	3,28569E-05	4,69E-05
19	4,4740E-05	0,74	1,32	3,3894E-05	4,59E-05
20	4,7095E-05	0,78	1,35	3,48851E-05	4,48E-05

A.2.4. Titration of isonicotinamide with $AgNO_3$

H-NMR titration of 19 x 0,03 ml AgNO3 in DMSO-d6 to a 0,75 ml solution of isonicotinamide (inicam) in DMSO-d6

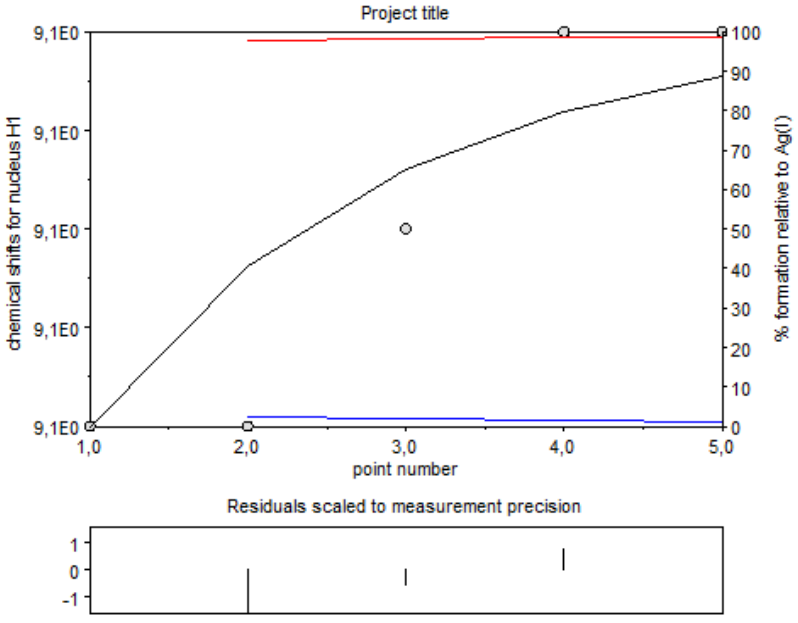
M(AgNO3):	**169,87**	g/mol	Vol. titrated in each step:	**0,03**	ml
m(AgNO3):	**0,01**	g	Moles added / titration:	**2,35E-06**	mol
n(AgNO3):	**5,89E-05**	mol			
ci(AgNO3):	**7,85E-05**	mol/ml			
M(inicam):	**122,12**	g/mol			
m(inicam):	**0,008**	g			
n(inicam):	**6,55E-05**	mol			
ci(inicam):	**8,73E-05**	mol/ml			

Titration #	Tot. am. of Ag(I) [mol]	Mol eq. Ag(I) to inicam	Tot. vol. of DMSO-d6 [ml]	conc. Ag(I) [mol/ml]	conc. inicam [mol/ml]
0	0,0000E+00	0,00	0,75	0	8,73E-05
1	2,3547E-06	0,04	0,78	3,0189E-06	8,40E-05
2	4,7095E-06	0,07	0,81	5,81418E-06	8,09E-05
3	7,0642E-06	0,11	0,84	8,40979E-06	7,80E-05
4	9,4190E-06	0,14	0,87	1,08264E-05	7,53E-05
5	1,1774E-05	0,18	0,9	1,30819E-05	7,28E-05
6	1,4128E-05	0,22	0,93	1,51919E-05	7,04E-05
7	1,6483E-05	0,25	0,96	1,717E-05	6,82E-05
8	1,8838E-05	0,29	0,99	1,90282E-05	6,62E-05
9	2,1193E-05	0,32	1,02	2,07771E-05	6,42E-05
10	2,3547E-05	0,36	1,05	2,24261E-05	6,24E-05
11	2,5902E-05	0,40	1,08	2,39835E-05	6,07E-05
12	2,8257E-05	0,43	1,11	2,54567E-05	5,90E-05
13	3,0612E-05	0,47	1,14	2,68523E-05	5,75E-05
14	3,2966E-05	0,50	1,17	2,81764E-05	5,60E-05
15	3,5321E-05	0,54	1,2	2,94343E-05	5,46E-05
16	3,7676E-05	0,58	1,23	3,06308E-05	5,33E-05
17	4,0031E-05	0,61	1,26	3,17703E-05	5,20E-05
18	4,2385E-05	0,65	1,29	3,28569E-05	5,08E-05
19	4,4740E-05	0,68	1,32	3,3894E-05	4,96E-05
20	4,7095E-05	0,72	1,35	3,48851E-05	4,85E-05

Appendix III – Results from HypNMR2008

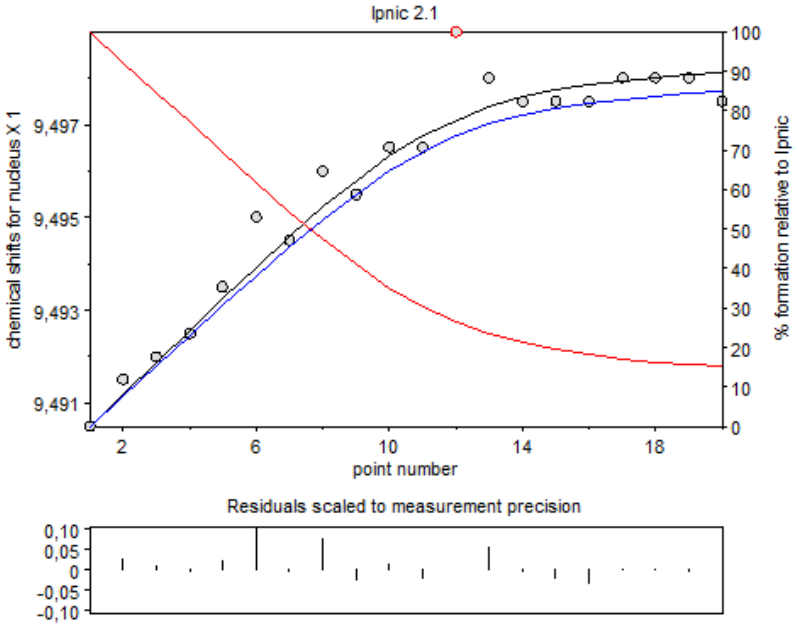
A.3.1. [Ag(5-nitroquinoline)$_2$]

```
Initial values
Log beta(Ag(I)(5-nqu)2)= 1
Initial sigma =  1,8946691221614

Iteration 1
                          relative
                          shift     new value
Log beta(Ag(I)(5-nqu)2)   0,5487    1,19
 new sigma =  1,8946643693683  (rel. change 0,000003)


…

Iteration 4
Marquardt parameter =   1,088726E-06
                          relative
                          shift     new value
Log beta(Ag(I)(5-nqu)2)   0,0000    1,176
 new sigma =  1,89466432298922  (rel. change 0,000000)

</iterations>

Converged in 4 iterations with sigma = 1,894664
**********************************
but Marquardt parameter is not zero
**********************************

                                    standard
                            value   deviation   Comments
 1 log beta(Ag(I)(5-nqu)2)  1.176   excessive   relative error on beta = 3371%
</results>
<shifts>

Chemical shifts for each nucleus (error on 4th decimal place)
             5-nqu    Ag(I)(5-nqu)2
H1        9,0820(0)      9,3249(0)
H2        8,8380(0)      9,4216(0)
H3        8,4400(0)      9,3754(0)
H4        7,9500(0)      9,3629(0)
H5        7,8140(0)      8,3556(0)
```



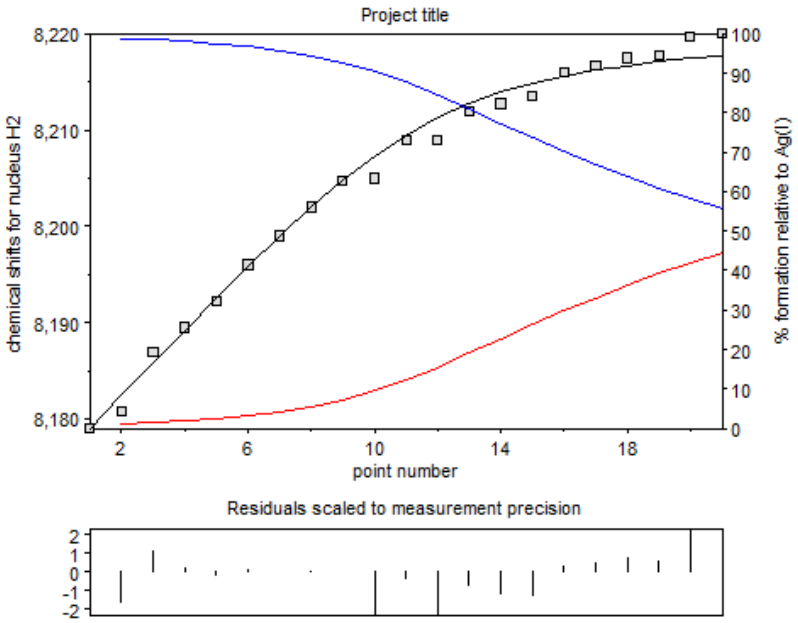
A.3.2. [Ag(isopropyl nicotinate)$_2$]

```
Initial values
Log beta(Ipnic2Ag(I))= 2
Initial sigma =  0,103439864217701

Iteration 1
                        relative
                        shift     new value
Log beta(Ipnic2Ag(I))  50,9307   3,7154
 new sigma =  6,45035033490876E-02  (rel. change 0,376415)


…

Iteration 9
                        relative
                        shift     new value
Log beta(Ipnic2Ag(I))  0,0000    4,4465
 new sigma =  0,0552357929253  (rel. change 0,000000)

</iterations>
<results>

Converged in 9 iterations with sigma = 0,055236

                                    standard
                          value     deviation   Comments
 1 log beta(Ipnic2Ag(I))  4.4465    0.1337      4.4(1)
</results>
<shifts>

Chemical shifts for each nucleus (error on 4th decimal place)
             Ipnic    Ag(I)(Ipnic)2
H1       9,4905(3)      9,4995(3)
H2       9,2253(2)      9,2275(2)
H3       8,6860(5)      8,7090(5)
H4       7,9785(6)      8,0052(6)
H5       5,5860(2)      5,5878(2)
```



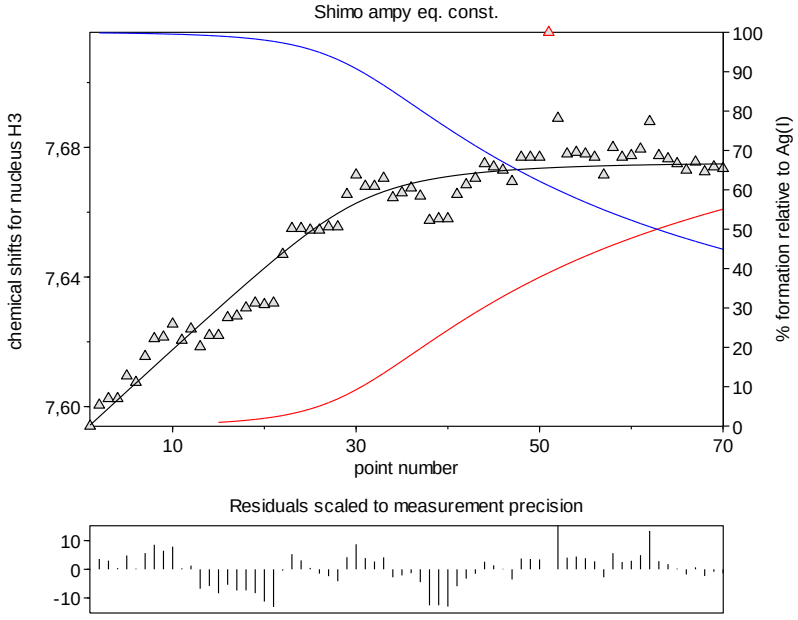
A.3.3. [Ag(isonicotinamide)$_2$]

```
Initial values
Log beta(Ag(I)inicam2)= 2
Initial sigma =  1,9716476460112

Iteration 1
                        relative
                        shift     new value
Log beta(Ag(I)inicam2)  20,2029   3,3264
 new sigma =  1,3099441049385  (rel. change 0,335609)

…

Iteration 8
Marquardt parameter =   2,038609E-02
                        relative
                        shift     new value
Log beta(Ag(I)inicam2)  0,0000    4,1286
 new sigma =  1,0379832435731  (rel. change 0,000000)

</iterations>
<results>

Converged in 8 iterations with sigma = 1,037983
***********************************
but Marquardt parameter is not zero
***********************************

                                    standard
                           value    deviation   Comments
 1 log beta(Ag(I)inicam2)  4.1286   excessive   relative error on beta = 39%

Chemical shifts for each nucleus (error on 4th decimal place)
              inicam   Ag(I)(inicam)2
H1        9,1300(28)    9,1377(28)
H2        8,1790(28)    8,2273(28)
```



A.3.4. [Ag(2-aminopyridine)$_2$]

```
Initial values
Log beta(Ag(I)(2-ampy)2)= 1
Initial sigma =  21,2882666338172

Iteration 1
                          relative
                          shift     new value
Log beta(Ag(I)(2-ampy)2)  713,8347  3,8542
 new sigma =  13,1025454716565  (rel. change 0,384518)


…

Iteration 22
                          relative
                          shift     new value
Log beta(Ag(I)(2-ampy)2)  -0,0001   6,2513
 new sigma =  7,2774872546461  (rel. change 0,000000)

</iterations>
<results>

Converged in 22 iterations with sigma = 7,277487

                                    standard
                             value  deviation   Comments
 1 log beta(Ag(I)(2-ampy)2)  6.2513  excessive   relative error on beta = 60%
</results>
<shifts>

Chemical shifts for each nucleus (error on 4th decimal place)
              2-ampy  Ag(I)(2-ampy)2
H1        6,7168(28)    6,8446(28)
H2        6,7990(58)    6,8262(58)
H3        7,5943(23)    7,6882(23)
H4        7,9883(19)    7,9781(19)
```



Appendix IV – Diffusion test data

A.4.1. Sample preparation of Ag(I)/5-nitroquinoline-mixtrue in DMSO

A.4.1.1. Tests against S. Aureus

AgNO3/5-nitroquinoline-DMSO-solution against S. Aureus:

AgNO3 solution (1)

Solute: AgNO3

Solvent: DMSO

5,040 mg AgNO3 in 1 ml DMSO

[AgNO3]: 5040 µg/ml

[Ag(I)]: 3200 µg/ml

5-nitroquinoline solution (2)

Solute: 5-nitroquinoline

Solvent: DMSO

21,740 mg 5-nitroquinoline in 1 ml DMSO

[5-nitroquinoline]: 21740 µg/ml

Total volume of all samples: 15 µl

Sample:	*V(1) [µl]*	*V(2) [µl]*	*V(DMSO) added [µl]*
AgNO3	10	0	5,00
blank*	0	3,33	11,67
1:3	10	5,00	0,00
1:2	10	3,33	1,67
1:1	10	1,67	3,33
1:0,5	10	0,83	4,17

A.4.1.2. Tests against P. Aerigunosa:

AgNO3/5-nitroquinoline-DMSO-solution against P. Aerigunosa:

AgNO3 solution (1)

Solute: AgNO3

Solvent: DMSO

1,260 mg AgNO3 in 1 ml DMSO

[AgNO3]: 1260 µg/ml

[Ag(I)]: 820 µg/ml

5-nitroquinoline solution (2)

Solute: 5-nitroquinoline

Solvent: DMSO

5,617 mg 5-nitroquinoline in 1 ml DMSO

[5-nitroquinoline]: 5617 µg/ml

Total volume of all samples: 15 µl

Sample:	*V(1) [µl]*	*V(2) [µl]*	*V(DMSO) added [µl]*
AgNO3	10	0	5,00
blank*	0	3,33	11,67
1:3	10	5,00	0,00
1:2	10	3,33	1,67
1:1	10	1,67	3,33
1:0,5	10	0,83	4,17

A.4.2. Sample preparation of Ag(I)/ethyl nicotinate-mixtrue in DMSO

A.4.2.1. Tests against S. Aureus

AgNO3/ethyl nicotinate-DMSO-solution against S. Aureus:

AgNO3 solution (1)

Solute: AgNO3

Solvent: DMSO

0,6458 mg AgNO3 in 1 ml DMSO

[AgNO3]: 645,8 µg/ml

[Ag(I)]: 410 µg/ml

ethyl nicotinate solution (2)

Solute: ethyl nicotinate

Solvent: DMSO

3,4818 mg ethyl nicotinate in 1 ml DMSO

[ethyl nicotinate]: 3481,8 µg/ml

Total volume of all samples: 15 µl

Sample:	*V(1) [µl]*	*V(2) [µl]*	*V(DMSO) added [µl]*
AgNO3	10	0	5,00
blank*	0	3,33	11,67
1:3	10	5,00	0,00
1:2	10	3,33	1,67
1:1	10	1,67	3,33
1:0,5	10	0,83	4,17

A.4.2.2. Tests against P. Aeruginosa:

AgNO3/ethyl nicotinate-DMSO-solution against P. Aeruginosa:

AgNO3 solution (1)

Solute: AgNO3

Solvent: DMSO

0,6458 mg AgNO3 in 1 ml DMSO

[AgNO3]: 645,8 µg/ml

[Ag(I)]: 410 µg/ml

ethyl nicotinate solution (2)

Solute: ethyl nicotinate

Solvent: DMSO

3,4818 mg ethyl nicotinate in 1 ml DMSO

[ethyl nicotinate]: 3481,8 µg/ml

Total volume of all samples: 15 µl

Sample:	*V(1) [µl]*	*V(2) [µl]*	*V(DMSO) added [µl]*
AgNO3	10	0	5,00
blank*	0	3,33	11,67
1:3	10	5,00	0,00
1:2	10	3,33	1,67
1:1	10	1,67	3,33
1:0,5	10	0,83	4,17

A.4.3. Calculated data utilised for graphical representation of the effect of increased reactant (Ag(I)) concentration on the formation of [Ag(ethyl nicotinate)$_2$].

Reactants in mixture			Silver containing products	
Ag(I) [µg/ml]	Ag(I) [mol/l]	Ethyl nicotinate [mol/l]	[Ag(5-nitroquinoline)$_2$] [mol/l]	Free Ag(I) [mol/l]
2	1,90E-05	3,80E-05	6,8855E-10	1,8999E-05
4	3,80E-05	7,60E-05	5,5084E-09	3,7994E-05
8	7,60E-05	1,52E-04	4,3995E-08	7,5956E-05
16	1,52E-04	3,04E-04	3,5022E-07	1,5165E-04
32	3,04E-04	6,08E-04	2,7461E-06	3,0125E-04
64	6,08E-04	1,22E-03	2,0373E-05	5,8763E-04
128	1,22E-03	2,43E-03	1,2899E-04	1,0870E-03
256	2,43E-03	4,86E-03	6,0859E-04	1,8234E-03
512	4,86E-03	9,73E-03	2,1066E-03	2,7574E-03
1024	9,73E-03	1,95E-02	5,8520E-03	3,8760E-03