

Exploration of Methods for Solubility Studies of Amyloid Beta

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June 2024

Abstract

The protein amyloid beta ($A\beta$) is a major component in the amyloid plaques associated with Alzheimer's disease. This project concerns the most common version of this protein, $A\beta_{40}$. In a supersaturated solution, $A\beta_{40}$ monomers can aggregate to form amyloid fibrils. The solubility of an amyloid protein is equal to the concentration of the monomeric peptide in solution at equilibrium. To study the solubility, with the ambition to better understand the driving forces of the fibril formation, there is a need for reliable methods to determine the monomer concentration at equilibrium. In this project, three such methods were explored.

The first quantification method utilised the fluorescent probe o-phthalaldehyde (OPA), which reacts with primary amines in the peptide. The second method was high-performance liquid chromatography with mass spectrometry (HPLC-MS), which gave results based on both UV absorbance and total ion count after separation based on hydrophobicity. Both OPA and HPLC-MS quantification required the monomers to be separated from the fibrils, prior to measurements. Additionally, the samples were incubated in plate readers with ThT prior to the separation and quantification. The third and final quantification method was nuclear magnetic resonance spectroscopy (NMR). In NMR, the concentration of the monomers was calculated both based on the initial concentration and on an external standard.

NMR reported the highest apparent solubility, OPA an intermediate value (with an increase as the total $A\beta_{40}$ concentration increased) and HPLC-MS reported the lowest apparent solubility. Kinetic traps, adsorption and protein degradation/modification were suggested to contribute to the differences in the results. Future experiments should include incubating with shaking, measuring with NMR and then calculating with external standard. Furthermore, it is necessary to repeat the experiments and evaluate their accuracy and precision.

Acknowledgements

I have many people to thank for being able to complete this project and I want to start with my supervisors. **Max**, thank you for all your guidance and endless discussions about why my results look as they do. **Jing**, thank you for all your help with the NMR preparations and data analysis. **Sara**, you are my academic role model. I am so grateful for my time in your group and for all your insights and support. I would also like to thank **Emma** for being an assistant supervisor and **Kristine** for being my examiner.

Zoltan, thank you so much for getting me through my first real encounter with NMR and TopSpin. **Johan**, I cannot thank you enough for figuring out what was wrong in my TopSpin processing. **All the members of Sara's group**, thank you for making me feel so at home and for sharing your experience and scientific curiosity. I also want to give my thanks to anyone else at **CMPS** who I have chatted with in the lab, at lunch or at fika. Also, thanks for the fika!

Finally, **my friends and family**, who have listened to both my excited ramblings and frustrated rants about this project. I love and appreciate you endlessly.

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1 Introduction

1.1 Background

The neurodegenerative disease Alzheimer's disorder (AD) is heavily associated with the presence of amyloid plaques in the brain, largely consisting of the protein amyloid beta (A β)[1]. A β is derived from the endogenous amyloid precursor protein (APP) through proteolysis and, depending on the cleavage sites, the peptide can vary in length with the 40 residues long peptide A β 40 being the most common one[2]. A β 40 is therefore highly interesting to study. In a lab setting, the peptide can be produced either synthetically or recombinantly. This project is concerned with recombinant A β (M1-40), which contains an extra N-terminal methionine (compared to the peptide A β (1-40)) and has previously been reproducibly expressed and purified from *Escherichia coli*[3]. For brevity, A β (M1-40) will be referred to as A β 40 in this thesis and its sequence is shown in Figure 1 below.



Figure 1: Sequence of A β (M1-40), the peptide studied in this project. Henceforth referred to as A β 40. Residues are coloured from most hydrophobic (red) to least hydrophobic (blue).

In its monomeric state, A β is an intrinsically disordered protein, but A β monomers are known to self-associate into various sizes of oligomers and can eventually aggregate into larger fibrillar structures, typically characterised by a stable core of stacked beta sheets[4]. This aggregation can be seen as a phase transition, where insoluble fibrils precipitate out from a supersaturated solution of A β monomers. The solubility of A β is then defined as the concentration of the monomer in solution at the phase equilibrium, where the monomers and fibrils coexist.

Understanding the relationship between temperature and the solubility of amyloid proteins will help elucidate the driving forces involved in their aggregation and associated pathologies. The previously reported solubilities of recombinant A β 40 (Table 1) are based on both experiments with A β (M1-40) and A β (1-40). The additional methionine has been estimated to decrease the solubility with a factor of 2.5 [5].

Table 1 Reported solubilities of recombinant A β 40.

Temperature (°C)	A β Sequence	Solubility (μ M)	Method
37	A β (M1-40)	0.36 ± 0.15 [6]	MS and MDS [6]
37	A β (M1-40)	0.2 ± 0.1 [5]	OPA [5]
37	A β (1-40)	0.5 ± 0.1 [5]	OPA [5]
26	A β (1-40)	1.6 ± 0.2 [5]	OPA [5]

1.2 Disposition and scope

The report below is divided into three main sections: methodology, results, and discussion. The methodology section elaborates some on current theory about A β 40. The methodology section also covers the methods and tools used in the experimental work. The monomer quantification methods used in this project fall in one of two categories: quantification with separation and quantification without separation. The separation requiring methods used in this project were the use of the fluorescent probe o-phthalaldehyde (OPA), high performance liquid chromatography (HPLC) and mass spectrometry (MS). Nuclear magnetic resonance

(NMR) was investigated as a quantification method not requiring separation. The experimental and processed data from the different methods is presented in the results section. In the discussion section, the results are critically reviewed and suggestions for future developments are presented.

The aim of this Master Thesis is to explore methods to determine the solubility of A β 40 at different temperatures. The task is therefore to form A β 40 fibrils at different temperatures and use different methods to determine the equilibrium concentration of the monomer in the solution phase. These methods should then be compared and evaluated.

2 Methodology

2.1 Monomers and fibrils as phases

The monomeric A β 40 in solution can be viewed as the soluble phase and the chemical potential of the monomer in dilute solution, μ_m , is given by Equation 1 [7], where μ_m° is the standard chemical potential of the monomer in solution, R is the molar gas constant, T is the absolute temperature and c_m is the concentration of free monomer, which is an approximation of the activity of the monomer.

$$\mu_m = \mu_m^\circ + RT \ln c_m$$

Equation 1

When the A β 40 concentration increases to a point of supersaturation, i.e., when the concentration increases above the solubility of the monomer, fibrils may form as a precipitate. Since the fibrils can be seen as a pure phase, their chemical potential is concentration-independent and can be approximated with the standard chemical potential [7]:

$$\mu_f = \mu_f^\circ$$

Equation 2

The precipitated fibrils coexist with the monomers in solution, forming a two-phase system. This system reaches a dynamic equilibrium when the chemical potentials for the two phases are equal: $\mu_m = \mu_f$. The concentration of the monomer, c_m , is then equal to the solubility, s , and s can be derived according to Equation 3.

$$\begin{aligned} \mu_m^\circ + RT \ln(s) &= \mu_f^\circ \\ \Rightarrow s &= e^{(\mu_f^\circ - \mu_m^\circ)/(RT)} \end{aligned}$$

Equation 3

A β 40 in solution exhibits what is known as metastability, where a range of monomer concentrations exceeding the solubility result in no observable fibril formation; this range of concentrations, the metastable zone, has been shown to decrease with increased incubation time[6].

2.2 Purification and sample preparation

Freeze-dried recombinant A β 40 was provided by members of the Linse lab at Lund University after being purified by the methods explained by Linse (2020)[3].

The freeze-dried A β 40 was further purified using size exclusion chromatography (SEC) in a fast protein liquid chromatography (FPLC) system, using a phosphate buffer (20 mM sodium phosphate, 0.2 mM EDTA, pH 7.5). The monomer concentration was calculated from the absorbance at 280 nm using the extinction coefficient 1440 M⁻¹cm⁻¹ of A β 40. An example of a SEC chromatogram can be found in the Appendix (Figure A 1).

If the samples were to be used in the separation experiments (i.e., with OPA and HPLC-MS), the eluate was then diluted, most often in duplicates or triplicates, to desired concentrations using the SEC buffer. The samples were prepared in non-binding tubes (MCT-150-L-C, Axygen) for cases where the temperature of interest was reached through incubation in a heating block, incubation cabinet, or chilled room. For some temperatures, a plate reader was used to control the temperatures and the samples were therefore prepared in PEGylated 96-well plates (further discussed below). When using tubes, the sample volume was 200 μ L, while in the plate wells the sample volume was 100 μ L. From the plates, samples with the same total A β 40 concentration were pooled pairwise into non-binding tubes for a sample volume of 200 μ L going into the separation.

The samples intended for NMR quantification were not diluted after SEC, since a very high starting concentration was desired for a strong and clear signal, as well as more rapid aggregation. Samples to be used in NMR underwent SEC using the same phosphate buffer as samples intended for OPA and HPLC-MS, but with 10% D₂O.

During incubations in plate reader, the formation of fibrils was monitored, described in the next subsection, and during NMR incubations the monomer depletion was monitored based on the decreasing intensity of the peaks (see NMR subheading). The aggregation occurring for samples incubated in tubes, however, was not monitored. Therefore, incubation time and starting concentrations were chosen as to minimise the likelihood of the samples being metastable and to increase the likelihood of reaching equilibrium.

2.3 Monitoring aggregation with ThT fluorescence

For temperatures where little was known about the kinetics of A β , the formation of fibrils was monitored during incubation. Thioflavin T (ThT) was used as fluorescent probe for this purpose. ThT binds to amyloid fibrils, causing a shift in the probe's fluorescence; excitation at 450 nm gives a fluorescence signal at 482 nm[8].

The protocol for the ThT fluorescence measurements was adapted from previous work of the Linse group[3]. A ThT concentration between 5 and 10 μ M was used. The higher end of this range is within what has been recommended in literature to achieve a high fluorescence signal at the aggregation plateau, which is proportional to the fibril mass concentration, and without ThT having substantial effect on the aggregation[9].

To minimise protein adhering to the surface of the wells, PEGylated polystyrene black clear-bottomed plates with half-area wells (Corning® 3881) were used, since PEG has been shown to decrease protein binding to surfaces [10, 11]. In the plate reader (FLUOstar Omega, BMGLabtech), the ThT fluorescence was measured through the bottom of each well (hence the clear bottomed plates) with an excitation filter of 440 nm and an emission filter of 480 nm.

The ThT fluorescence intensity for each sample was corrected with the intensity for buffer with the same ThT concentration, but no protein present. This is especially important as ThT has shown self-fluorescence at concentrations ≥ 5 μ M [9].

The ThT plateau being reached was used as an indication that samples had reached equilibrium. Although, this may not strictly be true – the ThT plateau may be reached before the system has equilibrated, even if there is no longer any noticeable increase in ThT fluorescence as more and more monomers become part of fibrils. Therefore, the incubation continued a few hours after plateau had been reached.

2.4 Quantification of monomers with separation

Two approaches that require separation of the monomers in solution from the fibrils were used: high performance liquid chromatography-mass spectrometry (HPLC-MS) and quantification with a fluorescent probe.

The incubated samples were separated through centrifugation for 60 min at 31500 g or 90 min at 20817 g, depending on which centrifuge was available. The supernatant samples were carefully taken from the top of the solution and transferred to PEGylated polystyrene, black-bottomed plates with half-area wells (Corning® 3686). 50 μ L supernatant was added to one plate for the probe quantification and 50 μ L supernatant was added to a second plate for the HPLC-MS assay.

The fluorescent probe used was the dye o-phthalaldehyde (OPA). OPA can bind covalently to primary amine groups, present at the N-terminal and lysine residues of peptides; this results in a fluorescent product[12]. The procedure for quantification of peptides from OPA fluorescence was adapted from previous research[5]. 5 μ L of OPA was mixed with 50 μ L of supernatant, incubated at room temperature away from light for 20 minutes. The fluorescence was then measured in a plate reader (ClarioStar, BMGLabtech) with top optics. The excitation wavelength used was 340 nm (with a 30 nm slit) and the emission wavelength was 440 nm (with a 40 nm slit).

For each measurement on sample supernatants, a dilution series of standard samples with A β 40 monomer at known concentration was prepared. The standards were prepared through dilution of frozen and thawed monomer samples from previous SEC purifications. OPA fluorescence was measured for the standards and a calibration curve was constructed from the linear region of the plot of standard concentration vs OPA fluorescence using MATLAB (R2022a). This calibration curve then allowed for quantification of A β 40 in the supernatant samples.

A reverse-phase column (BIOshell™ A160 Peptide CN, 2.7 μ m) was used in an HPLC-MS system (HPLC-MS 2020, Shimadzu), with acetonitrile as the mobile phase. As in SEC, the monomer can in HPLC be quantified through absorbance measurements at 205 nm and 280 nm. In the chromatograms, the peaks corresponding to the monomer were identified based on the retention time and through comparison to peaks in the MS chromatogram. The concentration was then determined from the area of the peaks, using calibration curves based on standards with known concentrations.

The MS component of the HPLC-MS system was a single quad mass spectrometer, which was used to collect a total ion count spectrum and perform a selected ion monitoring (SIM) scan for each sample. In the SIM scan the two ions used were: $m/z = 1448$ and $m/z = 1116$. These ions respectively correspond to a full length A β 40 peptide with a charge of $z = 3$ or $z = 4$. In MS, quantification was achieved using calibration curves based on standards with known concentrations.

2.5 Quantification of monomers without separation

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique and explaining the full theory and principles behind it is outside the scope of this report. For the purposes of this project, it is sufficient to understand two things. Firstly, in NMR spectra, different NMR active nuclei (such as protons and carbon-13 atoms) will show up as peaks at different chemical shift depending on their chemical environment, such as what they are bonded to. Secondly, when studying amyloid proteins, NMR is especially useful as it allows for detection of monomeric species, without first separating out the fibrils. This is because the

fibrils are large enough to tumble too slowly to be seen in the NMR spectra, while monomers tumble more quickly[13].

In this project an 800 MHz NMR magnet (Bruker Avance Neo 800 MHz 4-channel spectrometer with TCI 5mm Z-gradient CryoProbe 1H/13C/15N/2H.) was used. After purifying the monomeric A β 40 with 10% D2O buffer (as explained above), 500 μ L of the SEC eluate was transferred to the NMR tube and kept on ice until the NMR experiment was set up. The NMR experiments used the pulse program *zgesgpppe*, which consists of a 1D pulse sequence and uses excitation sculpting with gradients for water suppression. The incubation temperature was regulated and monitored in the NMR magnet, which was calibrated before each experiment with a methanol sample. A measurement was performed every 30 minutes (experiment time: 12 min) to collect a 1D ^1H spectrum.

The NMR data was analysed using TopSpin (4.3.0). In the data analysis, a set of peaks were identified as methyl groups of the monomers and another set were identified as the aromatic protons. The methyl peaks were integrated together and integral was used to track the change in monomer concentration over time. In the relative quantification, the initial monomer concentration was set to the concentration calculated from the SEC. By comparing the integral of the methyl peaks in each spectrum, relative to that in the first spectrum, the concentration at each time point could be calculated. This procedure could also be repeated for the aromatics to further strengthen the results.

In addition, an external calibration was performed using the salt potassium hydrogen phthalate (KHP) as an external standard. A ^1H spectrum for KHP was gathered prior to each incubation of A β 40. In the KHP spectrum, two multiplets of peaks (corresponding to a total of four aromatic protons) were integrated together to use as the reference signal [14]. The aim was to use this for absolute quantification of the monomers, by comparing the integral of a known number of protons in the sample to the reference signal from the KHP standard at the set temperature. The concentration in the samples were calculated both using Equation 4 and with the built-in tool Eretic2 in TopSpin.

$$\frac{I_S}{H_S} = \frac{I_R}{H_R} \Leftrightarrow \frac{I_S}{H_{AB40} * C_{AB40}} = \frac{I_R}{H_{KHP} * C_{KHP}}$$

$$\Rightarrow C_{AB40} = \frac{I_S}{H_{AB40}} * \frac{H_{KHP} * C_{KHP}}{I_R} = \frac{I_S}{H_{AB}} * \frac{4 * 100 \mu\text{M}}{I_R}$$

Equation 4

I: integral of the peaks in the sample (S) or reference (R); *H*: number of protons in the integrated peaks in total in the sample (S) reference (R) or per molecule of A β 40 or KHP; *C*: molar concentration.

To estimate the number of protons in the integrated methyl peaks in the ^1H spectra, an edited H1-C13 HSQC experiment was conducted. HSQC is a 2D NMR experiment, where cross-peaks for protons directly bonded ^{13}C are shown in the spectra. With this edited version of HSQC, it is possible to distinguish between CH₂ and CH₃ peaks. The chemical shifts of the methyl protons and carbons in the spectrum was compared to their literature values [15]. The number of protons in the methyl peaks was also estimated by comparing the integrals of the peaks from the aromatic and methyl protons.

3 Results

3.1 Method development

As explained in the methodology, the separation of monomers from fibrils was required for quantification with OPA and HPLC-MS. In a control experiment to investigate how much fibril remains in the sample after centrifugation, the second 50 μL supernatant was mixed with ThT. The ThT fluorescence was measured and, with a standard curve created from fibrillated samples with known concentrations (Figure A 2, in Appendix), the fibril concentration was quantified (Figure 2).

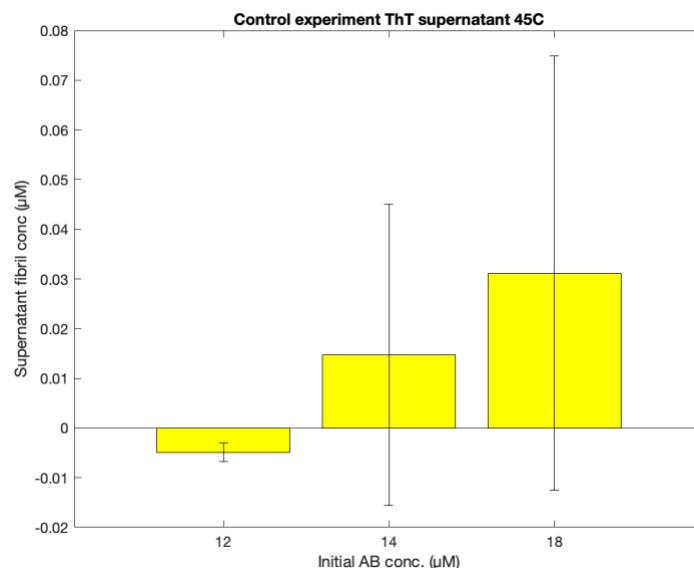


Figure 2: ThT quantification of fibril concentration in supernatant

Additionally, fibrillated samples with known concentrations were allowed to react with OPA and the fluorescence for these was measured. This allowed for construction of a standard curve relating fibril concentration to OPA fluorescence (Figure 3), which gives an indication of how sensitive OPA is to fibrils.

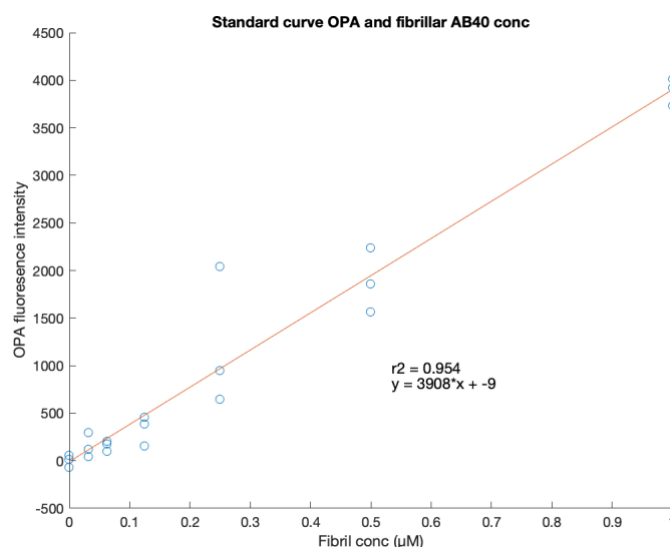


Figure 3: Standard curve for detection of fibrils with OPA fluorescence

The effect of buffer treatment was also investigated, by comparing the OPA fluorescence of incubated together with the samples and buffer kept at room temperature. An example of this can be seen in the experiment at 45 $^{\circ}\text{C}$ and can be found in the Appendix (Figure A 3). A one-

way ANOVA test of that example revealed that the means of the warm and room temperature buffer samples were different with a p-value of 0.08.

3.2 ThT fluorescence

To track fibril formation, ThT fluorescence was measured at 30 min intervals during the A β 40 self-assembly reactions in the plate reader. At each time point, an average ThT signal was calculated from the replicates with the same initial monomer concentrations, i.e., the same total A β 40 concentration. The averages were then normalised between 0 and 1 for easier comparison. The normalised average ThT fluorescence was plotted against aggregation time (Figure 4- Figure 8). Example of raw data for each replicate can be found in the Appendix (Figure A 4).

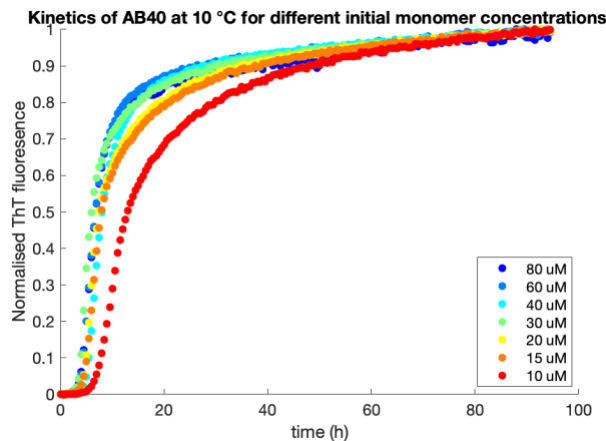


Figure 4: Normalised ThT fluorescence over time at 10 °C in plate reader

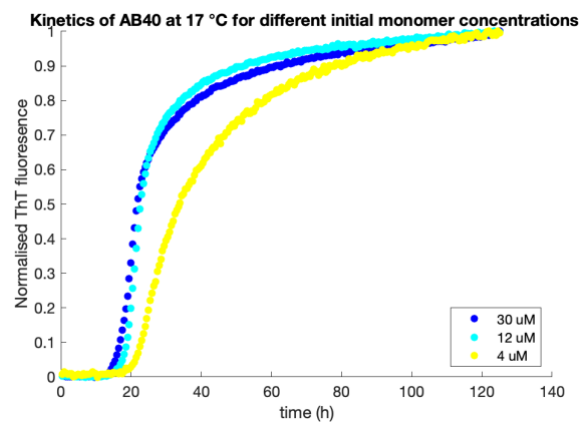


Figure 5: Normalised ThT fluorescence over time at 17 °C in plate reader

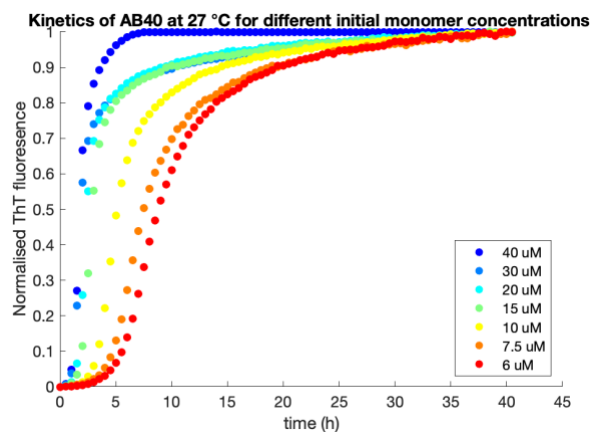


Figure 6: Normalised ThT fluorescence over time at 27 °C in plate reader

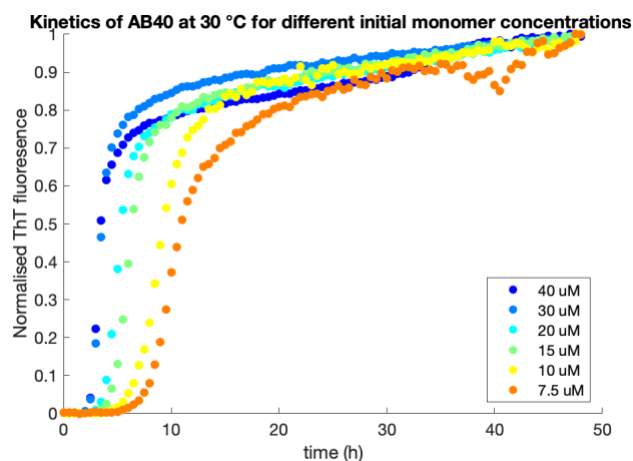


Figure 7: Normalised ThT fluorescence over time at 30 °C in plate reader

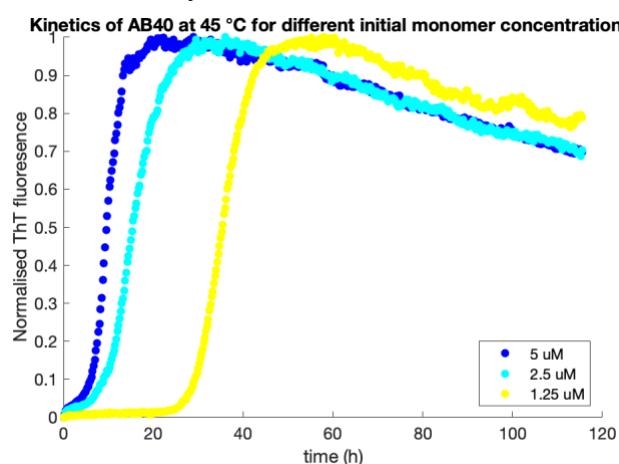


Figure 8: Normalised ThT fluorescence over time at 45 °C in plate reader

3.3 OPA and HPLC-MS

For the OPA quantification, a standard curve was created for each experiment (Figure A 6). The calculated supernatant A β 40 concentration was plotted against the total A β 40 concentration for each temperature (Figure 9). Results from each temperature can be found in the Appendix (Figure A 7 - Figure A 11).

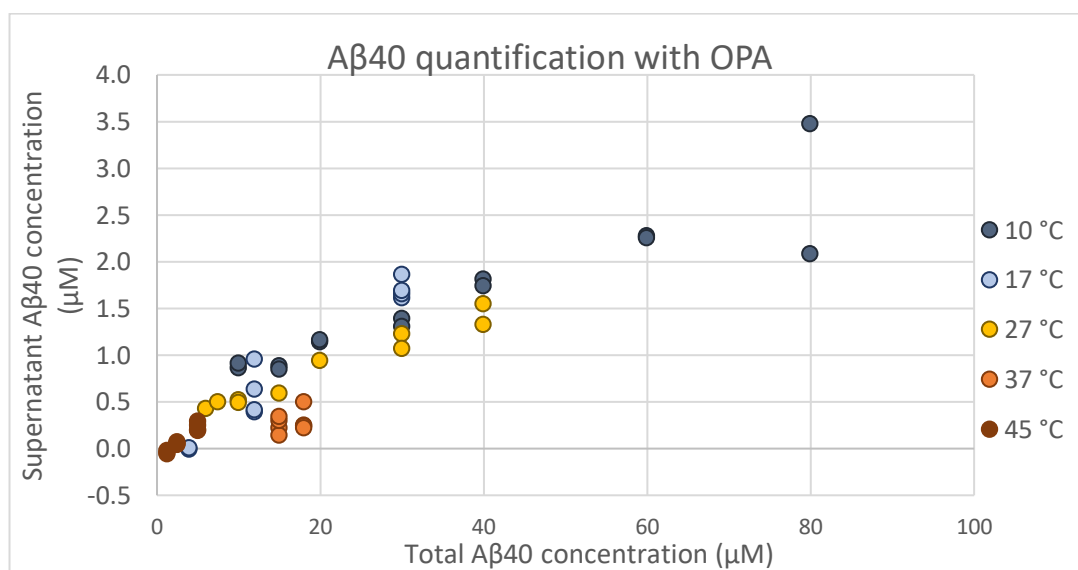


Figure 9: Results from quantification with OPA

In the HPLC-MS measurements, the same set of standard curves were used for each temperature (Figure A 12, Figure A 13 and Figure A 14). The LC quantification was performed using absorbance measurements at 205 nm and 280 nm. The 280 nm LC results are shown in Figure 10, while the TIC ($m/z = 1488$ and $m/z = 1116$) MS results are shown in Figure 11. The 205 nm LC results are shown in Figure A 15 in the Appendix. Examples of HPLC-MS chromatograms and spectra can be found in Figure A 16.

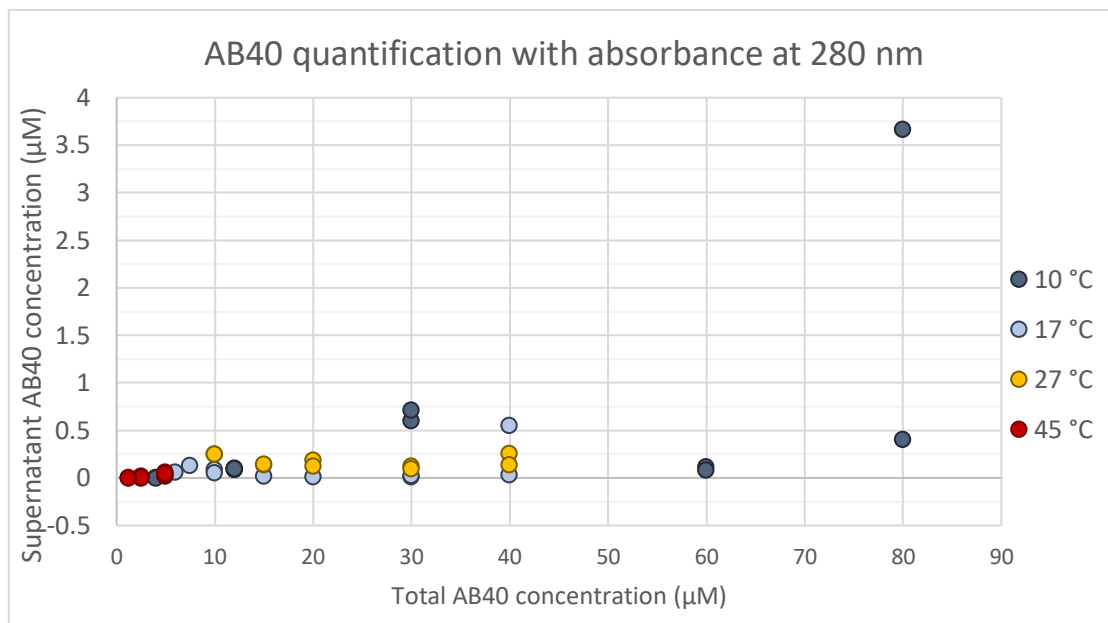


Figure 10: Results from quantification with absorbance at 280 nm

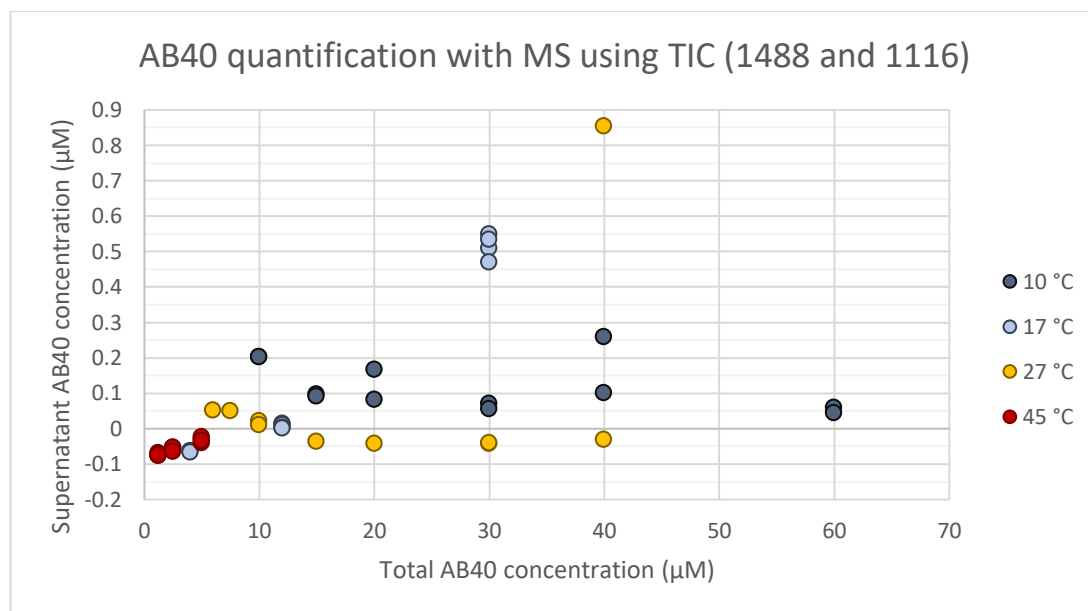


Figure 11: Results from quantification with MS using TIC ($m/z = 1488$ and $m/z = 1116$)

3.4 NMR: Relative quantification

Based on literature values for the chemical shifts of protons in amino acids[15] and comparisons with the HSQC spectra, methyl groups and aromatic groups were identified (Figure 18 for methyls and Figure A 18 for aromatics).

The integral of the methyl peaks (Figure 12) in the first spectrum of each NMR experiment was set to 1. The relative integrals of the methyl peaks in the subsequent spectra were plotted against the incubation time for each NMR experiment (Figure 13 - Figure 16). Due to time constraints, this process was not repeated for the aromatic peaks.

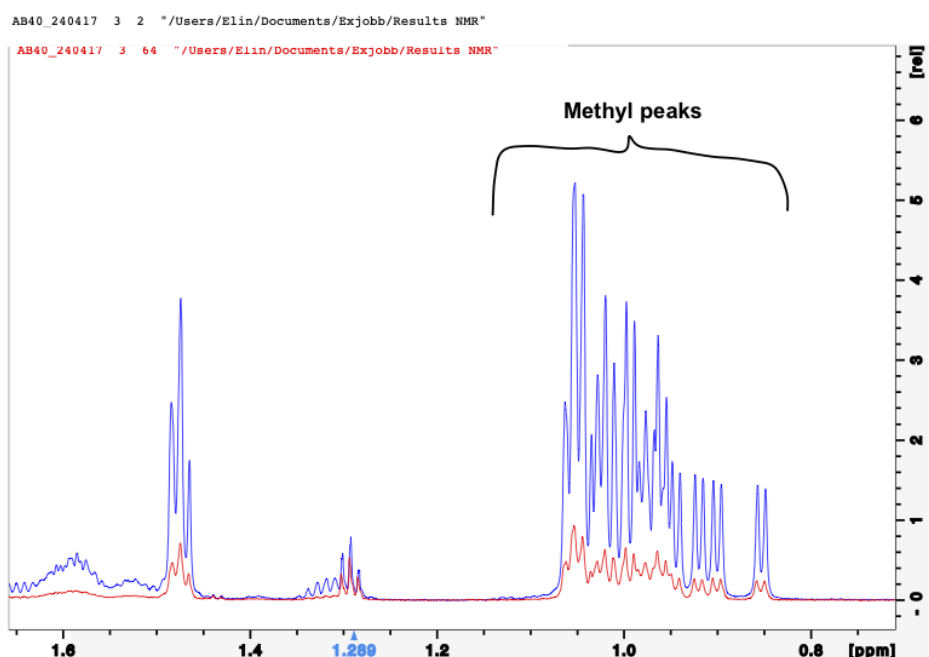


Figure 12: ^1H NMR spectra for $t = 0\text{ h}$ (blue) and $t = 31.5\text{ h}$ (red) at $45\text{ }^\circ\text{C}$.

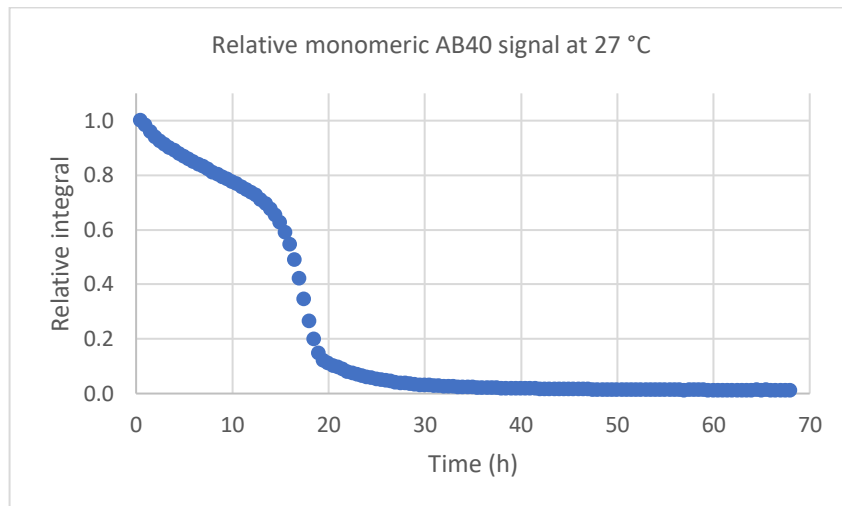


Figure 13: Change in monomer $\text{A}\beta_{40}$ signal in NMR experiment at $27\text{ }^\circ\text{C}$.

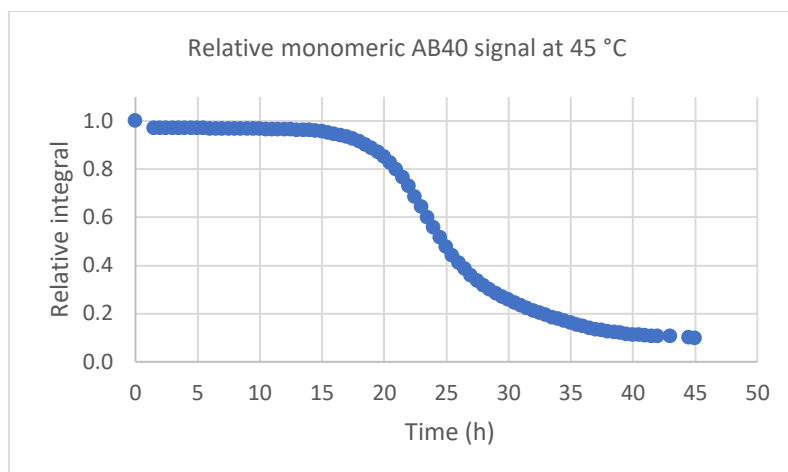


Figure 14: Change in monomer A β 40 signal in NMR experiment at 45 °C.

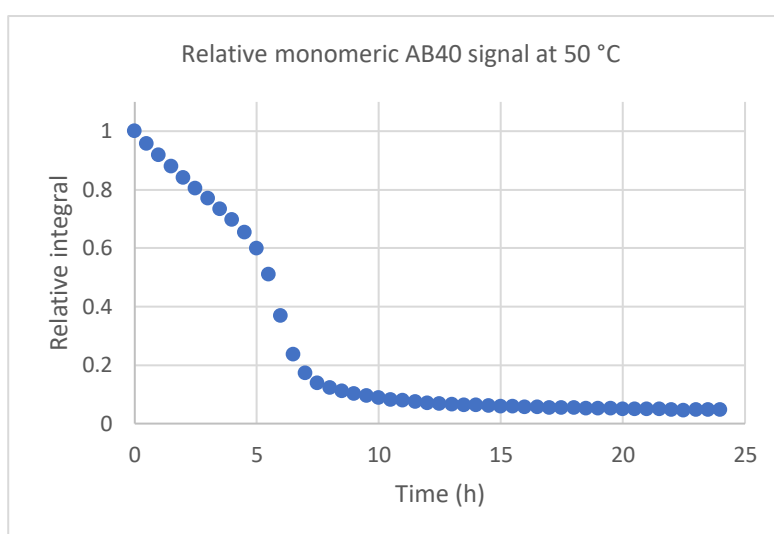


Figure 15: Change in monomer A β 40 signal in NMR experiment at 50 °C.

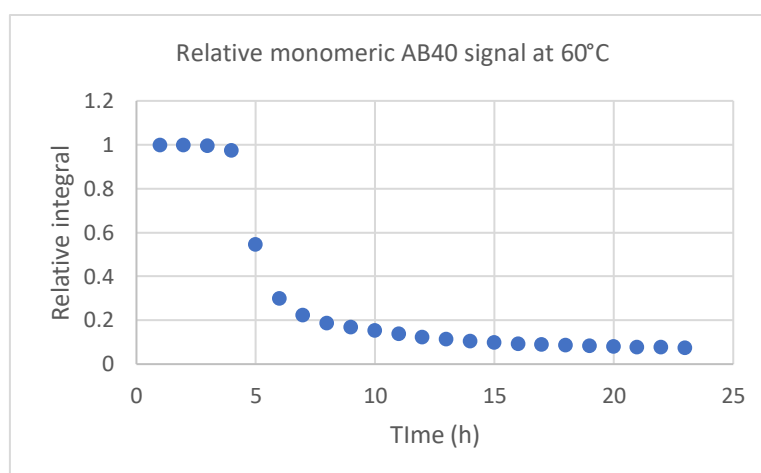


Figure 16: Change in monomer A β 40 signal in NMR experiment at 60 °C

The initial A β 40 concentration was calculated from the absorbance measurements in the SEC, allowing for calculation of the A β 40 concentration at the end of each NMR experiment, where all samples, except the 45 °C one (Figure 14), appeared to have reached a plateau (Table 2 and Figure 17). The sample at 45 °C is therefore excluded from Figure 17 and Figure 21.

Table 2: Quantification of A β 40 from relative NMR results

Temperature (°C)	Initial concentration according to SEC (μM)	Relative integral at plateau	Estimated concentration at plateau (μM)
27 °C	106	0.01	1.2
45 °C	101	0.10	9.9
50 °C	132	0.05	6.2
60 °C	101	0.07	7.1

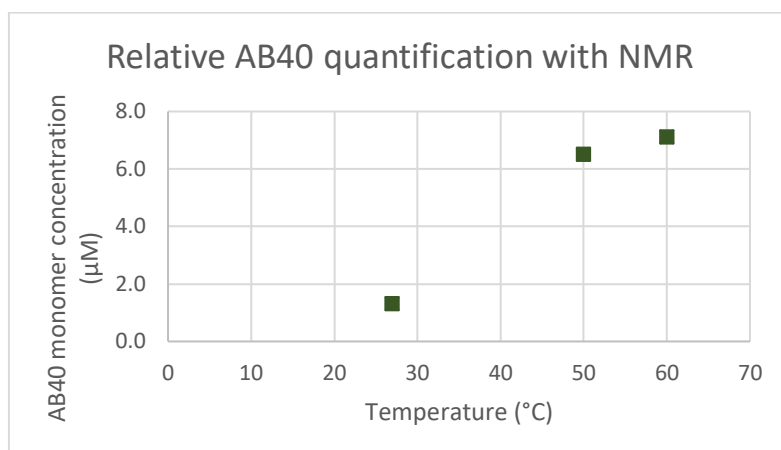


Figure 17: Plateau concentrations of A β 40 monomer in NMR experiments at different temperatures. Calculated using relative integral and starting concentration from SEC.

3.5 NMR: Absolute quantification

Absolute quantification proved more challenging and time consuming than expected. Due to time constraints, the data analysis necessary for quantification with an external standard was therefore only performed for the experiments at 27 °C, 45 °C and 50 °C.

The chemical shifts in the HSQC spectrum (taken at 45 °C), were compared to the average shifts of the protons and carbons in literature [15]. The methyl peaks were thus roughly assigned, and it was concluded that the peaks came from approximately 60 protons (Figure 18). The comparison between the integral of the methyl peaks and that of the aromatic peaks was made possible by the aromatic peaks being more well separated, with fewer protons giving rise to each individual peak. For the 45 °C experiment, this comparison supported the estimate of 60 protons, but for the 27 °C and 50 °C experiments the comparison to the aromatic peaks indicated that the methyl peaks respectively came from closer to 61 and 64 protons. The 50°C peaks are shown in

Figure 19 and Figure 20. For the purposes of this report, the number of protons used for all calculations was 60 (Table 3).



Figure 18: Methyls in HSQC at 45°C with calculation of protons in integrated peaks.

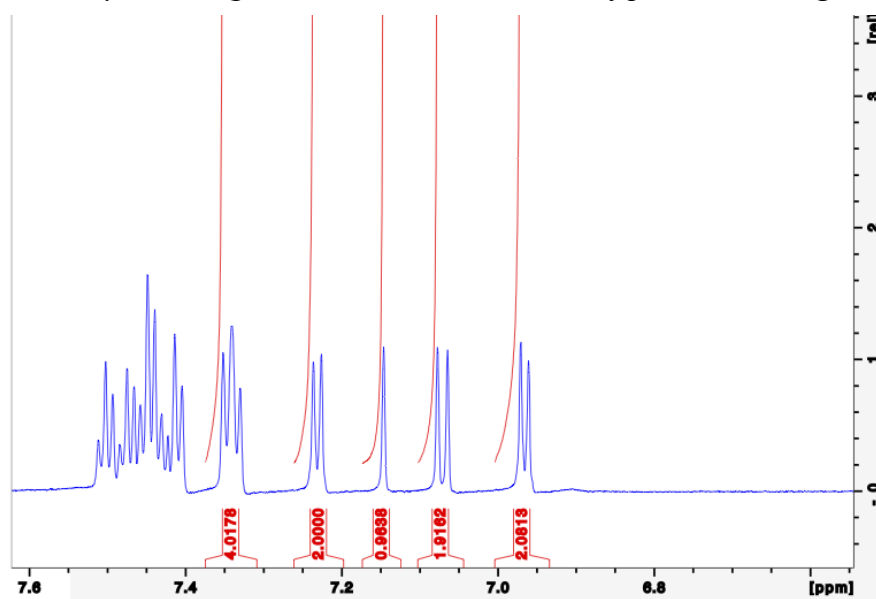


Figure 19: Aromatic peaks used to estimate number of protons (50°C)

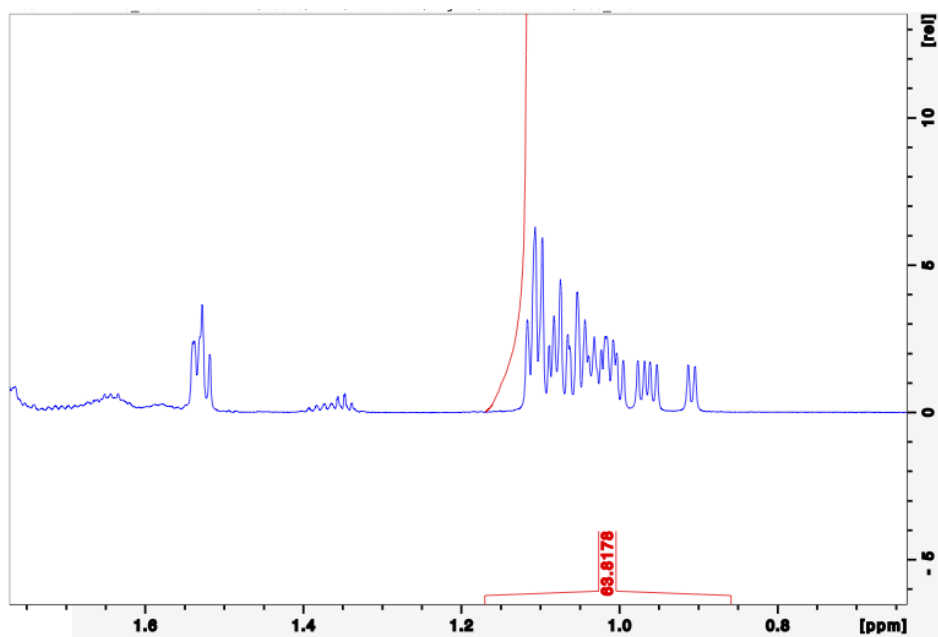


Figure 20: Methyl peaks with number of protons from comparison to aromatic peaks (50°C)

Table 3: Quantification with ^1H NMR using KHP as an external standard

Temperature (°C)	Starting concentration from SEC (μM)	Starting concentration, 60 protons (μM)	End point concentration, 60 protons (μM)
27	106	125	1.5
45	101	145	14
50	132	165	8

3.6 Solubility

To study the effect temperature has on the solubility of A β 40, the A β 40 concentrations was plotted against the incubation temperature (Figure 21). For the OPA and HPLC-MS results, the average supernatant concentration of samples with a total A β 40 concentration $\leq 20 \mu\text{M}$ was used. For the NMR results, the plateau concentration of equilibrated samples was used. All methods were used once for the determination of the solubility at 27 °C, these results are compiled in Table 4.

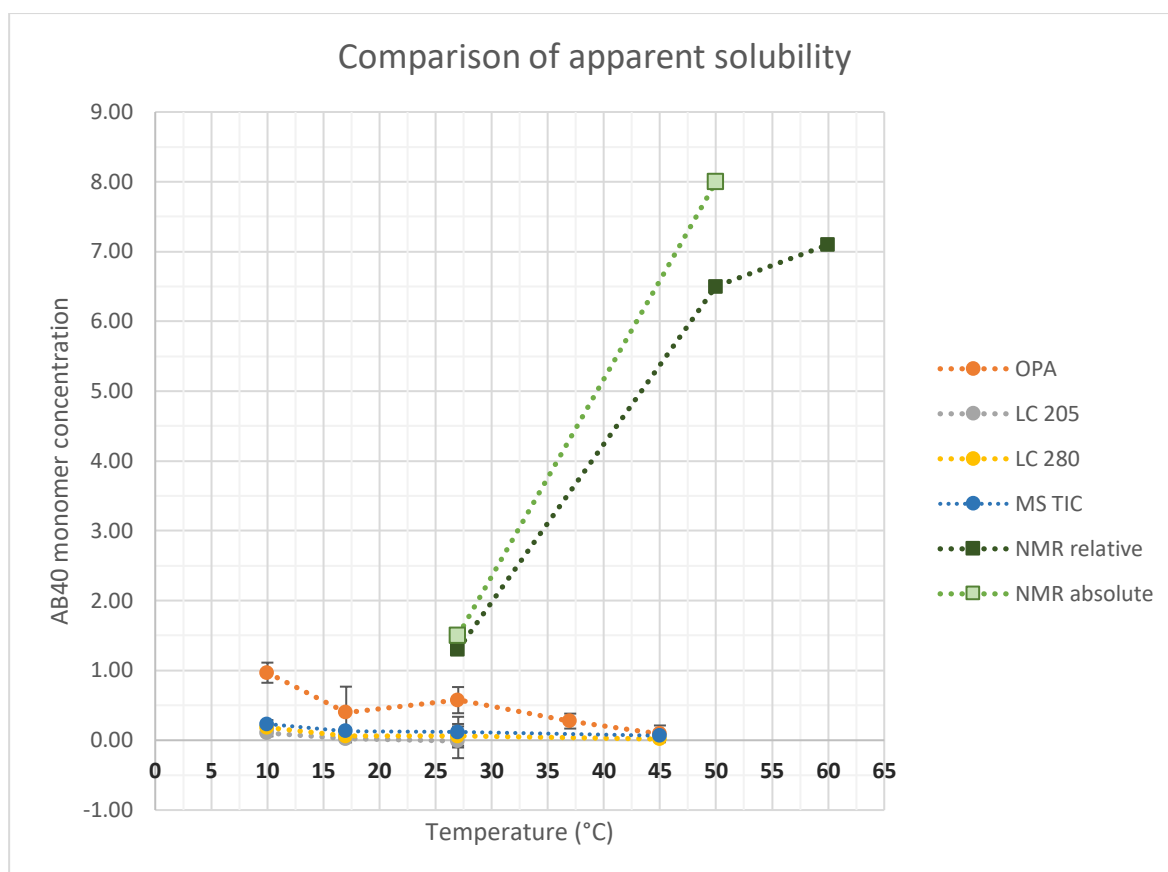


Figure 21: $A\beta 40$ concentration of equilibrium samples. Averages for OPA and HPLC-MS calculated from samples with total $A\beta 40 \leq 20 \mu\text{M}$.

Table 4: Comparison of 27 °C solubility

Method	Reported apparent solubility at 27°C (μM)
OPA	0.58
LC (280 nm)	0.06
MS (TIC 1448 and 1116)	0.01
NMR (relative)	1.2
NMR (absolute)	1.5

4 Discussion

4.1 Reaching equilibrium and monitoring the reaction

All measurements of solubility are dependent on the system reaching equilibrium. In this project, the way to equilibrium was monitored by measuring either the fibril formation (with ThT) or monomer depletion (in NMR) and allowing these to reach a plateau. Compared to tracking the monomer concentration, the tracking of fibril formation with ThT is a less direct method to monitor equilibrium. Apart from this monitoring, the aim was also for samples with different total $A\beta 40$ concentrations to reach the same monomer concentration, which would indicate they are at equilibrium. However, the upwards trend in supernatant monomer concentration with increased total concentration (in the OPA measurements, Figure A 7 -

Figure A 11) and, in some cases, a lack of replicates of the same total concentration (e.g. Figure A 9), made it difficult to state if the system was truly at equilibrium. An alternative method to confirm equilibrium in the OPA and HPLC-MS samples would be to measure the monomer concentration after different incubation times and compare the results.

The clear tracking of aggregation with ThT is also useful in solubility studies. The lowest initial concentration which allows for aggregation gives an indication of the highest possible value for the solubility; if the samples aggregate at a given initial concentration, then the solubility must be lower. However, by binding to the fibrils, the ThT must increase the stability of the fibrils, which would be expected to lower the solubility of the monomers. The size of this effect on the solubility should be further investigated. If it causes a noticeable change in solubility, then ThT should not be used in samples where one wants to determine the solubility of A β 40 alone.

Based on the change in monomer concentration, the NMR experiments showed that most samples did appear to reach equilibrium, except for the 45 °C experiment (Figure 14). Here the experiment was aborted too early; in future experiments it will be important to plan enough time for the samples to equilibrate fully when scheduling an NMR experiment. Figure 8 furthermore shows that the samples with an A β 40 concentration of 1.25 μ M did aggregate, suggesting an upper limit for the solubility. The 45 °C NMR experiment only reached a monomer concentration of 9.9 μ M according to the relative quantification (Table 2) and 14 μ M according to the absolute quantification (Table 3). Based on the ThT results, assuming the stabilising effect of ThT is negligible, neither of these concentrations can then be the solubility and the system cannot be at equilibrium.

Solubility can also be studied using a sample with fibrils and monomers at equilibrium and then altering the system so the monomer concentration is below solubility. This can, for example, be achieved through dilution of equilibrated samples or by increasing the solubility through changes in temperature, pH, or salt concentration. In this project, however, the focus was on method development and therefore only the reaction of supersaturated monomers to fibrils was studied, rather than investigating both directions.

4.2 Aggregation conditions

Different aggregation conditions were explored, including different ways to control the temperature during the aggregation. The use of a heat block was evaluated for 30 °C and 45 °C. It, however, became apparent that this was not a suitable heating method; the solution evaporated and visibly accumulated as condensation in the lid, since the lid was exposed to the room temperature air. When the samples then were centrifuged, this liquid diluted the remaining solution of fibrils and monomers. Instead, for the OPA and HPLC-MS experiments, incubation in the plate reader was preferred.

Using the plate reader during the aggregation process was beneficial due to the impact of mechanical action and sheer forces on aggregation rates, especially at temperatures below 37°C. For contrast, in the NMR experiments, the samples were kept entirely quiescent during the aggregation, while in the plate readers, the plate was moved for each measurement. NMR experiments at 10 °C and 17°C were attempted; the 10 °C sample was incubated only for 24 hours, with no decrease in monomer concentration, and the 17 °C was incubated 64 hours, but showed only a small decrease in monomer concentration (Figure A 19 and Figure A 20).

One drawback of using the plate reader to regulate temperature is the limited sample volume. To have sufficient volume of supernatant to safely take two 50 μ L from, samples from the plates had to be pooled. However, this may not be necessary if future control experiments of the separation show that it is possible to take that much supernatant from a smaller volume.

Alternatively, it could be avoided by opting to only perform either OPA or HPLC-MS and not both.

Towards the end of the project, it was noted that larger shake incubators (typically used for cell cultures) could be used to control the temperature of the aggregation process, with or without shaking. By incubating samples in tubes in these incubators, the process could be streamlined and allow for a higher throughput, with more replicates (including ones taken at different time points) and testing of multiple temperatures simultaneously.

4.3 Separation

As outlined in Method development, attempts were made to validate if the centrifugation provided sufficient separation of monomers and fibrils to quantify the monomer with OPA fluorescence and HPLC-MS. Figure 2 shows that approximately 30 nM of fibrils stay in the supernatant, which can be seen as negligible compared to the expected concentrations of monomer. However, this control experiment should be repeated. In part because the standard curve (Figure A 2) has a quite poor fit, but also because the samples used in this experiment were incubated in a heat block (see discussion above). When preparing the standard fibril samples, very careful pipetting is required to ensure correct concentrations. For more valid and reliable results, the control experiment should also be repeated for different temperature – in case the fibrils sediment differently depending on temperature. Furthermore, it should be repeated with samples with higher total A β 40 concentrations, since the trend appears to be that the fibril concentration increases at higher total concentration.

How large impact any remaining fibrils have on the quantification depends on how sensitive the OPA measurements are to fibrils. Figure 3 shows that the slope of the standard curve relating fibril concentration to OPA fluorescence was only 3900, which is almost a factor 6 lower than the slope 22800 shown by the curve relating monomer concentration to OPA fluorescence (Figure A 6). These measurements were taken at the same time, with the same incubation time with OPA and the same gain and focal height in the plate reader.

4.4 Evaluation of OPA results

In Figure 9, a trend with increasing A β 40 detected with OPA in the supernatant as the total A β 40 concentration increases. This trend is not seen in the HPLC-MS measurements (Figure 10 and Figure 11). Furthermore, OPA reports higher concentrations overall compared to HPLC-MS. Reactions with OPA is a less selective method and the increased signal could have multiple explanations.

The trend of increasing supernatant concentration with increased total concentration could be explained if the samples have not reached equilibrium but have instead been kinetically trapped at a local energy minimum with a higher concentration of monomers in solution. However, then the trend would be expected to show up in the HPLC-MS results as well.

An alternative explanation to the increasing OPA signal is if the samples are contaminated with something that is not intact monomeric A β 40. This could be fibrils from incomplete separation, proteolytic degradation products of A β 40 or external contaminants. For the contaminants to give increased signal, they would have to either have intrinsic fluorescence or react with OPA (i.e., contain primary amines). These sources would all have a higher impact in samples with higher total concentration. However, if the external contaminants entered the sample after dilution to the desired total concentration, e.g., in the OPA plate after separation, the effect of contamination would be independent of the total concentration.

4.5 Evaluation of HPLC-MS results

Compared to the other methods used in this project, HPLC-MS consistently reported the lowest apparent solubilities. One possible explanation for the low concentration results could

be that not all monomer elutes at the same time and is therefore not included when determining the peak area to compare to the calibration curve. For example, the HPLC-MS results for the 27°C experiment show a peak in both 205 nm and 280 nm absorbance can be seen at retention time 4.1 min, with a clear peak at $m/z = 1307$ (Figure A 17). It can be argued that this is modified (e.g., oxidated) A β 40, which elutes at a different time than the normal peptide. If this modification occurred after the equilibrium was reached, that fraction of the A β 40 population should still be counted towards the solubility. This could account for part of the “missing” A β 40 in the HPLC samples.

A similar effect might occur if the protein was degraded into shorter peptides after equilibrium, but no new equilibrium was reached with these new species. These would then not necessarily elute together with the intact monomer. Future experiments should include extra efforts to avoid contaminating the sample with proteases from the environment. Control experiments using synthetic A β 40 could also be performed, to evaluate if any bacterial proteins manage to remain in the sample. However, it has been suggested that A β 40 is auto-hydrolytic and that its oligomers and protofibrils are catalytic in this cleavage [16]. This could also explain why OPA reports increasing supernatant concentrations with increased total concentration. In future experiment, samples could also be sent away to be analysed with more detailed MS, such as MALDI-TOF, to further investigate the presence of degradation products or other contaminants.

It is possible that the HPLC-MS measurements could be improved by increasing the injection volume, if one suspects that the sensitivity may be an issue. However, if the solubilities are expected to be much higher than those shown by HPLC-MS, it is more likely that the concentration in the supernatant do not actually reflect the equilibrium concentration, rather than the HPLC-MS failing to accurately detect and quantify the monomers. Additionally, redoing the calibration curves with a narrower range of standard concentrations could improve the accuracy. The concentration could also be calculated directly by using the extinction coefficient of A β 40 and the results from the different calculation methods could be compared.

In the HPLC-MS system, the samples are kept at 10 °C during the experiment. If there are remaining fibrils in the sample after separation, these may dissociate into monomers if the solubility is higher at 10 °C than at the temperature investigated. However, this is expected to cause an increased signal, which the data in this project does not show. It is possible that the reaction rate for the dissociation is low enough for no noticeable change to occur while the sample is kept in the HPLC-MS system.

4.6 Evaluation of NMR results

One limiting aspect of using NMR to determine solubility is the sensitivity. Using NMR at temperatures close to 37 °C, where the solubility of A β 40 is $< 1 \mu\text{M}$, would make it difficult to get a good signal-to-noise ratio at the equilibrium. Worth mentioning is therefore that isotope labelling would increase the sensitivity, but a label-free quantification method is highly convenient.

The relative quantification with NMR relies on the assumption that the starting monomer concentration is known. The accuracy is therefore limited not only by the accuracy of the quantification in the SEC, but also by the stability of the protein prior to the start of the experiment. If the sample has already begun aggregating, then the monomer concentration will no longer be the same as it was when the sample was collected from the SEC. This issue was, however, minimised by keeping the sample on ice until the experiment started. In Figure 13 and Figure 15, the concentration appears to decrease immediately at the start of the 27°C and 50°C experiments, albeit at a lower rate compared to later in the incubation. This

contrasts with how flat the curve is at the start of experiments at 45°C and 60°C (Figure 14 and Figure 16).

One source of error in the absolute quantification with NMR is how correctly estimated the number of protons in the peaks was. The assignment of the peaks could be improved if more 2D spectra were collected. However, even when the highest possible number of methyl protons present in the peptide (75 protons) was used, the absolute quantification still overshoot the concentration. The high starting concentration suggested by the absolute quantification does not indicate that the aggregation has started before the experiment (as discussed above), but the large error in the absolute quantification makes it difficult to confidently state this.

The error from deviations in the KHP concentration could also be improved by more careful weighing and pipetting when preparing the standard. Beyond trying to minimise the errors, a more rigorous quantification of the errors and error propagation would be required when using this method. This includes validating the accuracy and precision of the external calibration through measurements on standard samples with known concentrations and more well studied spectra. One could also use the integral of the aromatic peaks for the concentration calculations and compare the results. If the absolute quantification was made more reliable, it might be possible to incubate the samples with shaking, outside of the magnet, and only collect spectra and quantify the monomer at the end of the incubation.

In Table 3, it is noticeable that the absolute quantification seems to overestimate the starting concentration compared to the SEC results. However, it should be noted that the SEC results could be said to have a 10-20% experimental error. This is a limiting factor in the accuracy of the relative quantification and makes it difficult to evaluate the accuracy of the absolute quantification.

In this project, only one sample and temperature were studied per NMR experiment and no repeated experiments with the same temperatures were performed. This limits the reliability of the NMR results. However, the results in this project do support the previous findings that NMR is useful in monitoring monomer populations. Future repeats of the experiments performed in this project, with improvements and adjustments as needed, could potentially generate a more reliable value for the solubility of A β 40 at different temperatures.

4.7 Solubility

As seen in Figure 21 and Table 4, the different methods reported different apparent solubility at 27 °C. Out of these, the values given by NMR and OPA are the most in line with what would be expected at 27°C [5], compared to the very low values reported by HPLC-MS.

In addition to the possible explanations for higher values reported by NMR, the lower values given by OPA and HPLC-MS could be in part explained by monomers being lost somewhere in the process of sample preparation and separation for the OPA and HPLC-MS experiments – possibly through adsorption to surfaces. In the NMR experiments, the SEC eluate was measured on directly after only one transfer to the NMR tube. For comparison, the OPA and HPLC-MS samples were diluted, transferred to plates for the ThT measurements, transferred to tubes for the centrifugation and then transferred to plates for OPA or HPLC-MS measurements. This process includes many opportunities for the peptide to adsorb to surfaces in the wells or pipette tips. The increased amount of sample handling in the OPA and HPLC-MS experiments also increases the risk of contaminations, compared to the NMR experiment.

The NMR results do appear to indicate a higher solubility of A β 40 at a higher temperature. Similar to how ordered, globular, proteins may be destabilised or denatured at higher temperatures, the fibrils may become less stable and thus reach an equilibrium with a higher concentration of monomers.

4.8 Other effects of temperature and initial concentration

The OPA fluorescence measurements revealed that the incubation at different temperatures influenced the properties of the buffer, causing a change in the detected fluorescent compared to the room temperature buffer (Figure A 3). All samples were therefore corrected with the incubated buffer (negative control) instead of the blank used to construct the standard curve. This correction also minimises the risk of interference of batch differences between the buffer used in the samples and the one used in the standard curve.

Total A β 40 concentration has been shown to increase the aggregation rate[4]. However, in Figure 4-Figure 8 this effect appears to be saturated when concentrations are increased above 12-15 μ M. Proper comparison of the kinetics at the different temperatures and with different initial concentration would require more data fitting and analysis, which lie outside of the scope of this project. Interesting discussion about the reaction rates and shapes of the kinetic curves could be had in future endeavours but will not be included in this report.

4.9 Conclusions

The aim of this project was to explore different methods to determine the solubility of A β 40 at different temperatures. This was achieved through the quantification of the A β 40 monomer using OPA fluorescence, HPLC-MS and NMR. However, there is still much work left to establish ways to systematically study this relationship between temperature and A β 40 solubility. As of now, the limitations and error sources of the methods used in this project prevent a reliable, precise and reproducible determination of the A β 40 solubility at the different temperatures. Additionally, in future analysis of the effect of temperature on A β 40 solubility, more rigorous and systematic statistical analysis should be performed.

Incubating in a plate reader allows for real-time monitoring of fibril formation with ThT fluorescence, but including this in protocols for solubility measurements introduces more error sources due to the increased sample handling. Furthermore, ThT may influence the solubility and the ThT plateau may not fully correspond to the equilibrium of the reaction.

Shaking appears to be beneficial in trying to reach the equilibrium faster, which is good for improving the throughput.

OPA fluorescence as a method to quantify A β 40 has limited selectivity and is sensitive to degradation of the peptide, incomplete separation, and contaminations of the sample. However, OPA is a cheap reagent and allows for high throughput of solubility measurements.

HPLC-MS has high sensitivity and comparison of LC and MS chromatograms and spectra allows for better identification of what is being quantified. The throughput of these experiments is quite high, due to the use of auto-injection from 96-well plates.

Quantification with NMR holds great promise and has the advantage of real-time monitoring of the monomer population. However, absolute quantification with NMR remains difficult. The use of an external standard should be further investigated, together with improved ways to determine and verify the starting concentration. The NMR experiments are also limited to one sample at a time. Furthermore, not every lab may be equipped with NMR magnets of sufficient sensitivity.

In conclusion, all methods for measuring A β 40 solubility covered in this project should be further investigated as they all have both advantages and disadvantages. Together, they can paint a clearer picture of the driving forces behind the aggregation of A β 40, which can hopefully aid in the understanding and treatment of Alzheimer's disease.

5 Appendix

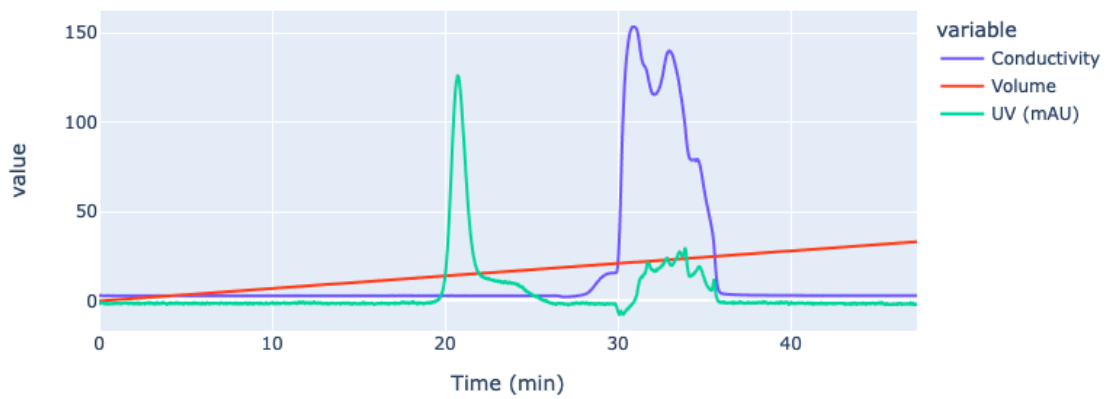


Figure A 1: SEC chromatogram for absorbance at 280 nm. $A\beta_{40}$ peak elutes at approximately 20-21 min.

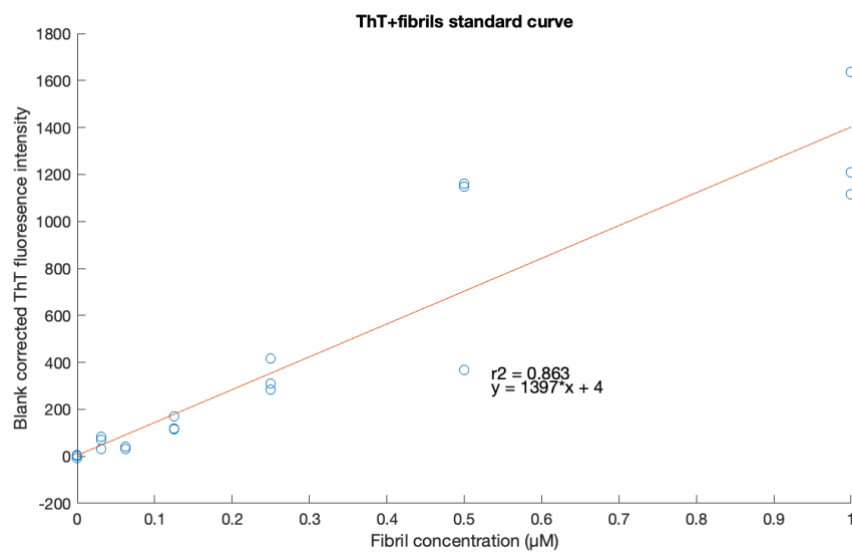


Figure A 2: Standard curve relating fibril concentration to ThT fluorescence intensity

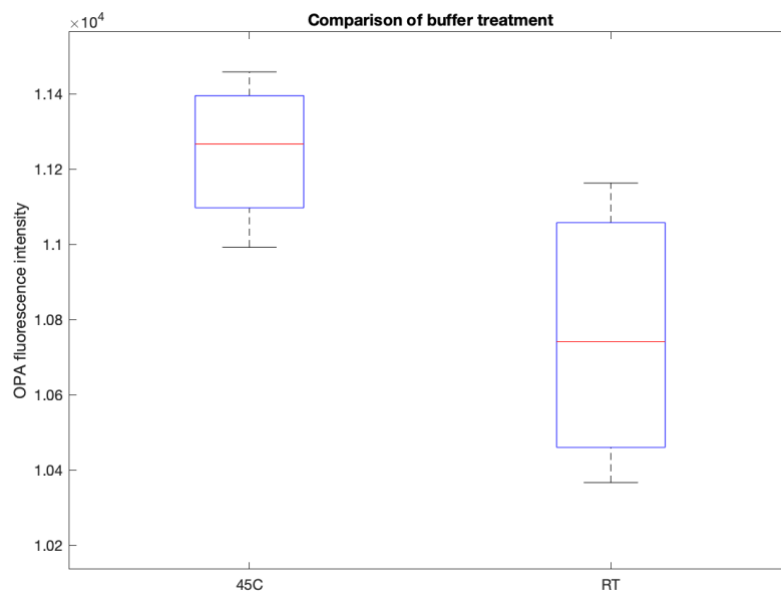


Figure A 3 Difference in OPA fluorescence based on buffer treatment.

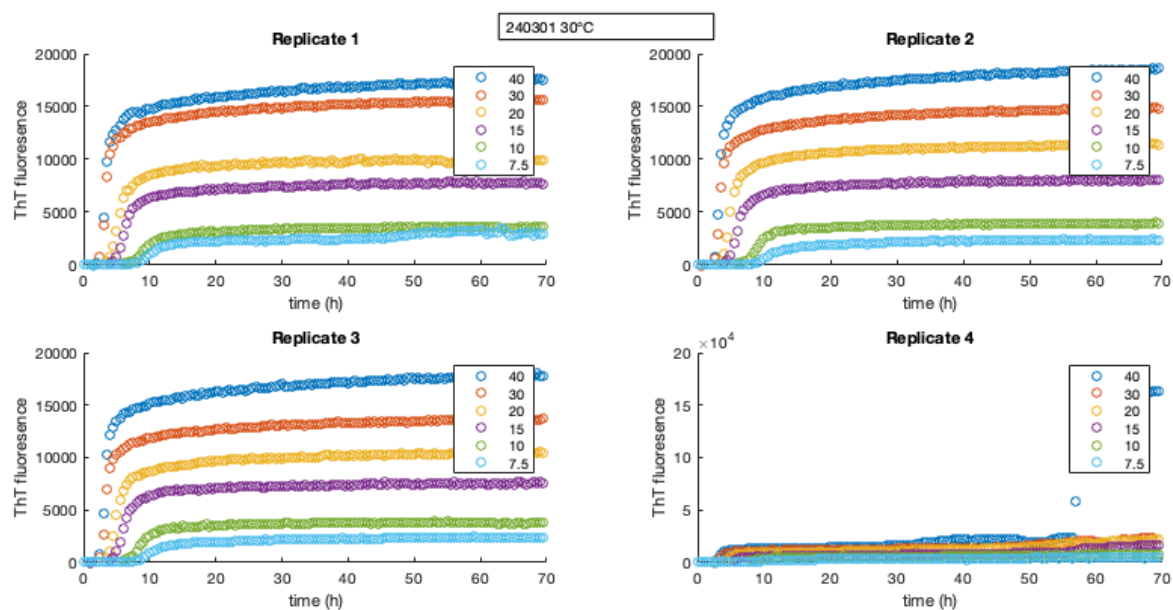


Figure A 4 Example of ThT fluorescence of replicates (30 °C)

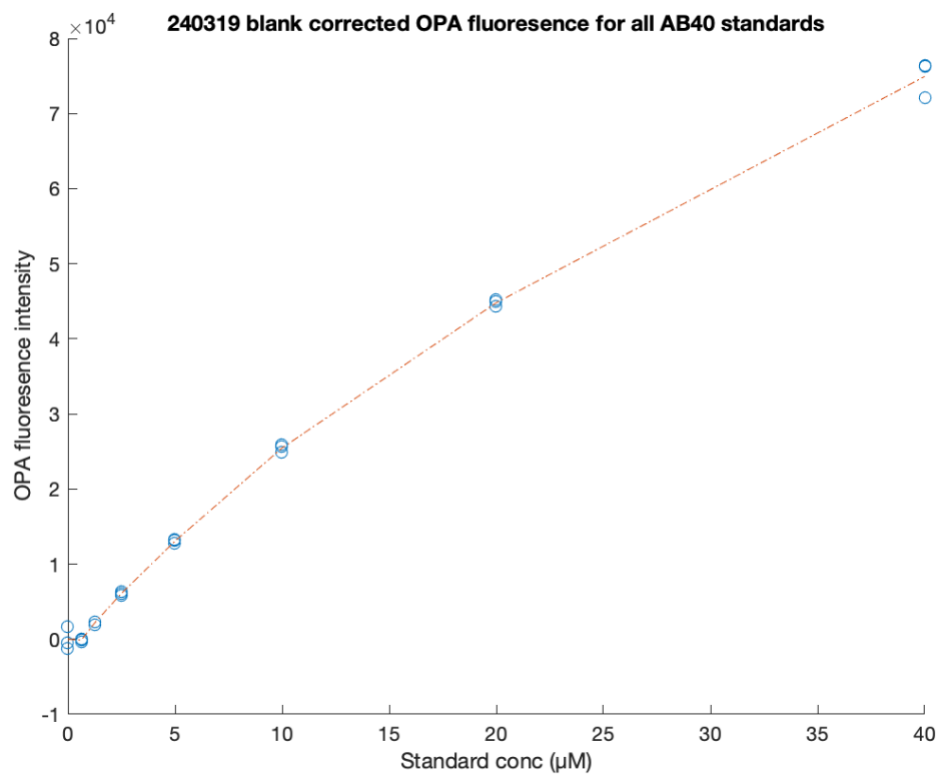


Figure A 5: Standards of A β 40 plotted against measured OPA fluorescence intensity at 10 °C.

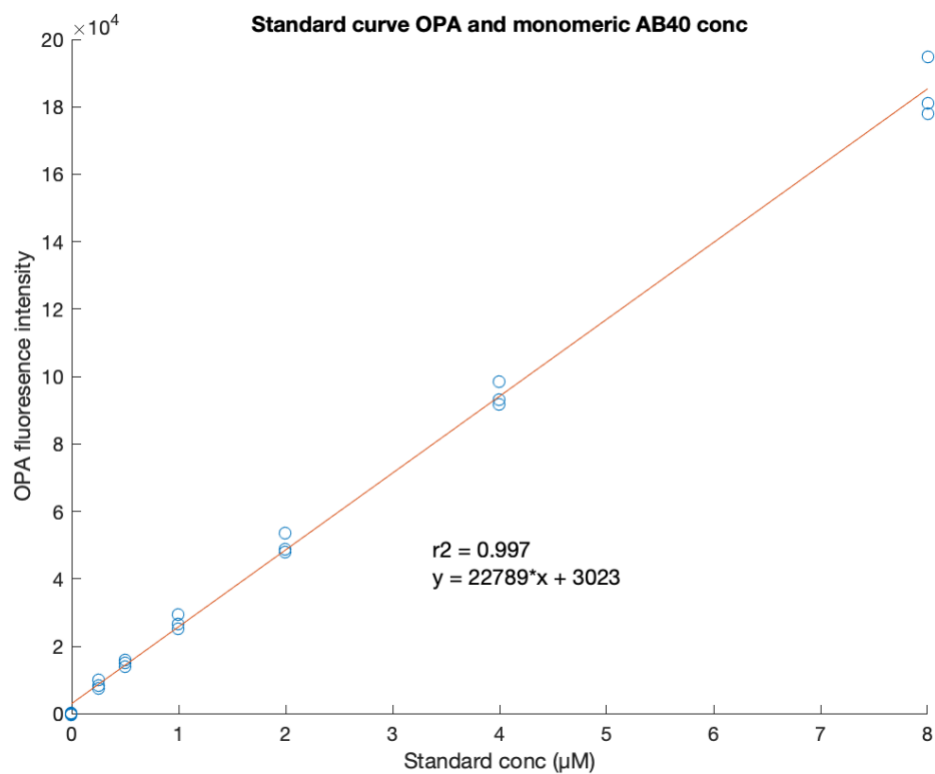


Figure A 6 Example of standard curve for OPA quantification of Aβ40 monomers.

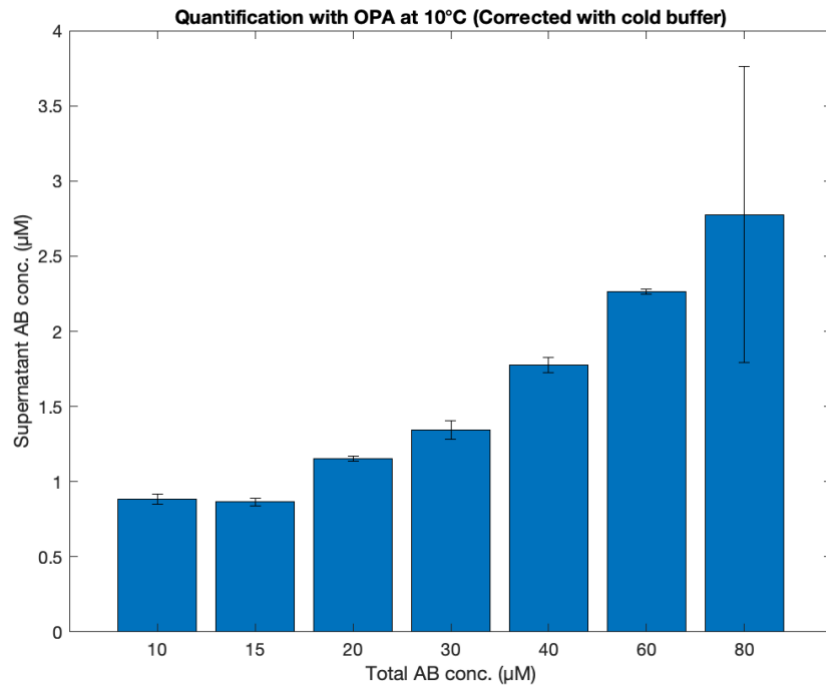


Figure A 7 OPA quantification at 10 °C

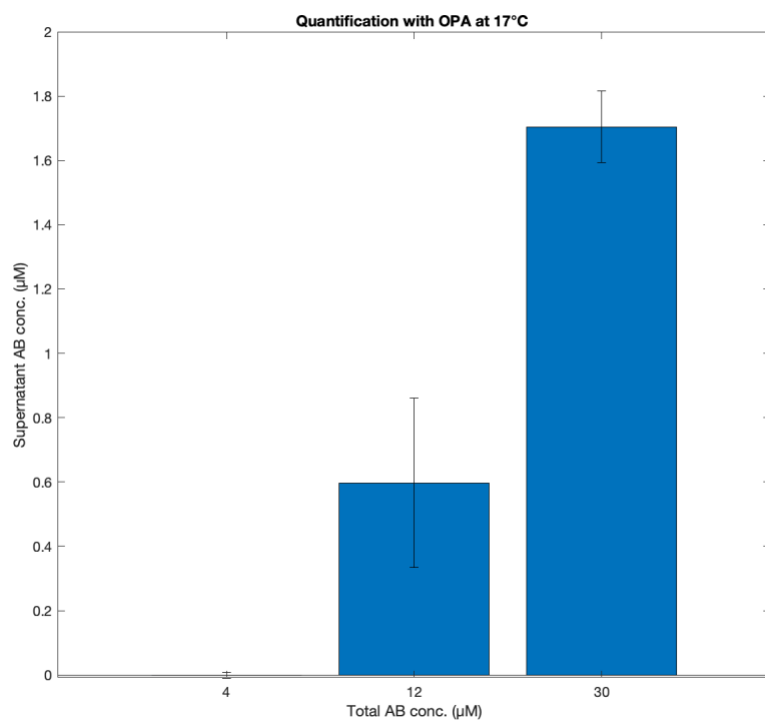
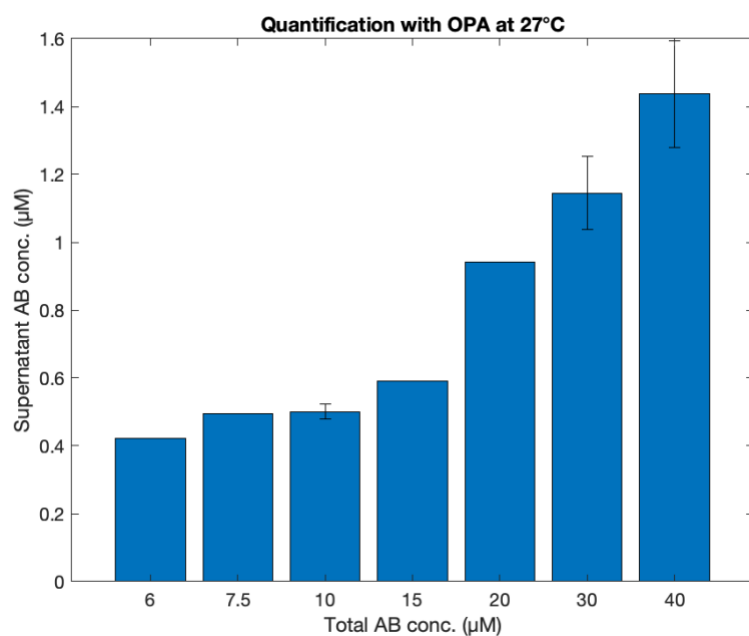


Figure A 8: OPA quantification at 17 °C.



[17]

Figure A 9: OPA quantification at 27°C

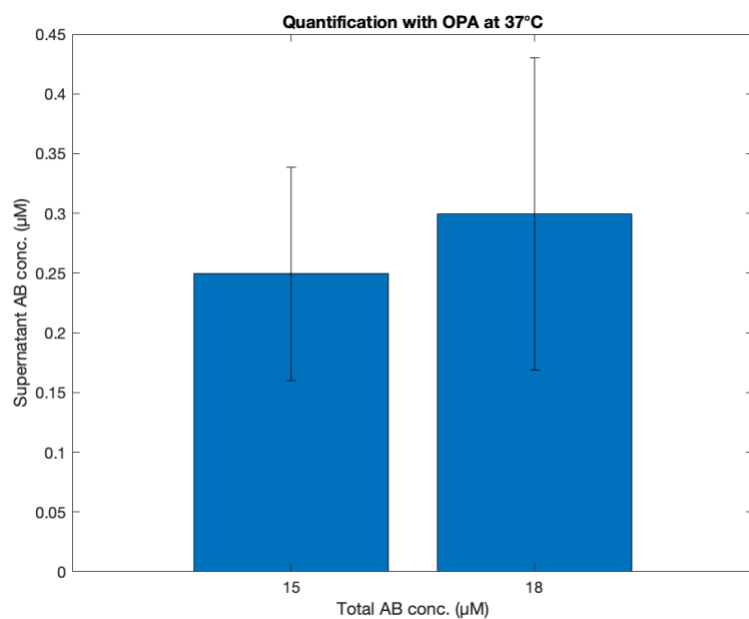


Figure A 10: OPA quantification at 37°C

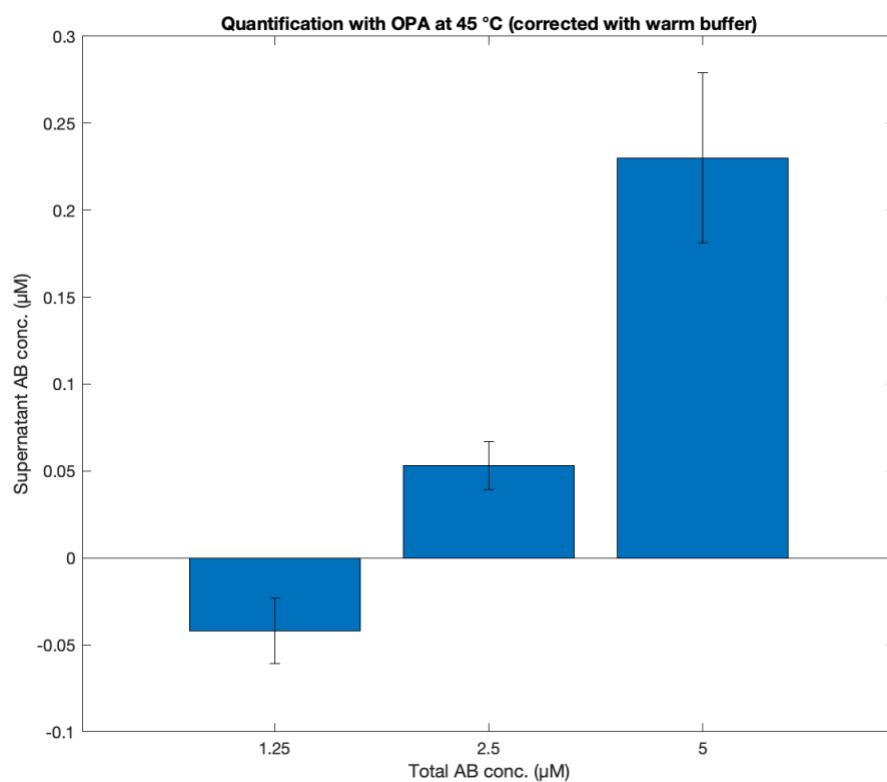


Figure A 11: OPA quantification at 45 °C

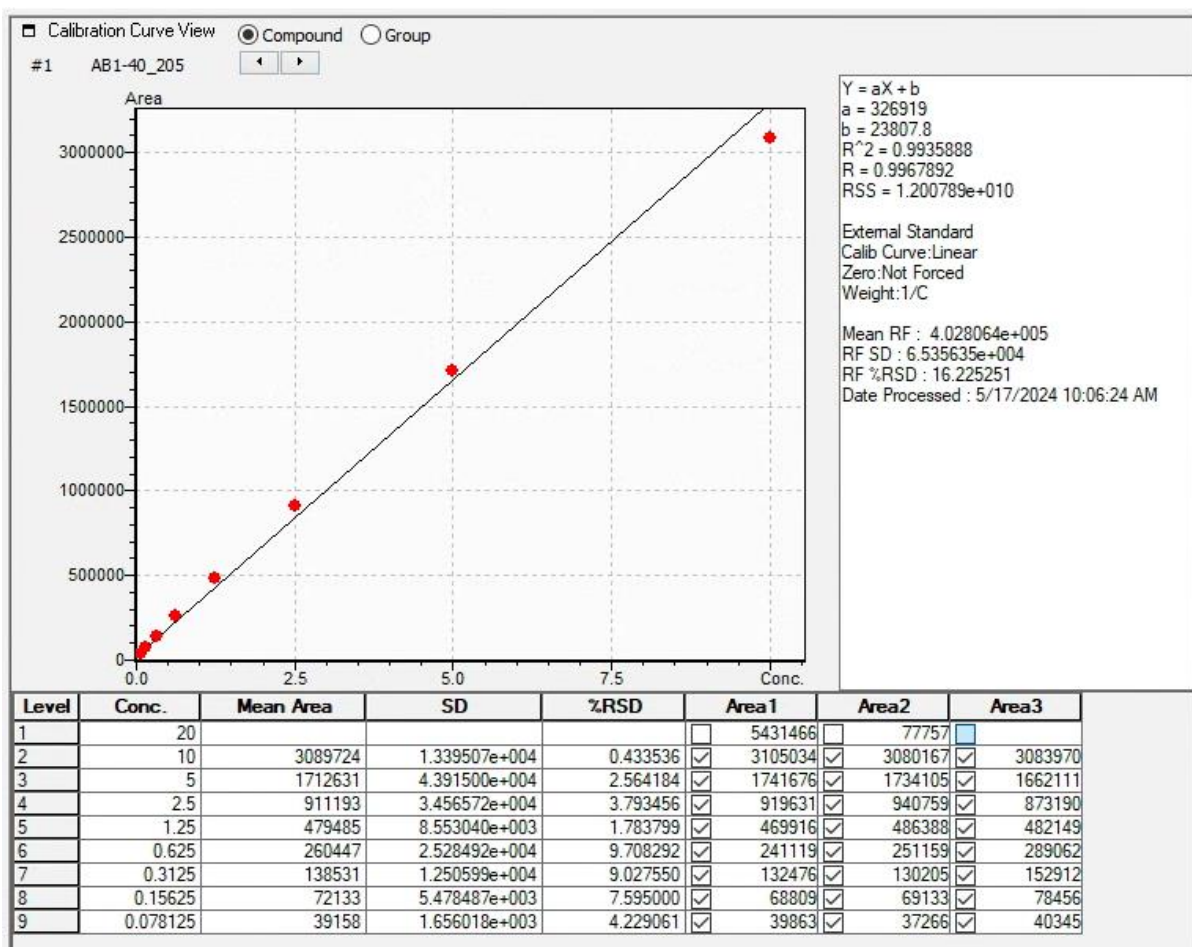


Figure A 12: Standard curve for absorbance at 205 nm

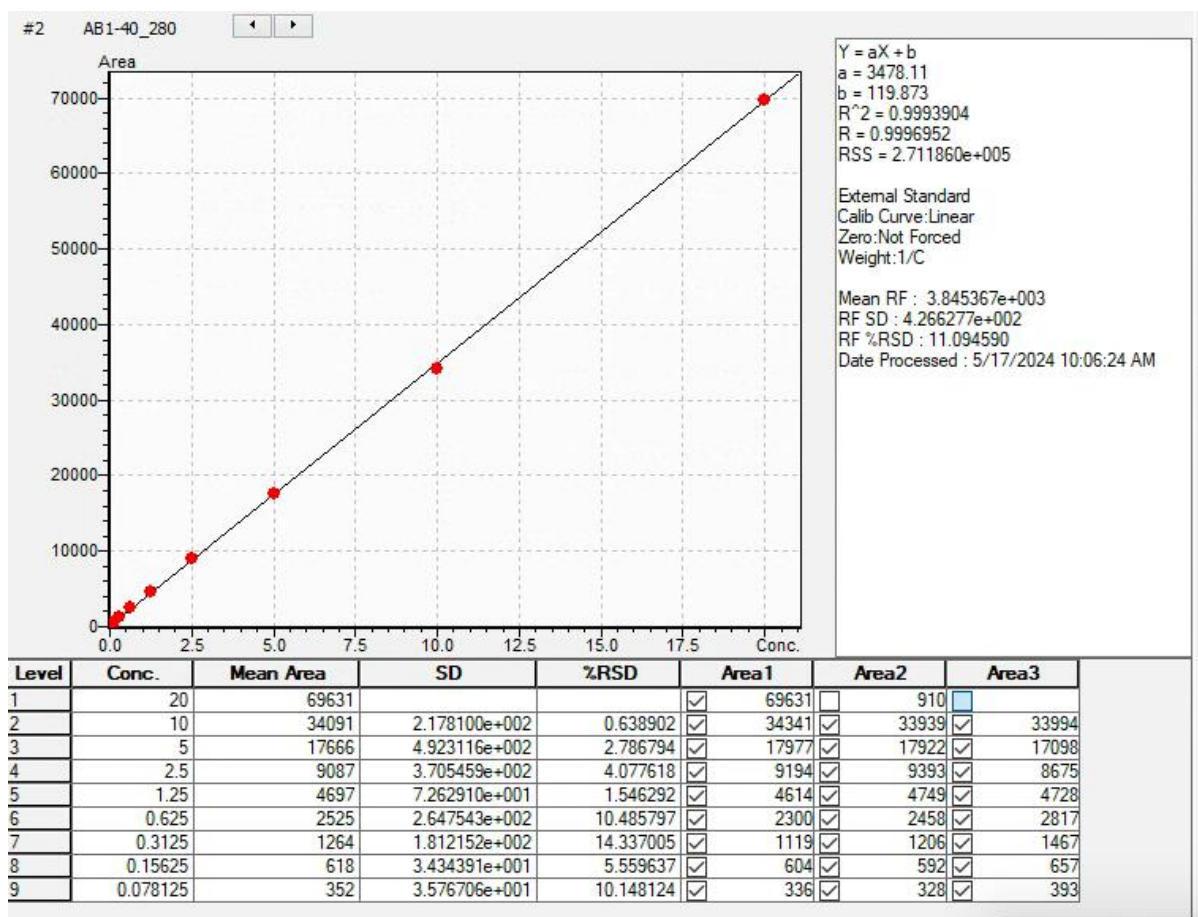


Figure A 13: Standard curve for absorbance at 280 nm

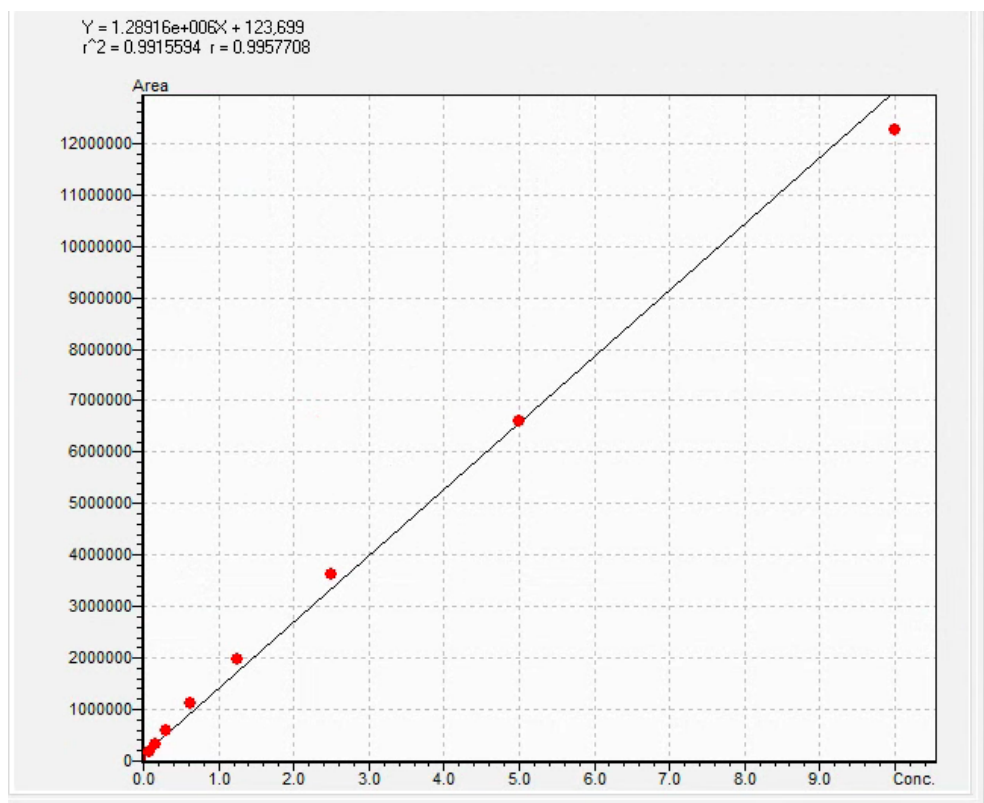


Figure A 14: Standard curve for TIC (1488 and 1116) in MS

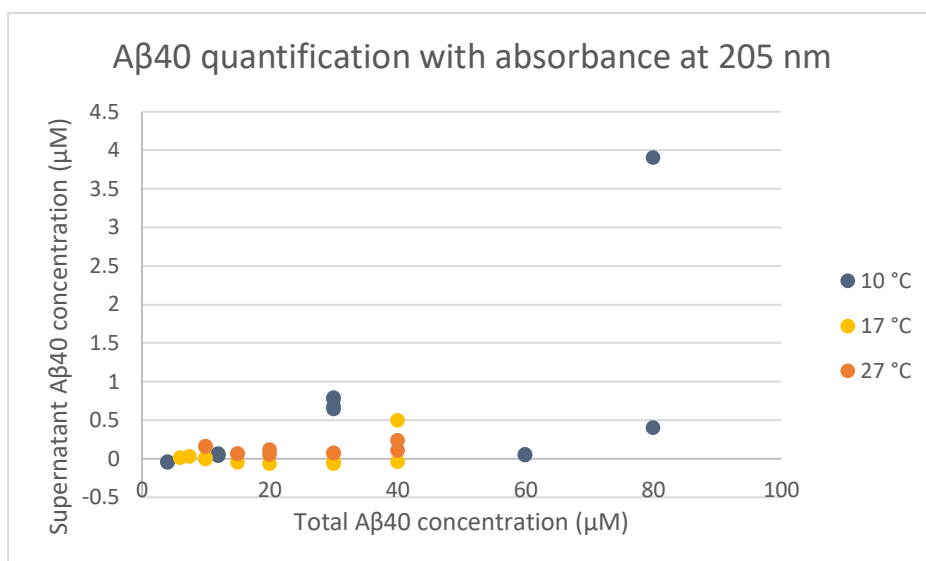


Figure A 15: Results from absorbance at 205 nm.

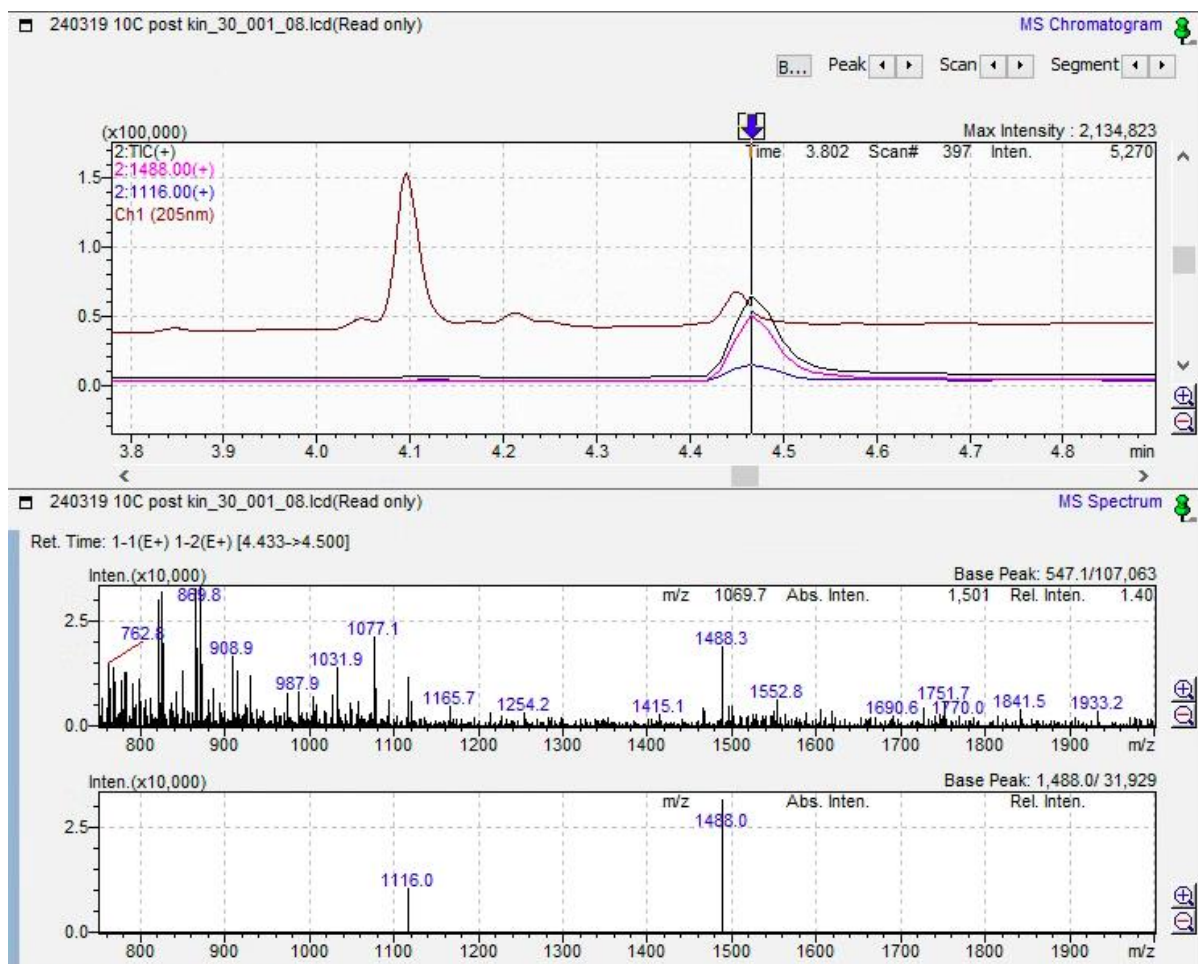


Figure A 16: LC and MS chromatograms (top) with 205nm absorbance multiplied by a factor 100. MS spectra for retention time 4.43-4.50 min (bottom).

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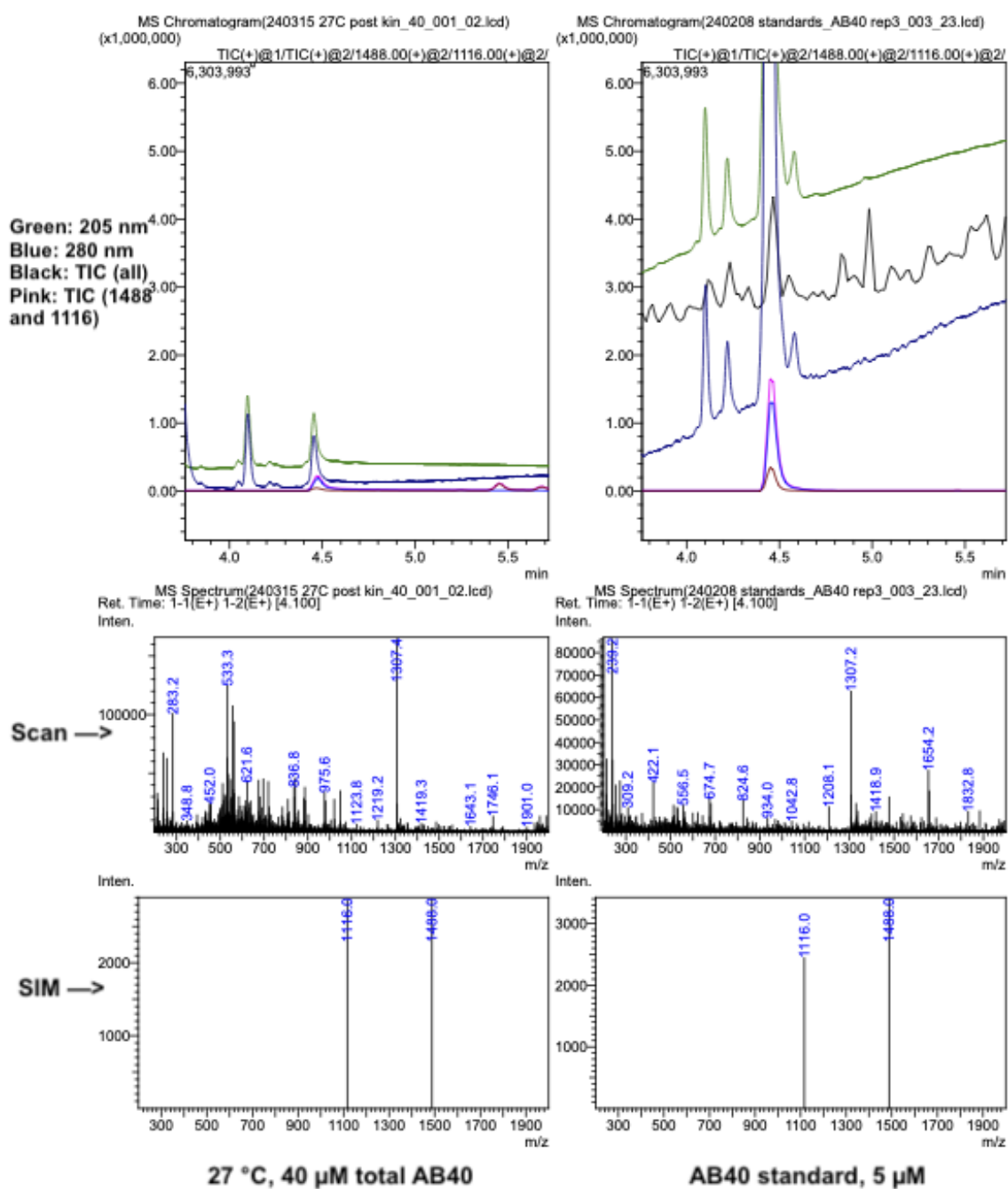


Figure A 17: HPLC-MS results. MS spectra are for retention time 4.1 min

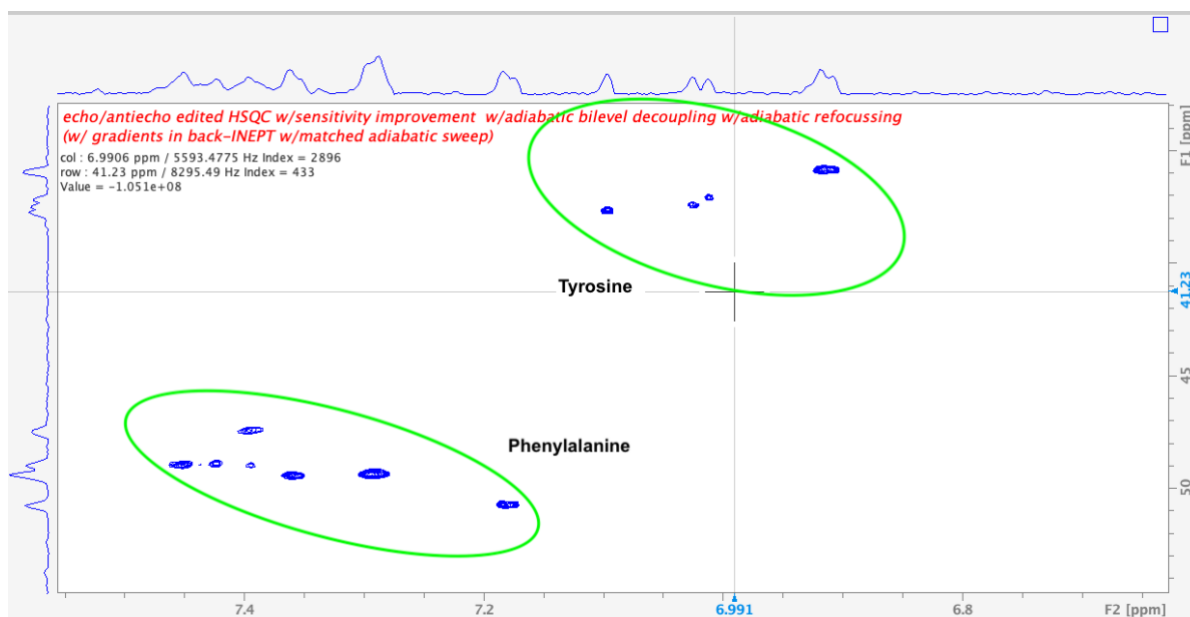


Figure A 18: HSQC peaks for aromatics at 45°C

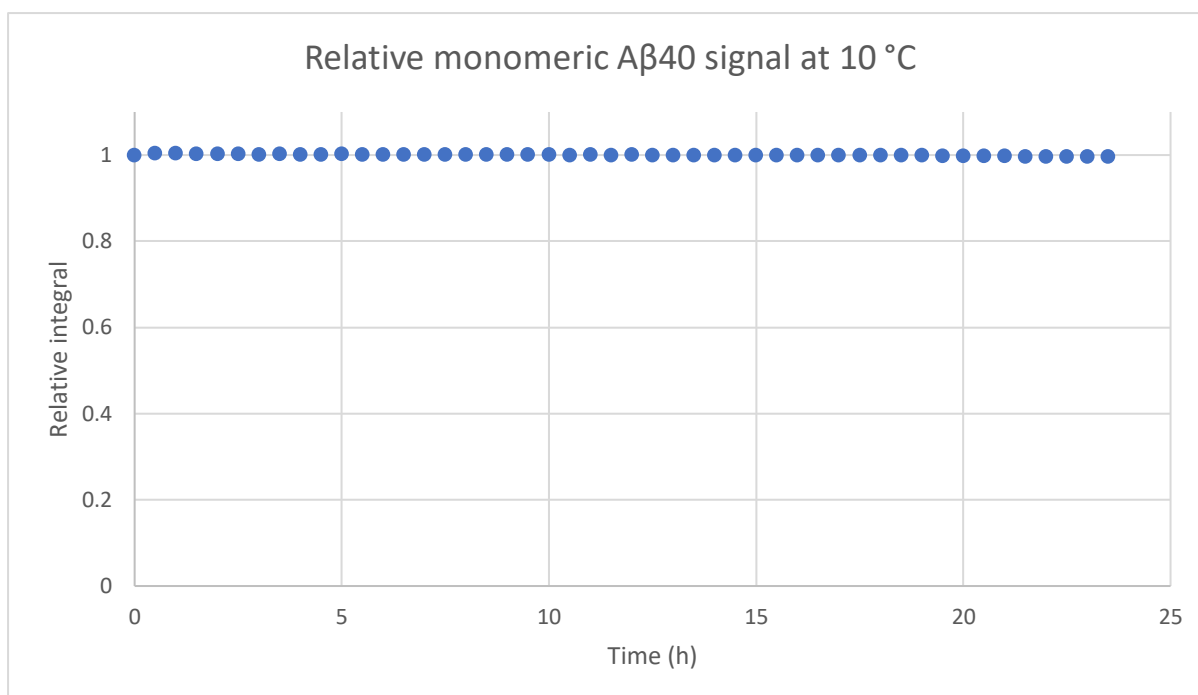


Figure A 19: NMR experiment at 10°C

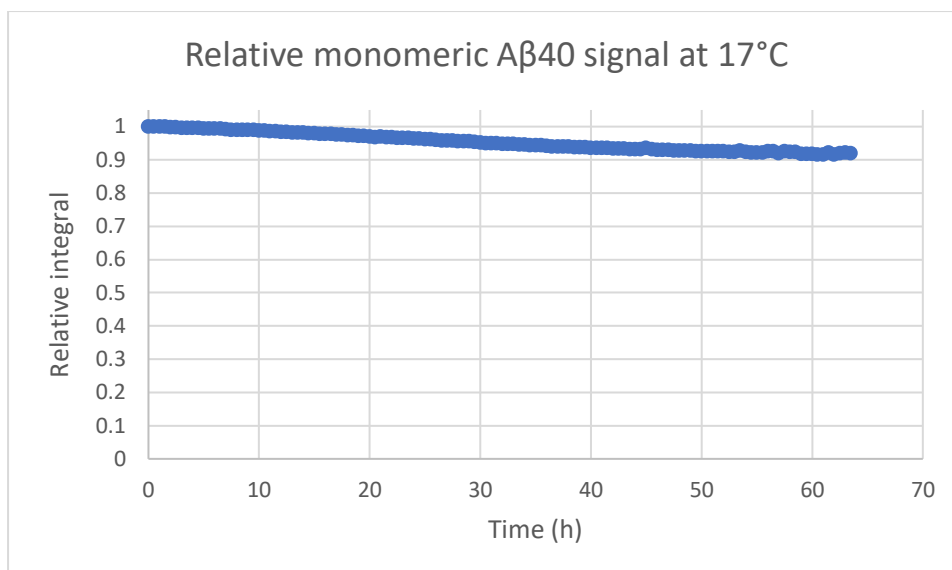


Figure A 20: NMR experiment at 17 °C

6 References

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