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ABSTRACT:

Acetylcholine (ACh) is a vital neurotransmitter in the central and peripheral nervous systems, playing crucial roles in synaptic transmission and neuromuscular signaling.

To obtain ACh, on an industrial scale, methods are limited to bioprocesses or chemical syntheses that combine acetyl chloride with choline in the presence of a base. The main drawbacks of these processes rely on process complexity and high production costs.

This project aims the development of a simple and cheaper process for ACh sythesis in just one step. For this purpose, choline chloride and acetic anhydride are used as precursors. This reaction is performed in batch where parameters such as stirring and temperature are controlled while operating under pressure. A first screening determined the conditions required to carry out the reaction, while a kinetic study allow for a full description of the reaction behavior.

As a result, the reaction was performed optimally after 6 hours at 150 °C, achieving 79% conversion and 80% ACh yield in the solid part of the final heterogeneous mixture .

RESUM:

L'acetilcolina (ACh) és un neurotransmissor vital en els sistemes nerviosos central i perifèric, jugant un paper crucial en la transmissió sinàptica i la senyalització neuromuscular.

Per obtenir ACh a escala industrial, els mètodes es limiten a bioprocessos o síntesis químiques que combinen clorur d'acetil amb colina en presència d'una base. Els principals inconvenients d'aquests processos es basen en la complexitat del procés i els alts costos de producció.

Aquest projecte té com a objectiu el desenvolupament d'un procés senzill i més barat per a la síntesi d'ACh en només un pas. Per a aquest propòsit, s'utilitzen clorur de colina i anhídrid acètic com a precursors. Aquesta reacció es realitza en lots on es controlen paràmetres com l'agitació i la temperatura mentre s'opera sota pressió. Un primer cribratge va determinar les condicions necessàries per dur a terme la reacció, mentre que un estudi cinètic permet una descripció completa del comportament de la reacció.

Com a resultat, la reacció es va realitzar de manera òptima al cap de 6 hores a 150 ºC, aconseguint un 79% de conversió i un 80% de rendiment en ACh a la part sòlida de la mescla heterogènia final.

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INTRODUCTION:

Acetylcholine (ACh) is a neurochemical that has a great number of functions of the brain and other organ systems of the body. It is a neurotransmitter in the peripheral nervous system, particularly acting in the autonomic nervous system, for the sympathetic and parasympathetic nervous system.[1]

Beyond its significance in neurobiology, ACh has gathered attention in industrial applications such as in medication development and neurological research that deals with neuronal function and communication, in the production of certain biosensors and other biotechnological applications within the same field, and some formulations of acetylcholine and derivatives used in agriculture as pesticides.

In the last decades, the demand of acetylcholine (ACh) has increased [2], driven not only by pharmaceutical industry but also by neurobiological research, and biotechnology. In 2020, global sales of ACh were around 500 tons and in the data recorded in 2023 there had been an increase of 30%, reaching more than 650 tons.[3] Values are expected to continue growing in the coming decades.[2] ACh is used in pharmaceutical industry to treat neurological disorders like Alzheimer's disease [4] [5] and myasthenia gravis.[6] [7] Its role in neuromuscular transmission allows ACh to be used for developing anesthetics and muscle relaxants used during surgeries. Additionally, ACh can be used to regulate cognitive functions and memory[8] as cognitive enhancers and nootropics.[9]

In the agricultural sector, acetylcholine analogs are used in developing pesticides that target the nervous systems of pests.[10]

On the market, ACh is sold as acetylcholine chloride (CAS-No 51-84-3), since the molecule needs a counterion to stabilize. It is sold in glass jars and its cost can vary between €1 and €5 per gram.[11]

ACh is produced in the nerve endings of neurons by the reaction between acetyl coenzyme A and choline, catalyzed by the enzyme choline acetyltransferase. [12] To carry out this enzymatic synthesis industrially, this process is replicated using genetically-modified bacteria-mediated fermentative reactors. Whilst the availability of process descriptions is limited in the literature because of intellectual property, those defined report quite efficient procedures without many unwanted by-products.[13] It is probably quite efficient and without many unwanted by-products. However, transgenic products or genetic manipulation have found some social rejection. [14] In ACh case, enzymes must be produced by genetically modified microorganisms grown in bioreactors, which requires specialized equipment and expensive culture media. [15] The culture of genetically modified organisms to produce the enzymes can take weeks or even months. Large amounts of resources are required, including culture media, and energy to maintain optimal growing conditions and subsequently the reaction. Furthermore, the purification of enzymes to obtain the required activity is expensive and complex generating significant chemical and biological wastes, which must be properly managed to minimize their environmental impact. [16]

Currently, there is a lack of detailed information and specific cost assessment associated with the production of this biomolecule by biotechnological companies.

As an alternative, this project aims to develop a non-bio-based laboratory-scale synthesis procedure for the production of Ach. This is achieved by using a simple, one-step and more efficient process. This approach should reduce costs and enhance productivity by the reduction of synthesis time, thus improving the profitability of the final product. In addition, the aim was to design a method that minimized the environmental impact, optimizing the use of resources and reducing the generation of waste.

METHODOLOGY:

ACh was intended to be synthesized by acetylation:

It is a chemical reaction in which an acetyl group is added into the molecule. To this aim, acetic anhydride was used since it contains two acetyl groups bonded to an oxygen. [17]

The oxygen of the hydroxyl group in choline chloride acts as a nucleophile, attacking one of the carbonyl carbons of acetic anhydride (Figure 1). An unstable intermediate is formed expelling acetic acid, which acts as a leaving group. An ester is obtained, thus generating the desired ACh.

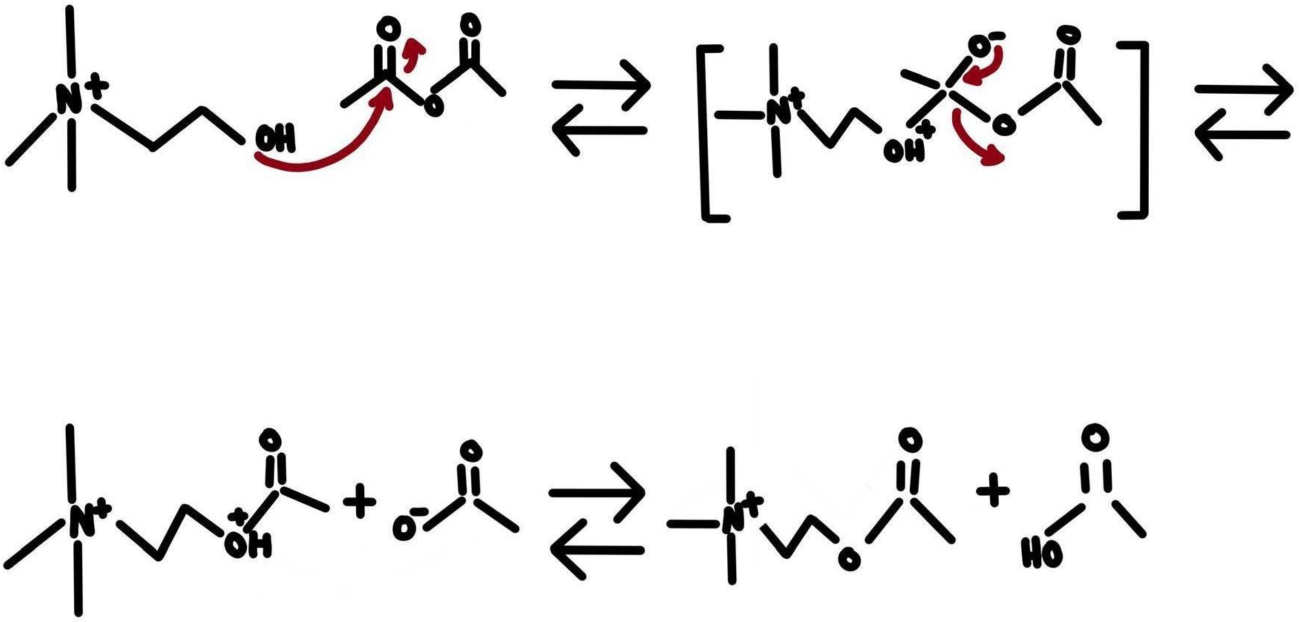

Figure 1: Choline acetylation

To describe the optimal reaction conditions a study of the reaction kinetics at different temperatures, molar ratio of reagents, stirring speed and reaction times is performed.

MATERIAL AND METHODS

Acetylation was performed without additional solvents, mixing choline chloride in solid state and dissolving it in acetic anhydride.

The first, chemically represented as trimethyl-2-hydroxyethylammonium (CAS-No 67- 48-1), was used in solid state. This compound is partially soluble in acetic anhydride (CAS-No 108-24-7) even at relatively high temperatures. So, despite theoretically needing an equimolar ratio (1:1), an excess of acetic anhydride of (1:30) was used. Consequently, 90 milliliters of acetic anhydride (0.9521 mol) and 4.4314 grams of choline chloride (0.0317 mol) were used to obtain a homogeneous mixture.

For scale up synthesis, the mixture was placed in a 100 mL round-bottom flask in a reaction block for round bottom flask and heated using a hot plate at the corresponding temperature. The mixture was stirred using a cross-shaped magnet at the corresponding speed. The system was equipped with a reflux condenser, which allowed the vapors to be condensed back to the reactor. From this reactor, 3 mL samples were taken for every elapsed time using a needle and a syringe from the center of the reactor (Illustration 2). The quantification of the evolution of the species in the mixture was followed using Nuclear Magnetic Resonance (NMR) (Mercuryplus NMR spectrometer system Varian 400, and vnmrJ-4.2 software for data processing), using deuterium oxide (D_2O) as solvent and malenic acid as internal standard. Once the set time was reached, the heating plate was turned off and the contents were allowed to cool slowly up to 5 ºC for just over 3h leading to supposed ACh crystallization, as the mixture was already saturated.

Consequently, the purification process included vacuum filtration of the crystals. To this aim, the mixture was deposited on a paper filter placed in a Büchner funnel and a vacuum pump was activated so that the liquid was filtered into a Kitasato. The solid crystals formed corresponded to ACh and chloride choline, which at room temperature, remains solid. The rejected liquid was mainly acetic anhydride and acetic acid. This mixture can be recycled and used again as a precursor, separating it from acetic acid, in a new reaction, thus reducing waste. The latter is out of the scope of this project.

To perform the reaction screening a reduced amount of the mixture with the corresponding molar ratio was tested using 10 mL closed screw caps tubing. The melting point of choline chloride is 302°C[18]. Nevertheless, the reflux system was set to a maximum temperature of acetic anhydride's boiling point, of 139°C. [19]

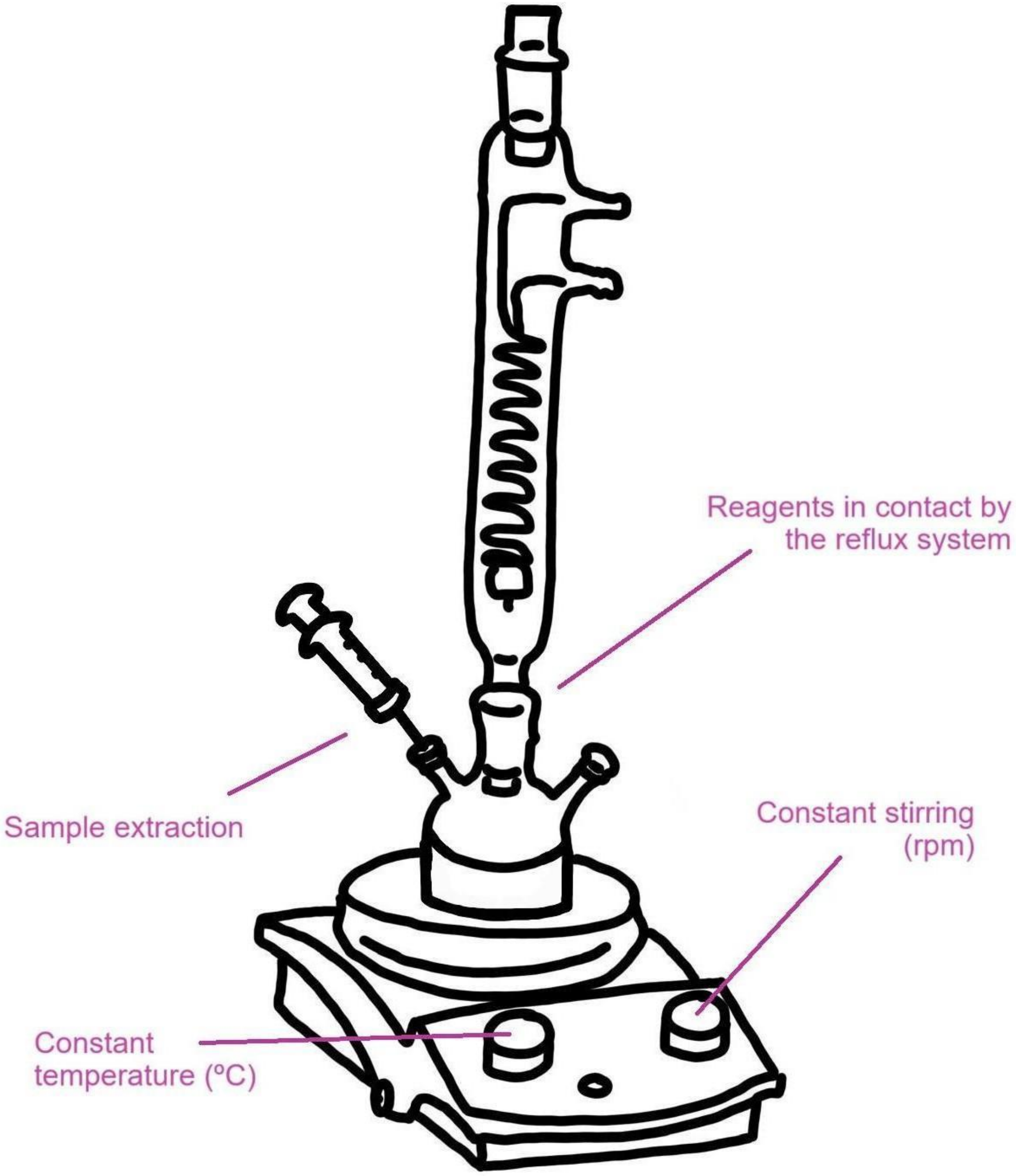

Figure 2: Reflux system diagram

The heating plate was adjusted to the desired temperature, placing on top a heating block appropriate to the type of reactor used. Once the desired mixture was prepared, it was quickly introduced into the reactor and the whole was placed on the heating block.

Three replicates were made for each experiment removing them from the heating plate according to the time interval required

To stop the reaction, the extracted samples were deposited directly into a tray with ice.

Then, they were transported to the refrigerator to continue at low temperatures, thus achieving the crystallization of ACh. Then the solid part formed and the liquid part were taken and sent to NMR to quantify their content.

RESULTS DISCUSSION

In the ^{1}H-NMR spectrum of ACh, the following chemical shifts are observed:

First, a 3H singlet is identified that appears in the range of 2 to 2.1 ppm, corresponding to the protons of the methyl group attached to the carbonyl of the ester ($COCH_3$). Next, a 2H triplet is detected in the range of 3.6 to 3.8 ppm, which corresponds to the methylene protons adjacent to the quaternary nitrogen (NCH_2). Another 2H triplet is found in the range of 4.2 to 4.4 ppm, attributable to the methylene protons adjacent to the oxygen of the ester group (CH_2O). And finally, a singlet of 9H is observed in the range of 3.1 to 3.3 ppm, corresponding to the protons of the three methyl groups attached to the quaternary nitrogen ($(CH_3)_3N$) (Illustration 3).

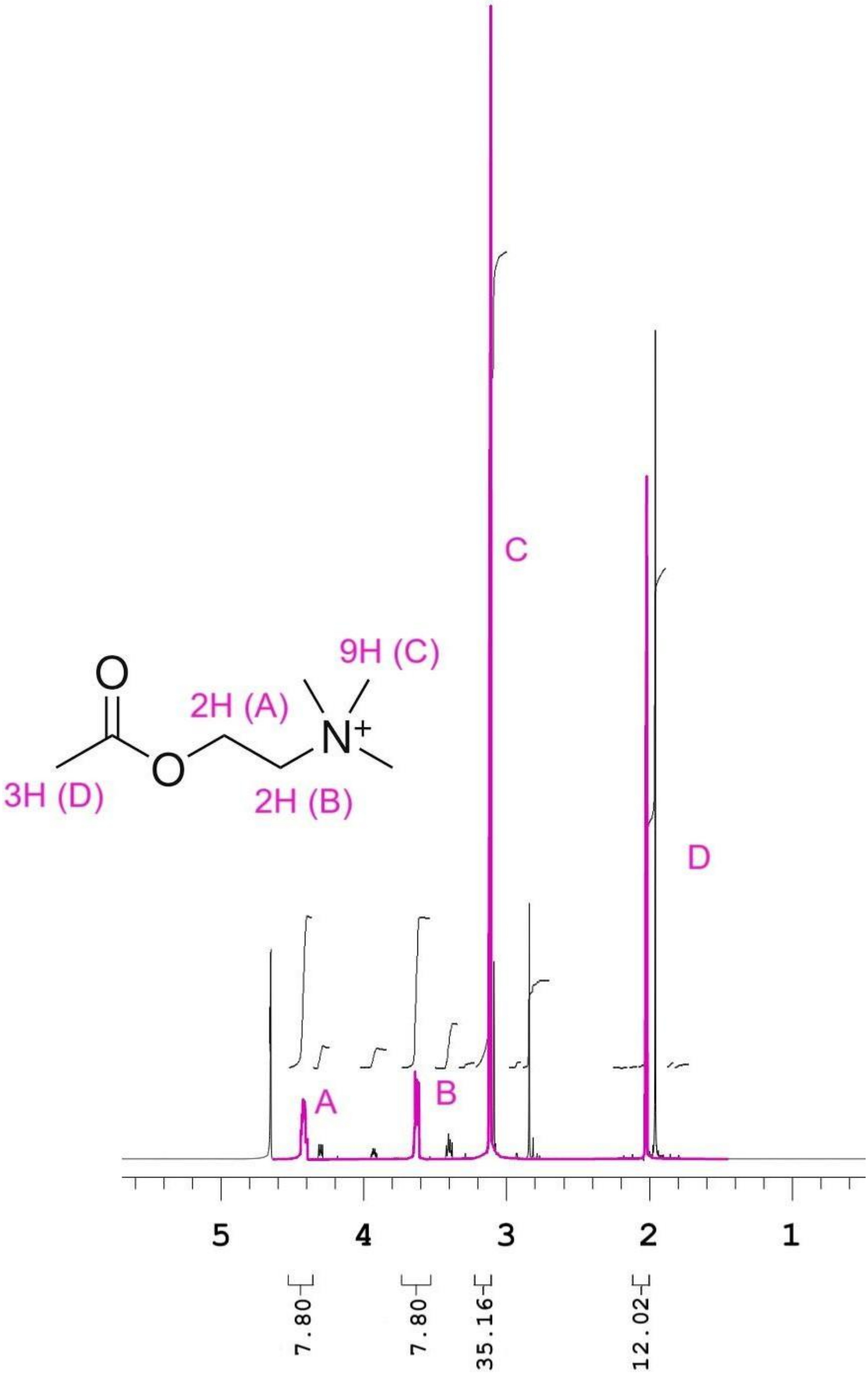

Figure 3: Acetylcholine chloride identification

A logarithmic trend curve was calculated from the values obtained from the NMR spectra (Appendices 1-15) that were made from the solid part formed in each sample. Each graph interprets a mixture (1:30), with a stirring of 500 rpm, at different temperatures (Illustration 4).

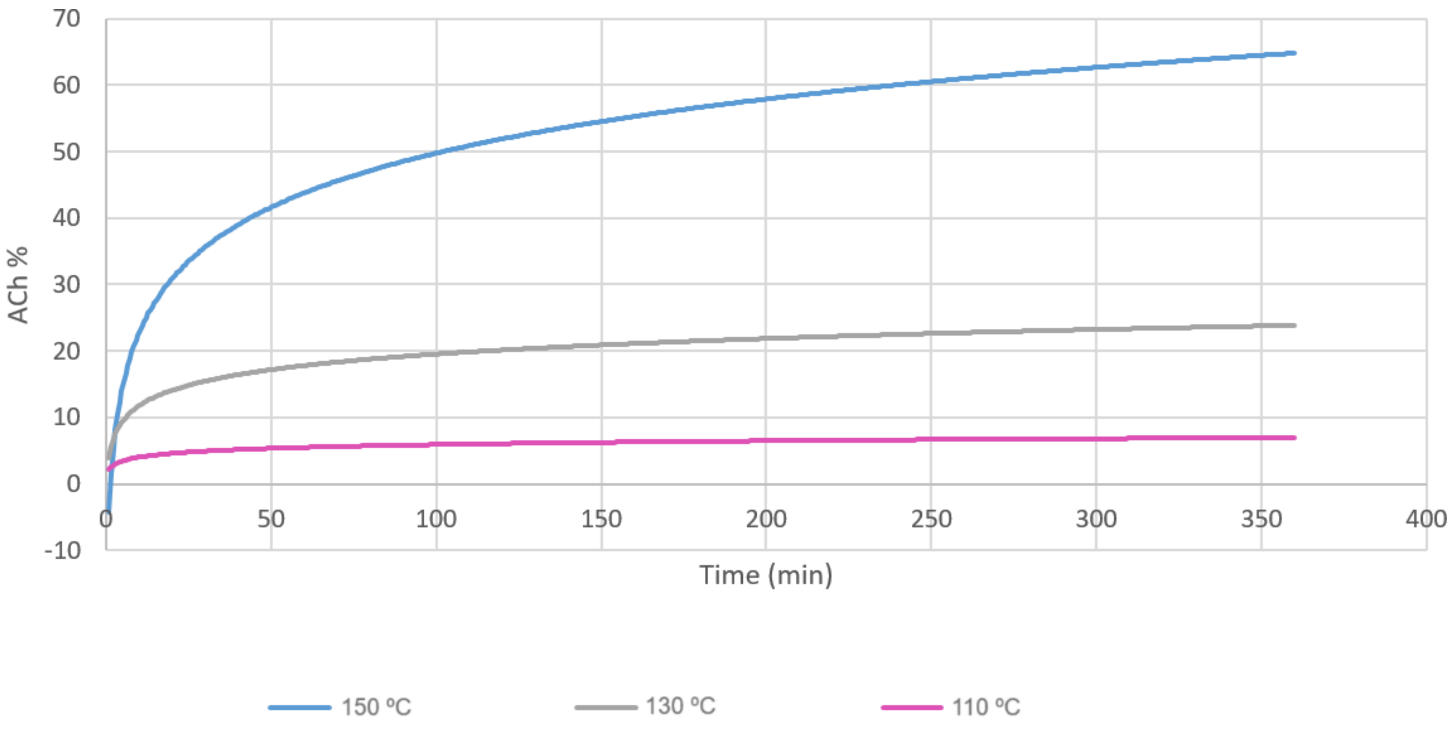

Figure 4: Reaction kinetics at different temperatures. % of ACh present in the sample vs time (min) in which the sample was extracted.

At high temperatures (Appendices 1-6) the percentage of ACh is between 50 and 60% and choline chloride is seen with values between 14-17%. These are the main components of the solid part. The rest, between 15-18% is acetic anhydride, between 17-19% is acetic acid and the rest may be some impurity or byproducts.

Meanwhile, at relatively low temperatures (Appendices 7-15), the percentage of ACh becomes between 6-18% and there is nothing visible of choline chloride. On the other hand, when moist, it also contains between 60-70% acetic anhydride and between 11- 14% acetic acid.

For the test case (1:30) at 150°C, the conversion was nearly 80% because it is estimated that there was a total of 0.91 grams of choline chloride including solid and liquid phases, which corresponds to exactly 79.49 % relative to the initial 4.43 grams.

The dried crystals, recovered in a solid amount of 5.39 grams after filtration, had a molar ratio of almost 4:5 for ACh and the remainder for choline chloride. From this mixture, 4.57 grams are ACh, and theoretically, based on the amount of 0.032 moles of choline chloride used in the reaction, 5.76 g of ACh are obtained, leading to 79.36 % yield.

Similarly, in the (1:30) test at 130°C, practically no choline chloride was found, indicating a conversion of almost 100%. However, a minimal dry residue of 1.63 g were recovered, resulting in a yield of 28.26%.

Overall, the reaction at 150°C proves to be better. Although the conversion rate is slightly lower compared to that at 130°C, the yield is significantly higher. This suggests that the former reaction is more efficient in obtaining the desired product in a purer form and in substantial quantity. Moreover, the higher yield implies effective minimization of undesired byproducts and losses during the purification process.

CONCLUSIONS:

The reaction conducted at 150°C and 500 rpm for 6 h exhibited a conversion of 79.5 % and a yield of 79.3 %, denoting high selectivity. There was nearly complete signal localization in the spectra, indicating fairly clear results. However, purification methods require significant improvement as there is still difficulty in isolating ACh in its pure form.

The solvent-free approach in the reaction has shown promising results, especially with the potential for recycling excess materials. Nevertheless, it is proposed to explore methods that reduce the use of acetic anhydride as a solvent, opting for a solvent in the dissolution of choline chloride as a precursor. Although water, as previously attempted, rapidly interacts with acetic anhydride via hydrolysis, reducing its availability, the use of volatile solvents such as methanol or ethanol in minimal quantities could enhance outcomes. The new hypothesis aims to use a precise amount of solvent that does not compete with the intended acetylation process, facilitating rapid dissolution of choline chloride and acetic anhydride for a homogeneous mixture without excessive acetic anhydride. Additionally, a volatile solvent offers the advantage of evaporating at the end of the reaction. However, a relatively higher proportion of acetic anhydride compared to choline chloride should be added to optimize the acetylation of choline, as there might also be concurrent acetylation of the solvent due to its hydroxyl group.

Future works would include the production in continuous flow. It is crucial to assess its functionality on an industrial scale and conduct a detailed analysis to ensure a smooth transition to factory applications.

We thank the Universitat de Lleida (UdL) for providing the material and space necessary to carry out this project. Likewise, we thank Mireia Oromi, who performed the NMR with the provided samples. The guidance of the tutor is acknowledged, as is the help of members of the campus chemistry laboratory. Finally, we appreciate the family for their unconditional support and for providing resources for the drives to the laboratory.

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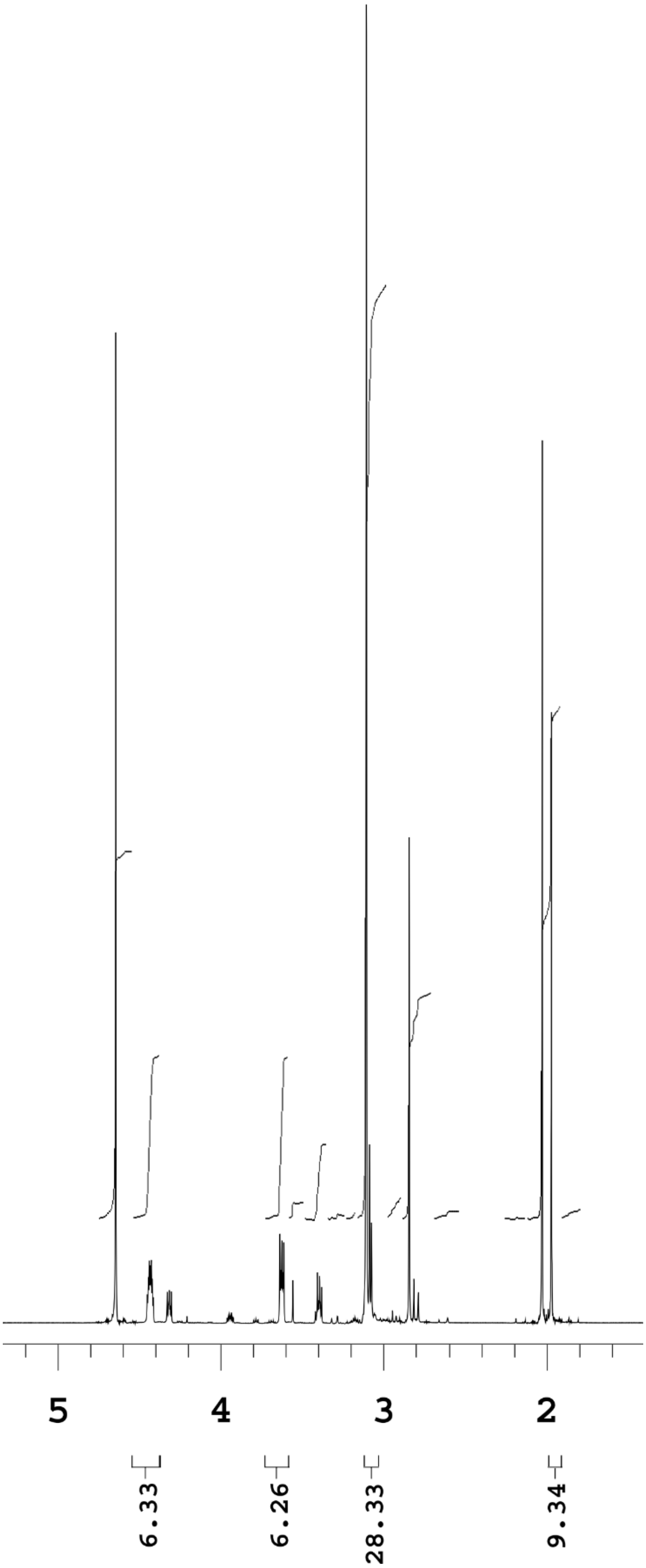
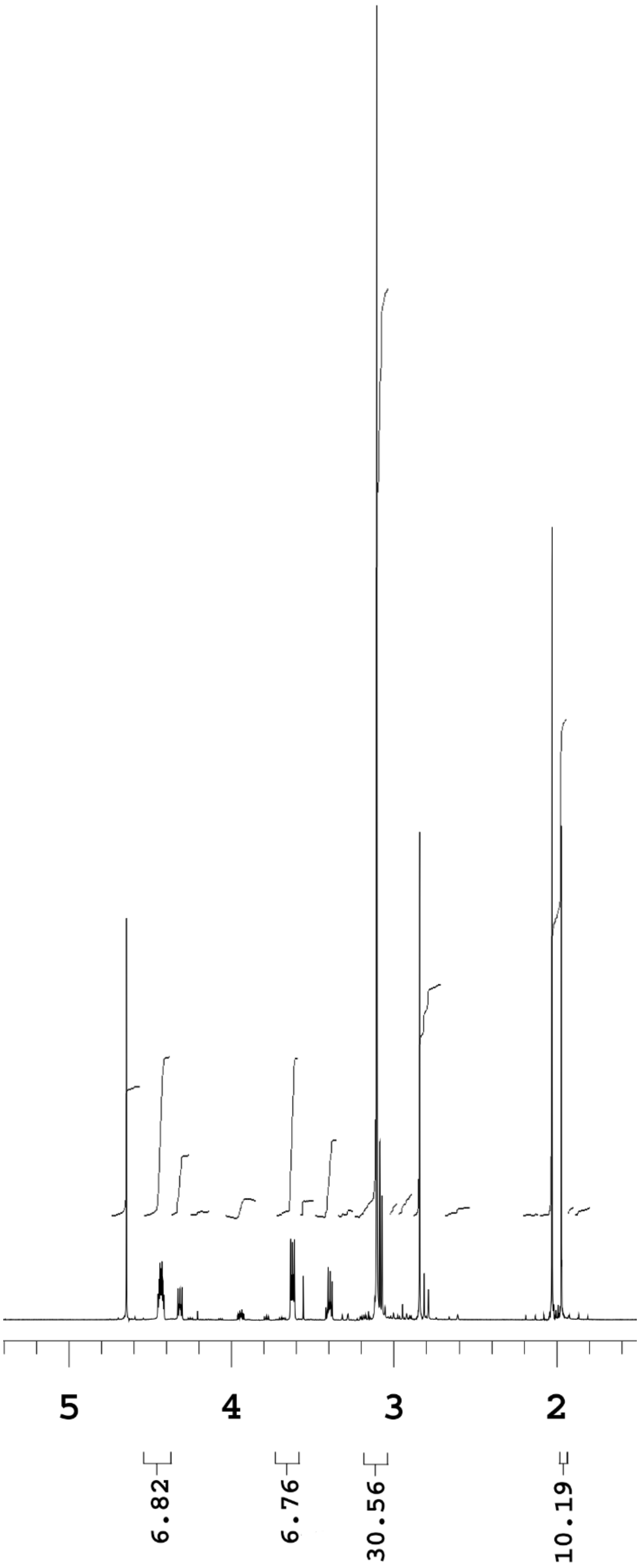
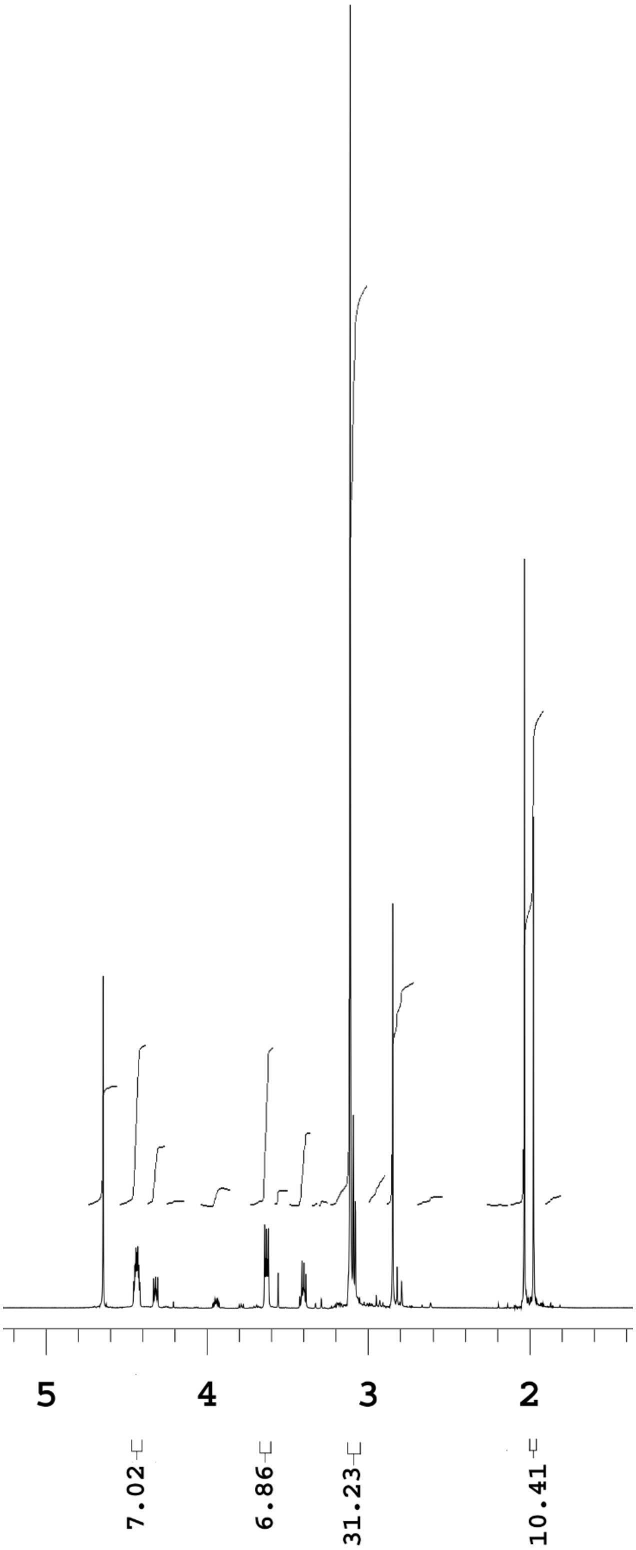


Appendix 1: kinetic curve of 150ºC point



Appendix 2: kinetic curve of 150ºC point



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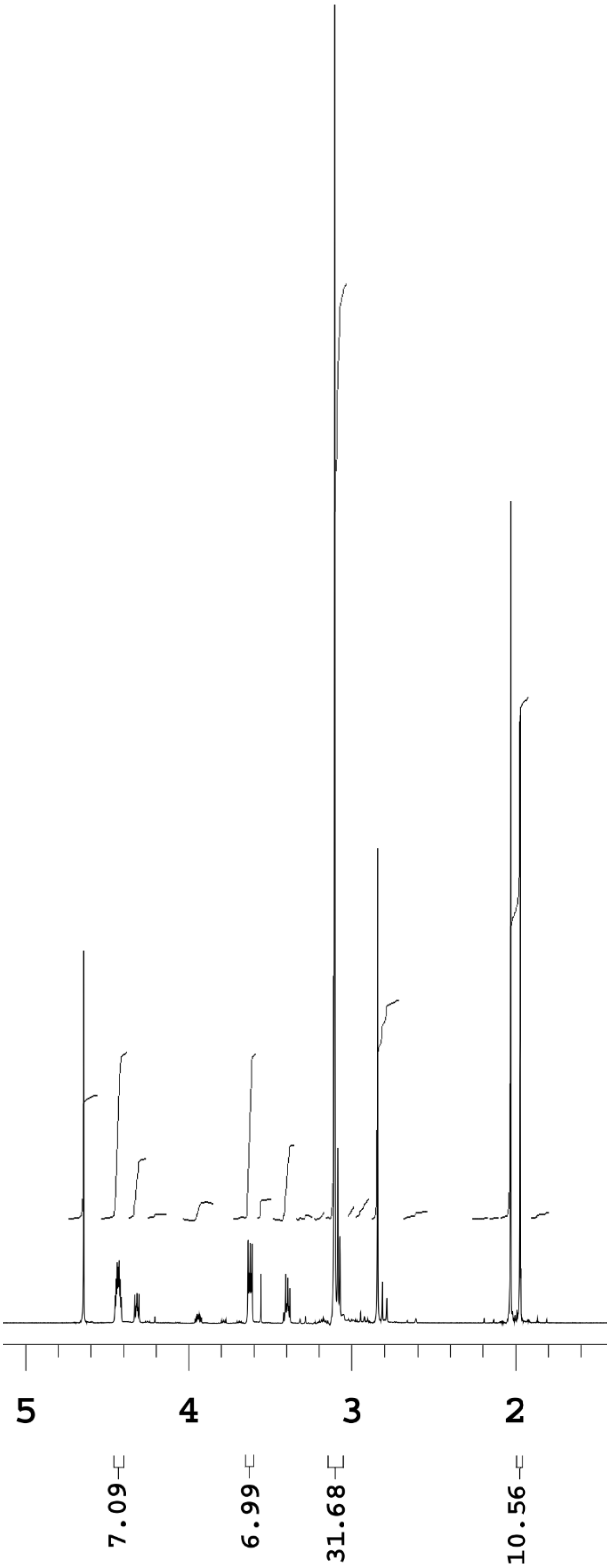

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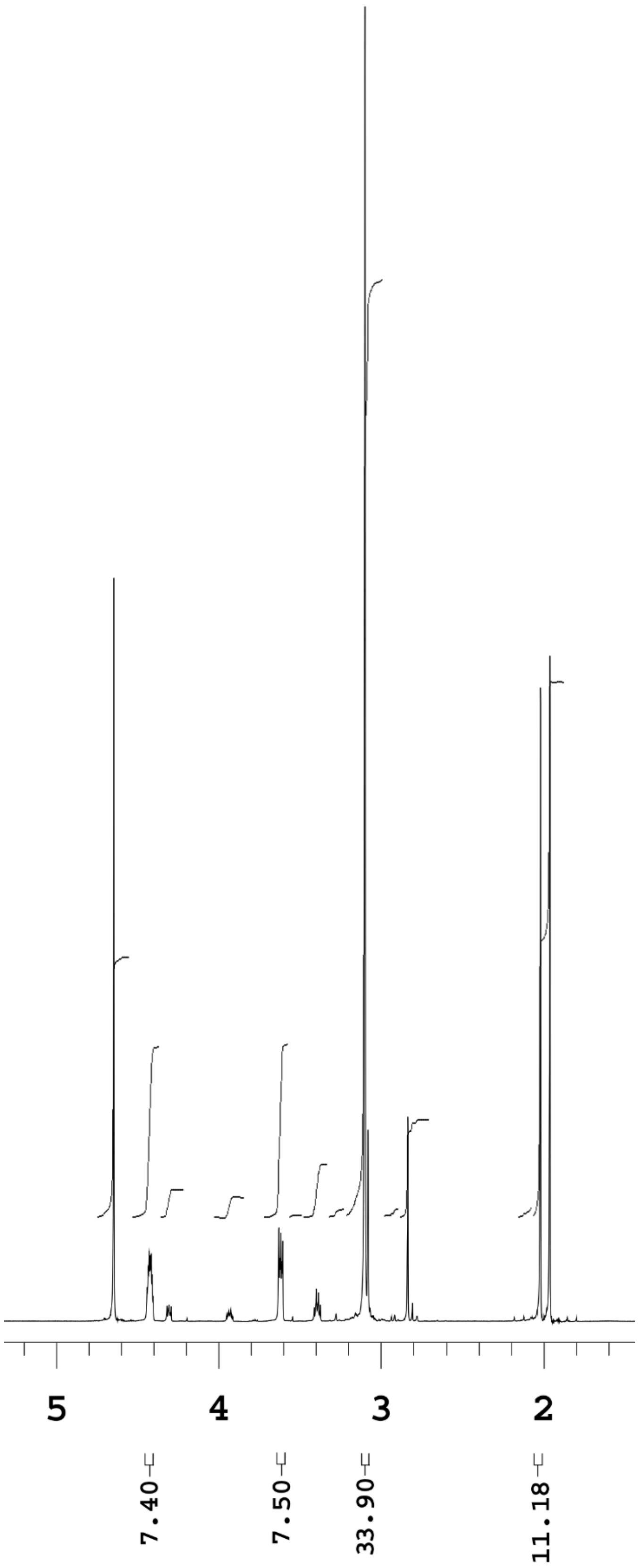
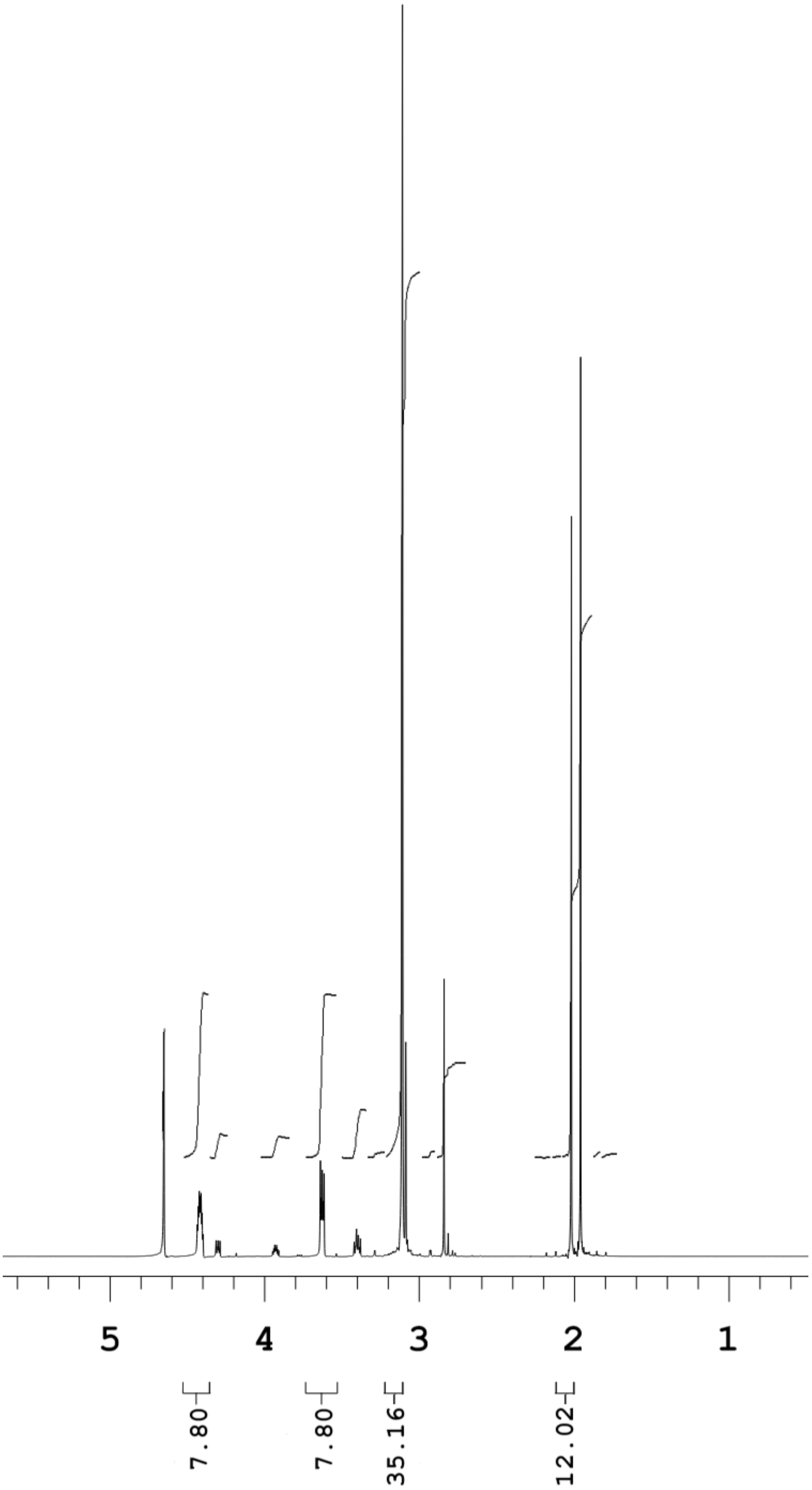
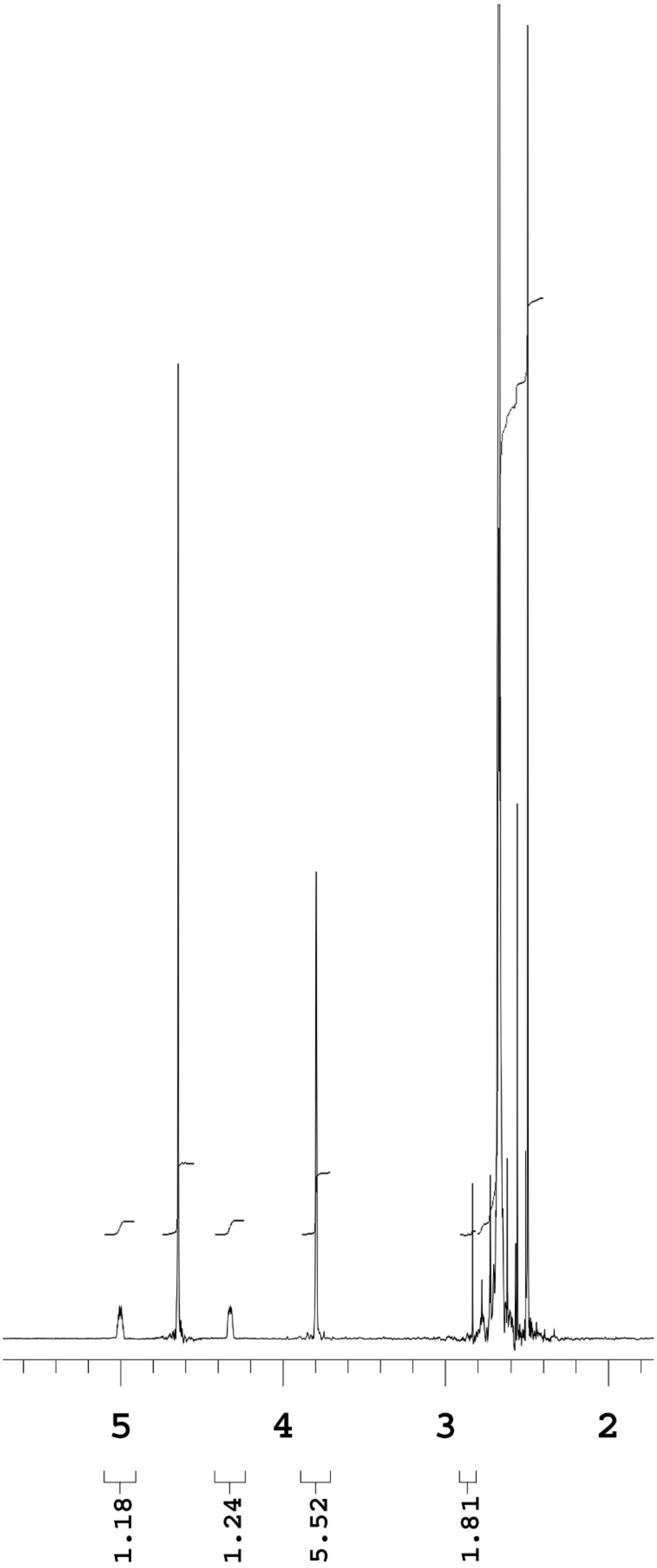


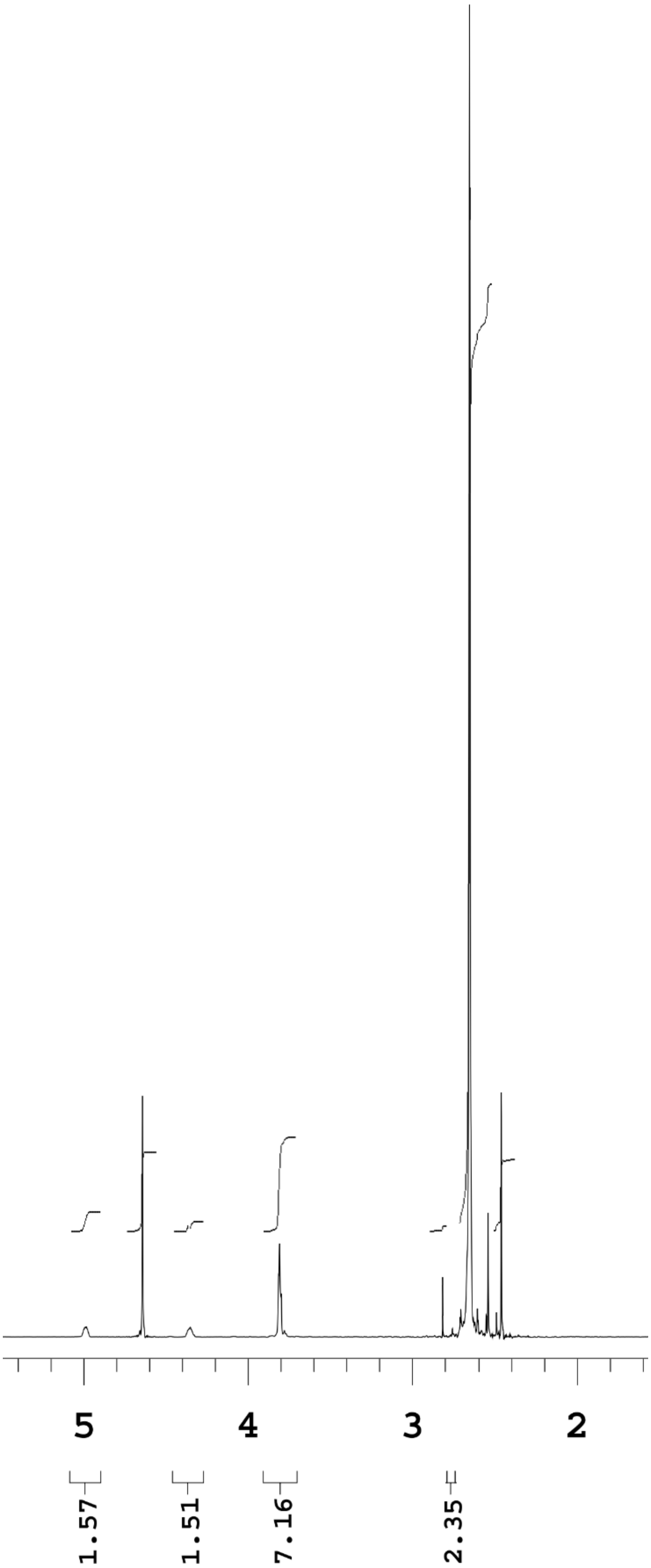
Appendix 5: kinetic curve of 150ºC point



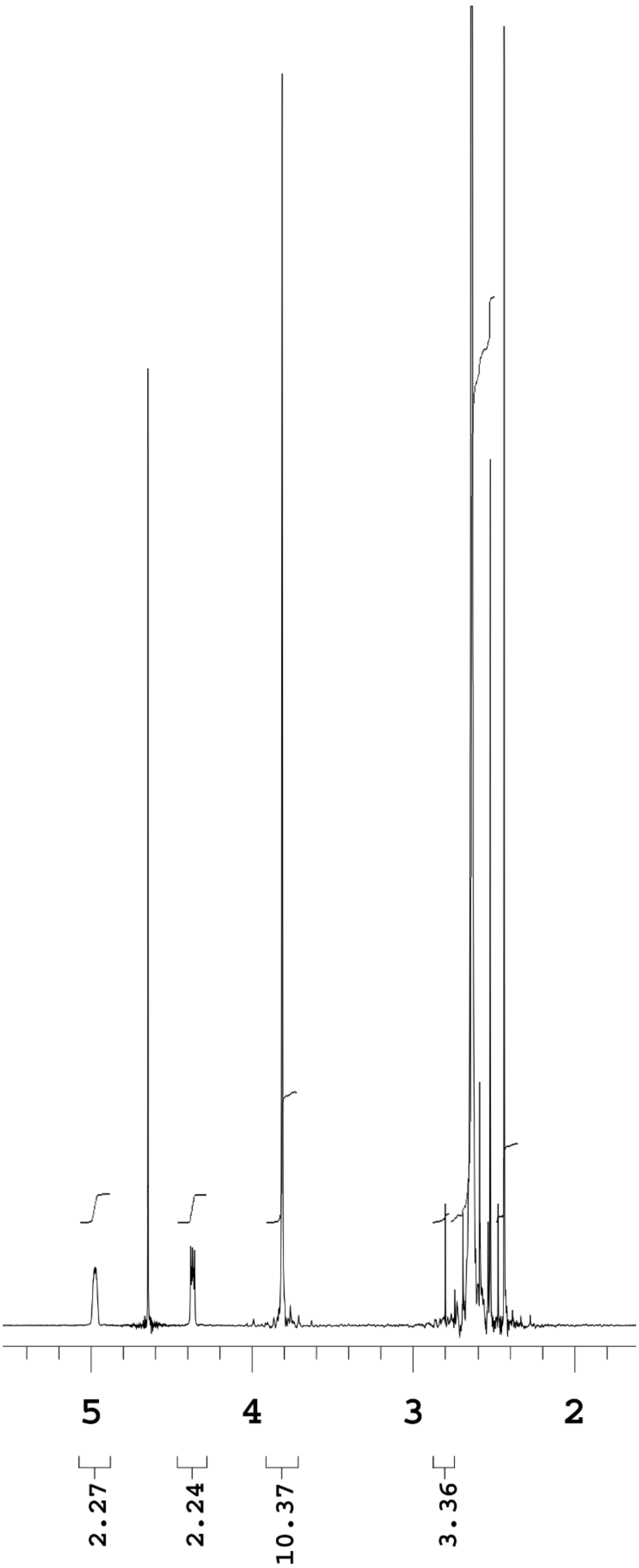
Appendix 6: kinetic curve of 150ºC point



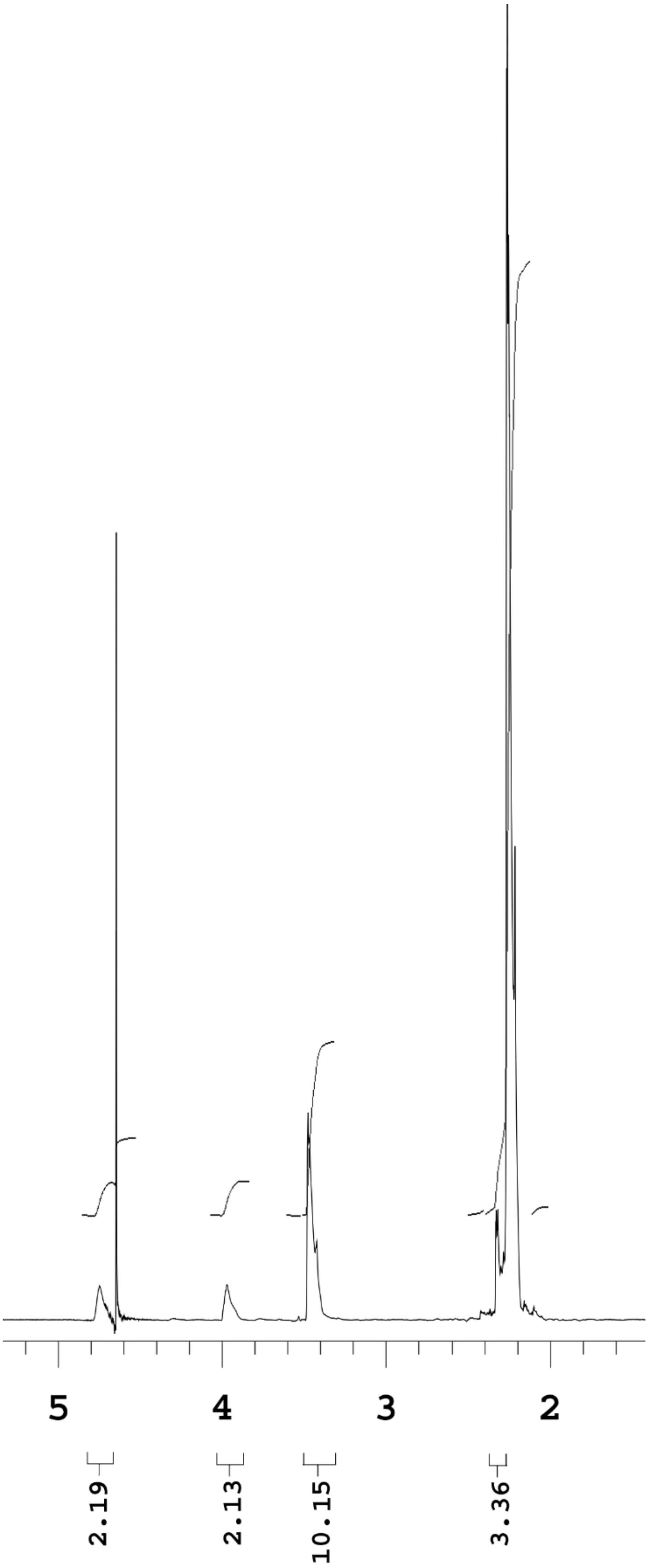
Appendix 7: kinetic curve of 130ºC point



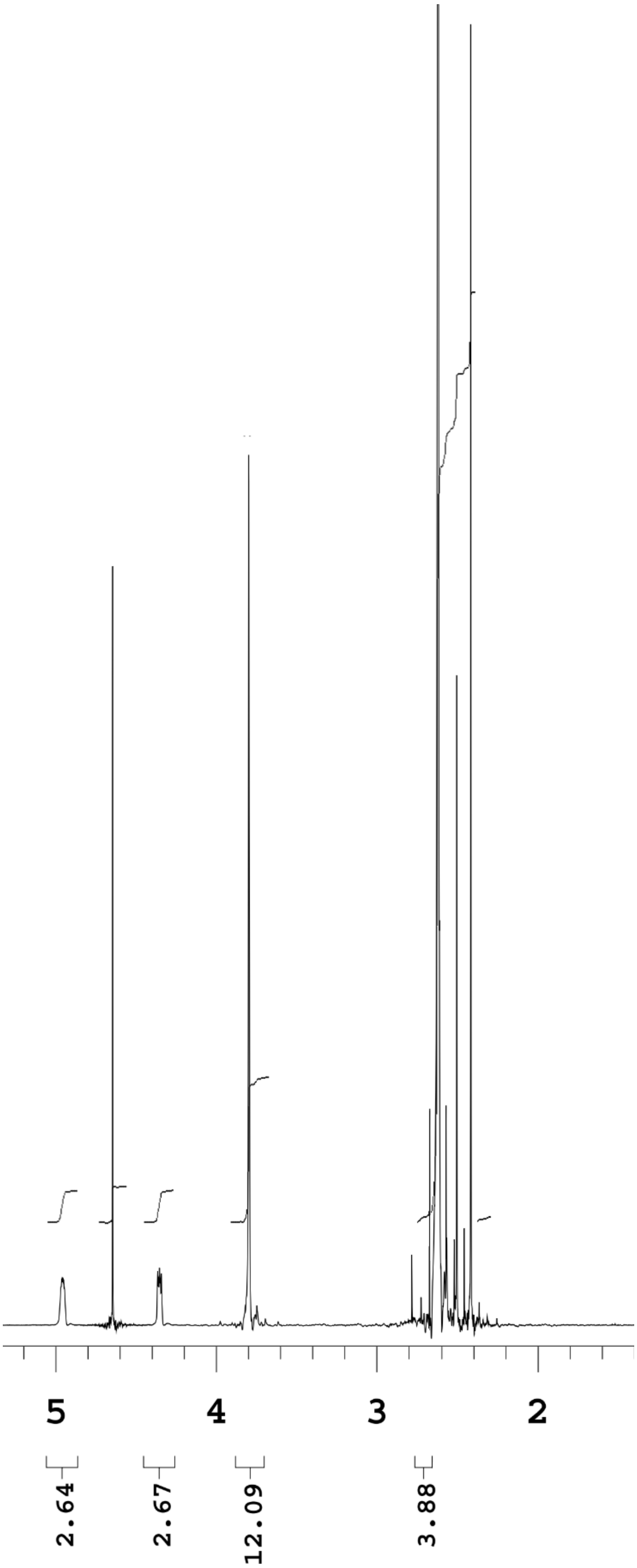
Appendix 8: kinetic curve of 130ºC point



Appendix 9: kinetic curve of 130ºC point



Appendix 10: kinetic curve of 130ºC point



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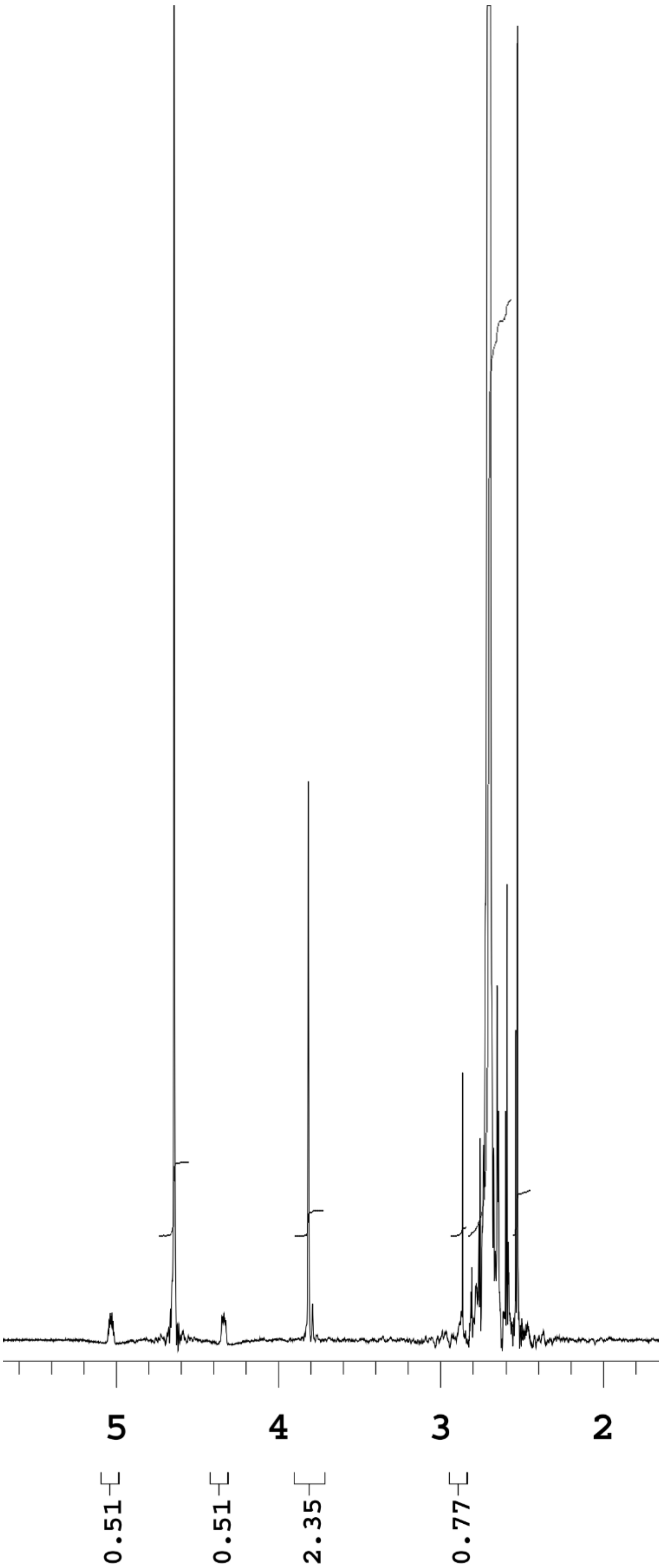
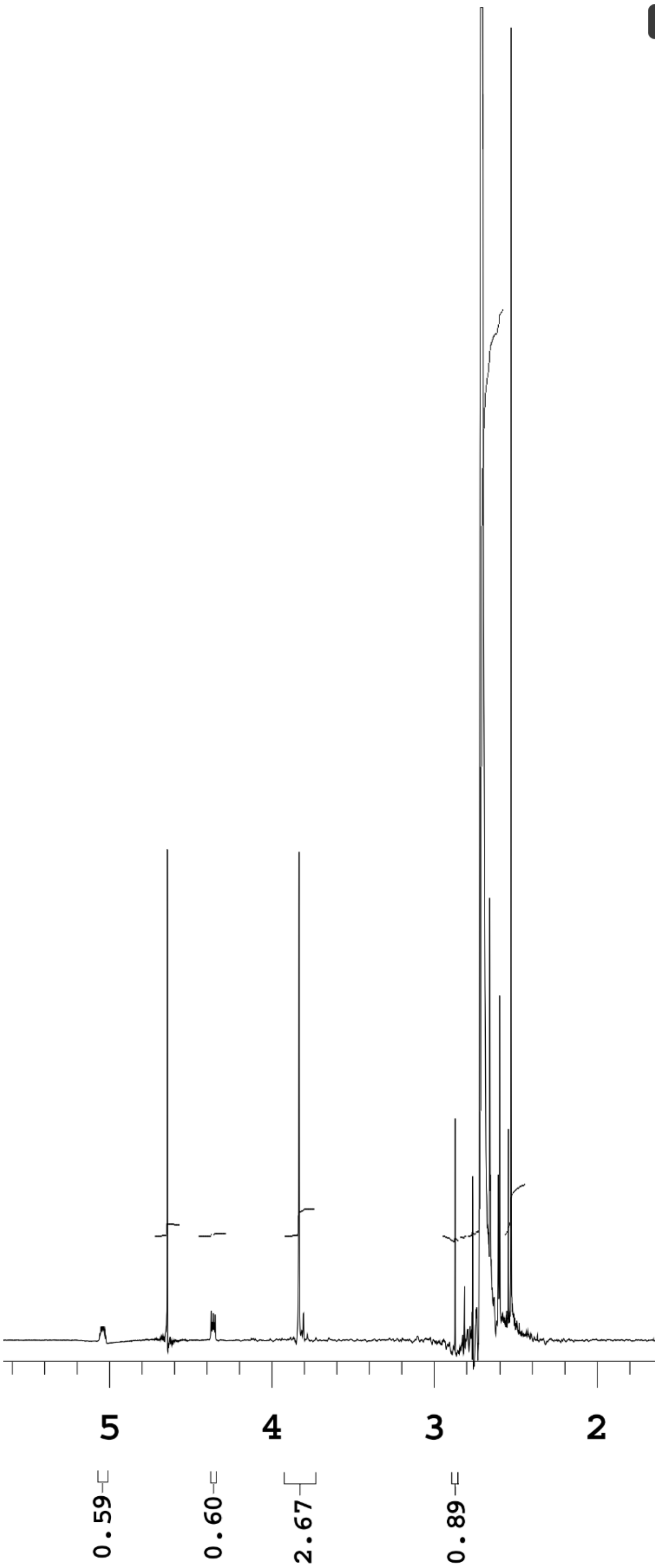
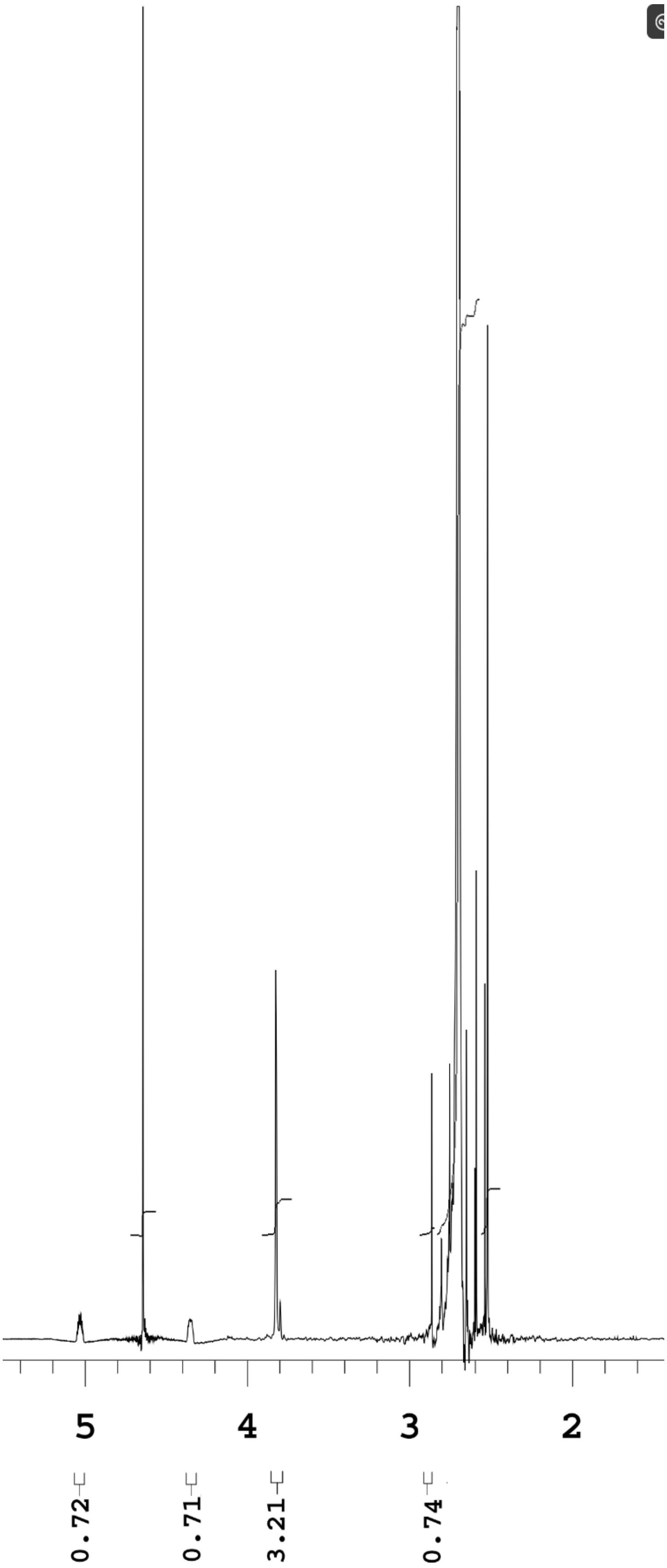


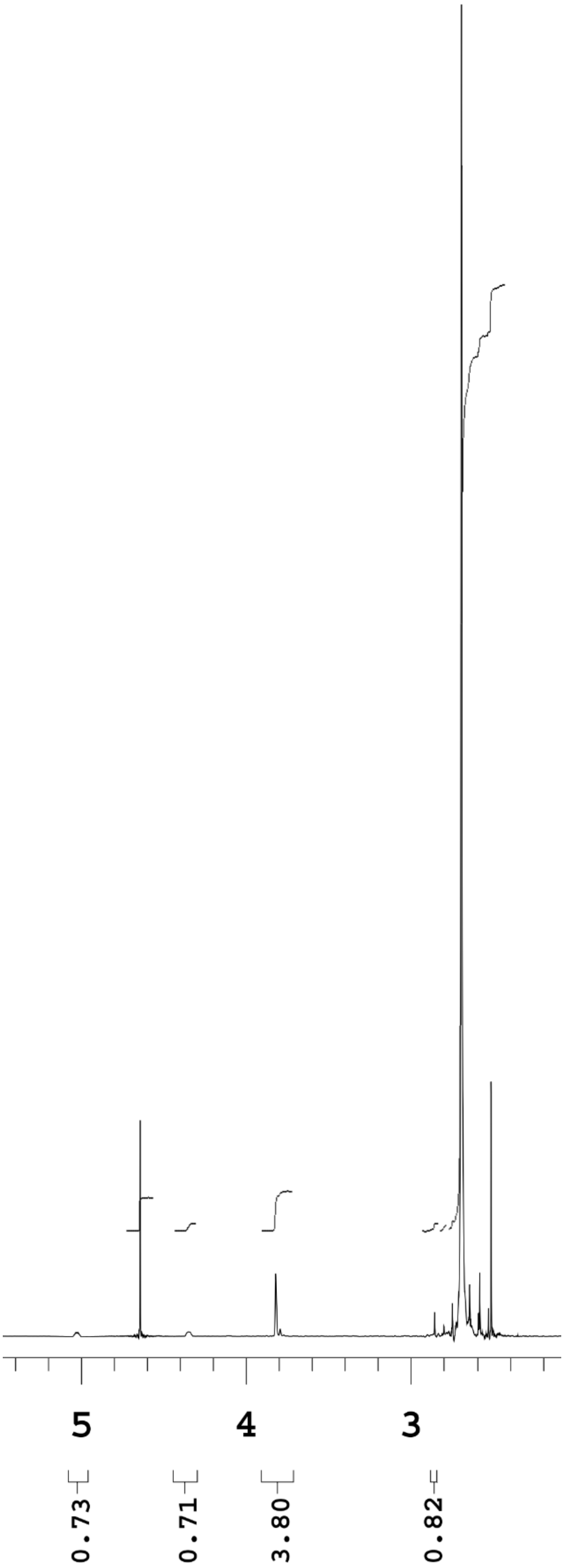
Appendix 12: kinetic curve of 110ºC point



Appendix 13: kinetic curve of 110ºC point



Appendix 14: kinetic curve of 110ºC point



Appendix 15: kinetic curve of 110ºC point