

Time-temperature Indicators

Based on the

Acidic Permanganate-oxalate Reaction System

by

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ABSTRACT

The kinetic behavior of acidified aqueous reaction systems based on the oxidation of sodium oxalate by potassium permanganate was investigated by UV-Vis absorbance spectrophotometry as part of a wider project to develop time-temperature indicators based on such systems. Reaction systems in various eutectic salt solutions as well as salt-free aqueous solutions were prepared and measured to establish the precision and accuracy of four different reaction systems designed to run for specified periods of time at 25°C. Reaction progress was also monitored in reaction systems previously subjected to prolonged periods of storage at -80°C, as well as systems subjected to repeated freezing and thawing, to determine the effect of preliminary freeze time or multiple freeze-thaw cycles on reaction run time. Eutectic-free aqueous reaction systems showed a noticeable decrease in run time from freshly frozen to 2 week frozen, but minimal to no decrease with increasing preliminary freeze time. Perchlorate-based reaction systems showed minimal to no decrease in reaction time with increasing preliminary freeze time, and were found to be capable of tolerating up to three <5-minute excursions into room temperature before reaction time was significantly affected. It was also found that all reaction systems studied were capable of reaching their target run times within the intended $\pm 10\%$ limit of accuracy, as average run times for each system ranged from 0.4% to 6.9% off from the target run time. These systems were found to be reproducible with considerable precision as well, with the average coefficient of variation for each system ranging from 2.6 to 5.5.

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CHAPTER 1

INTRODUCTION

Time-temperature indicators (TTIs) are devices that record the thermal history of the environment they have been exposed to, usually by undergoing some irreversible change after a particular temperature threshold is reached and held for long enough (Wang et al., 2015). Such devices are commonly used in supply chains for perishable goods such as food and pharmaceuticals to indicate prolonged time outside of cold storage, and thus warn of potential risk of spoilage in the former or loss of efficacy in the latter.

Chemical TTIs operate on the principle of the temperature dependence of reaction kinetics, utilizing reaction systems in which the rate of the reaction is decreased at lower temperatures and increased at higher temperatures, corresponding to a prolonged reaction time at lower temperatures and a shortened reaction time at higher ones. Chemical systems used for TTI applications usually undergo a visible change such as color change to indicate reaction completion and thus exposure to elevated temperatures, though because noticeable changes in reaction kinetics usually require significantly elevated temperatures it is usually the case that chemical TTIs find use in high-temperature applications instead of cold-chain applications.

A cursory look through the literature finds numerous examples of novel TTI designs whose operating principles are based on different chemical processes. Galagan and Su (2008) for instance report on a chemical TTI based on the oxidation of fadeable ink by atmospheric oxygen, where the rate of oxidation is controlled by the permeability of the polyacrylate coating over the printed ink. This property is dependent on both the composition of the polyacrylate and whether the ambient temperature is below or above

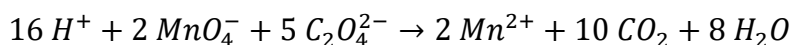
the polymer's glass transition temperature, which in the case of their applications could range from -5.5°C to 112.3°C . Another example is furnished in the work of Wanihsuksombat et al. (2010) in which the temperature-dependent process is the diffusion of lactic acid vapor through an agar gel dyed with pH indicators, which slowly change color as the vapor permeates the gel.

TTIs of these and other designs find ample application in the packaged food industry as safeguards against spoilage of frozen or refrigerated foods, but also serve in a less well-known but equally important capacity in clinical settings. Refrigeration is essential for the transport and storage of pharmaceuticals like drugs and vaccines, which frequently lose their efficacy when exposed to elevated temperatures, making the ability to track the thermal history of such items highly desirable for manufacturers and health services alike. This also applies to clinical specimens of biological matter such as blood (Shabihkhani et al., 2014), tissue samples (Yu et al., 2017), and even organs for transplant, for which cold storage is of great importance.

Exposure to temperatures above those recommended for storage may have ramifications for specimen integrity. The ensuing acceleration of the normal oxidative and enzymatic degradation processes is liable to produce altered concentrations of various biomolecules (Betsou et al., 2013), including those that serve as markers of disease. This opens up the possibility of questioning the results of tests conducted using such specimens, whether in screening for disease or testing drug efficacy, on the basis of such changes. Consequently, it is of great interest for researchers to develop time-temperature indicators capable of indicating such excursions into elevated temperatures during specimen processing, transport, and storage.

Most commercial TTIs are not suitable for such clinical applications, having been developed to track the time-temperature history of food in cold-chain transport and storage. The temperatures at which refrigerated or frozen foods are transported and stored are well above those at which biospecimens are stored, necessitating the development of TTIs that operate well below 0°C. Developing such TTIs that are inexpensive and therefore capable of being attached to samples at the individual vial level is the aim of the current project.

Among the various temperature-dependent chemical processes that could serve as the basis for developing a functional TTI, the acidic permanganate-oxalate reaction



was chosen for this project for the visible color change from vivid pink to colorless it displays. This reaction has been extensively studied (Pimienta et al., 1995; Kovacs et al., 2004), which allows one to model the effect of changing reaction parameters on theoretical run time, based on the reaction mechanism (Figure 1) and rates of individual steps, enabling greater control of reaction conditions. It is also autocatalyzed by its own reaction product Mn(II), allowing the system to maintain a pink color for a relatively long period of time before making a rapid transition to colorless.

This reaction between potassium permanganate and sodium oxalate has long been used in titrimetry to standardize solutions of potassium permanganate for redox titrations (McBride, 1912) and is still used today for the same purpose. The reaction system serves as its own indicator as the purple or pink permanganate is reduced to colorless Mn(II) when added dropwise to aqueous sodium oxalate, with a persistent pink indicating a lack of oxalate to oxidize and therefore the endpoint of the titration. However, titrations are set up to run under conditions of near-instantaneous equilibrium, as can be observed in the rapid

disappearance of color within seconds as each drop of aqueous permanganate titrant is added to the aqueous oxalate standard. This is generally not the case for the TTI systems under consideration, which are intended to come to equilibrium minutes or even hours after the mixing of the reactants.

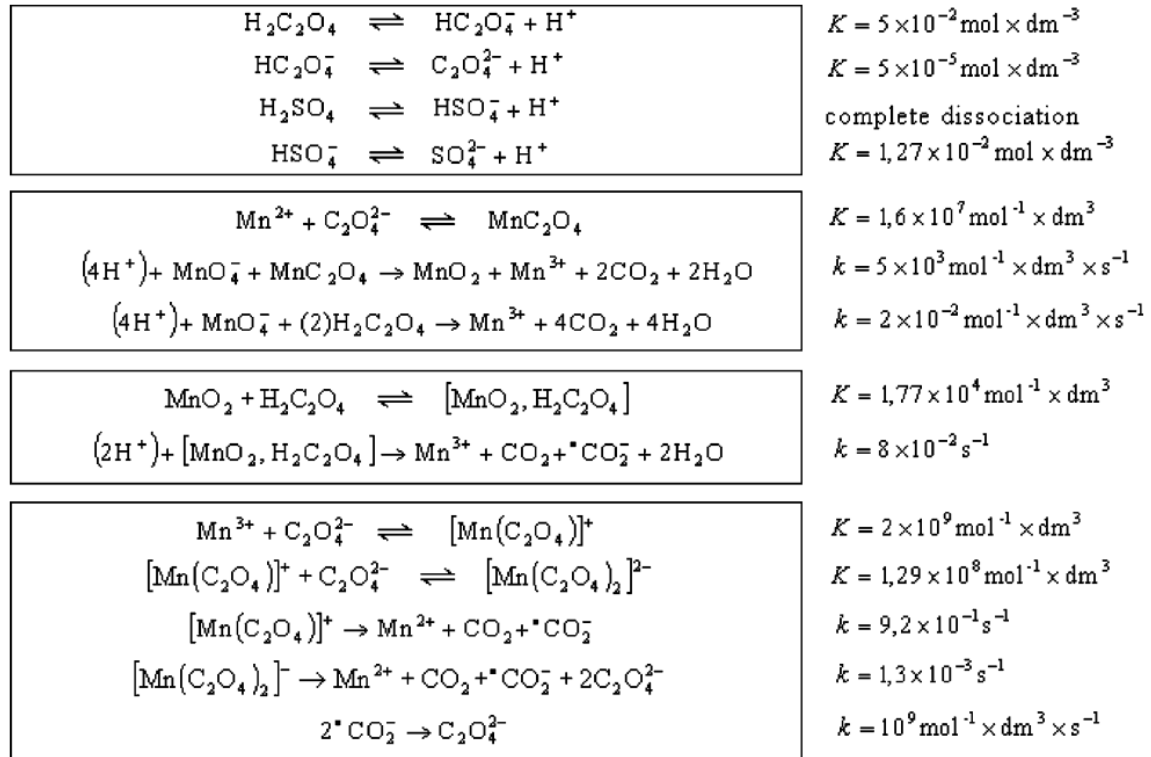


Figure 1. Reaction mechanism proposed by Pimienta et al. (1995). The final product Mn(II) functions as an autocatalyst by forming a complex with oxalate which is then oxidized by permanganate, forming intermediates which consume oxalate to produce more Mn(II). This forms a positive feedback loop: as more Mn(II) is produced, the rates of the succeeding steps increase and the overall reaction speeds up.

The overall goal of this project is to investigate the feasibility of a TTI system based on the permanganate-oxalate reaction for monitoring biospecimen integrity as well as other ultracold storage needs. This encompasses the implementation of antifreeze systems consisting of eutectic compositions of perchlorate salts that depress the melting point of the reaction solution to specific temperatures between 0 and -80 °C, development of systems with starting compositions that match targeted run times at a particular temperature,

validating the reproducibility, precision, and accuracy of run times belonging to replicate reaction systems of the same starting composition, documentation of freeze-thaw behavior, and examination of long-term stability in the frozen state.

To this end, the progress of the reaction will be thoroughly monitored spectrophotometrically to build an understanding of how the concentrations of the various aqueous species involved change over time. As the intended application of these indicators will encompass temperatures well below the freezing point of water, sub-zero investigations of reaction behavior will also be carried out. In addition, reaction behavior in eutectic compositions of perchlorate salts will also be examined to ascertain any effect high salt concentrations may have on reaction time.

Perchlorate salts were chosen for consideration, as the highly-oxidized perchlorate ion is inert to the oxidizing action of permanganate but itself does not oxidize Mn(II) (Urbanska et al., 2011). Chevrier et al. (2009) provides the phase diagrams for sodium perchlorate-water and magnesium perchlorate-water systems, as well as their associated eutectic points. For sodium perchlorate this point is 236 K (-37°C) at 52% NaClO_4 by weight, while for magnesium perchlorate it is 206 K (-67°C) at 44.0% $\text{Mg}(\text{ClO}_4)_2$ by weight. Davidian et al. (2014) provides the eutectic composition for lithium perchlorate, 2.94 M (3.15 *m*), while the eutectic point for the same is given by Marcus (2017) as 255 K (-18°C). This range of eutectic temperatures is intended to allow the development of TTI systems with a broad range of working temperatures, from applications for temperatures just below freezing to ultracold temperatures suitable for pharmaceutical and biospecimen sample storage.

However, since perchlorate salts are hygroscopic and can exist in various differently hydrated forms as well as in the anhydrous form, the actual concentration of perchlorate in solution may not match that given through calculations based on weighing out a solid perchlorate with an assumed formula. Quantifying the perchlorate concentration through potentiometric titration with an ion-selective electrode (Selig, 1979) circumvents this limitation and allow for greater reproducibility. This reaction is based on the precipitation reaction between perchlorate ion and hexadecyltrimethylammonium bromide (also known as cetyltrimethylammonium bromide, or CETAB). As CETAB is slowly added to a solution of aqueous perchlorate, it forms a precipitate with the ion thus removing it from solution, gradually raising the potential which is measured with a perchlorate-specific ion-selective electrode.

The aims of this project can be broken down into two overarching goals. One of these is to examine how reproducibly reaction systems of a given composition can reach their target run time to within 10% of the target, as consistency of run time is a necessary prerequisite to the commercial viability of a TTI system. The other is to examine the behavior of these reaction systems in the frozen state, namely, whether the length of time spent in the frozen state prior to reaction initiation affects the length of run time, and what effect repeated freezing and thawing may have on the run time of these reaction system. Adjacent to these principal project goals is the need to set up a reliable protocol for accurately quantifying the concentration of perchlorate in stock solutions used to prepare these reaction systems, using the aforementioned potentiometric titration proposed by Selig.

CHAPTER 2

REPRODUCIBILITY STUDIES

2.1 Introduction

In order to ascertain the commercial viability of the permanganate-oxalate reaction for use in a time-temperature indicator system, it is not enough to create a system composition that runs for a specific length of time at a specific temperature. It must also be shown that the run time achieved for a given composition is repeatable both among replicates of a given batch as well as between batches of the same system composition. For this purpose reproducibility studies were carried out to determine whether the various reaction systems under development are capable of achieving consistent, repeatable run times, within an error margin of $\pm 10\%$ of the target run time.

It is also necessary, in order that perchlorate-based systems may be accurately prepared to eutectic composition, that a method for quantifying perchlorate concentration in stock solutions be adopted for this project. To this end, the potentiometric titration method by Selig (1979) was used to quantify the concentration of perchlorate in stock solutions prepared by weighing the solid and diluting to a known volume.

As mentioned in the previous chapter, the hygroscopic nature of perchlorate salts makes it unreliable to prepare stock solutions with an accurately knowable concentration of perchlorate, necessitating the need for quantification. Selig's method answers this need by measuring the potential of the sample solution with an ion-selective electrode as the perchlorate ion is precipitated out of solution by the slow addition of CETAB titrant. As it is not possible to indicate the end of precipitation using conventional indicators, the end point is obtained by calculating the second derivative of the potential versus volume graph.

2.2 Methods

Stock solutions of sodium oxalate, perchloric acid, and potassium permanganate were prepared in clear snap-cap 2 mL Eppendorf tubes, while reaction systems were prepared in 96-well micropipette plates as well as in Eppendorf tubes. All materials used for preparation were pre-rinsed with ultrahigh purity 2% nitric acid then thoroughly dried to minimize contamination from Mn(II), and all stock solutions and reaction systems were prepared using 18.2 megaohm-cm deionized water. Stock solutions of potassium permanganate were prepared fresh on the day of each experiment.

Stock perchlorate solution was prepared from solid perchlorate salt (sodium, magnesium, or lithium perchlorate) dissolved in deionized water and diluted to the mark of a 50.00 mL volumetric flask, with the solution being weighed so that the density of the solution could be calculated from the known volume of the solution. Perchlorate concentration was then quantified according to the potentiometric method devised by Selig (1979), performed in triplicate.

Briefly, a 1.00 mL aliquot was taken and quantitatively diluted to 500.0 mL, and a 20.00 mL aliquot of the diluted solution was then taken as sample solution, to which 30 mL of deionized water and 1.00 mL of ionic strength adjustor solution was added. The sample was then titrated with an approximately 0.025 M solution of CETAB previously standardized against 50.00 mL of a 1000 ppm perchlorate reference solution from Cole-Parmer, the potential being measured with a perchlorate-specific ion-selective electrode. From this, the numerical second derivative of the potential vs volume graph was computed to find the endpoint, and from there stoichiometrically calculate the exact molarity of the

stock solution. Mass percent could then be calculated from molarity using the molar mass of the perchlorate salt used and the previously obtained density of the solution.

Reaction systems composed of deionized water or stock perchlorate salt solution, perchloric acid, sodium oxalate, and potassium permanganate, were prepared in Eppendorf tubes and in 96-well plates, everything being homogenized before permanganate solution was added and the reaction system homogenized once more. This was taken as the initiation of the reaction. Kinetic absorbance measurements were carried out in triplicate in 96-well plates using a Thermo Scientific Multiskan Go UV-Vis spectrophotometer. Absorbance measurements were taken at the permanganate λ_{max} of 525 nm at regular time intervals to monitor the reaction progress over time.

These reaction systems were prepared to the following compositions. For antifreeze-free aqueous systems, target run time 1 hour at 25°C: 0.01 M perchloric acid, 1.38 mM sodium oxalate, 0.50 mM potassium permanganate. For sodium perchlorate-based systems, target run time 5 minutes at 25°C: 52% w/w NaClO₄, 0.1 M HClO₄, 1.38 mM Na₂C₂O₄, 0.14 mM Mn(ClO₄)₂, and 0.50 mM KMnO₄. For magnesium perchlorate-based systems, target run time 5 minutes at 25°C: 44.0% w/w Mg(ClO₄)₂, 0.1 M HClO₄, 1.38 mM Na₂C₂O₄, 13.5 μ M Mn(ClO₄)₂, and 0.50 mM KMnO₄. For lithium perchlorate-based systems, target run time 5 minutes: 25.1% w/w LiClO₄, 0.1 M HClO₄, 1.38 mM Na₂C₂O₄, 35 μ M Mn(ClO₄)₂, and 0.50 mM KMnO₄. All reaction systems were prepared to a final volume of 200 μ L.

Reproducibility studies were conducted by preparing reaction systems in batches of six replicates, according to the procedure previously described, on four separate occasions, for a total of 24 runs. Trajectory analysis using root-mean-square calculations

was then performed in MS Excel to gauge the variability of individual runs in a single batch and their deviation from the batch mean, as well as variation between different batches of the same reaction system and their deviation from the overall mean.

The individual time points of each run were lined up to the nearest 10-second interval (the nearest 1-minute interval in the case of the 1-hour system) and a mean trajectory was created by taking the average of the absorbances at each time point across all the runs for a given day's batch (N=6). For each run, the residual for each time point was obtained by taking the square of the difference between the actual absorbance y_t and the mean trajectory's absorbance \hat{y}_t :

$$(y_t - \hat{y}_t)^2$$

Following this, the normalized root mean square deviation with respect to the day's average was obtained for each run using the formula

$$NRMSD = \frac{1}{\bar{y}} \sqrt{\left(\frac{1}{T}\right) \sum_{t=1}^T (y_t - \hat{y}_t)^2} \times 100\%$$

where T is the total number of time points and \bar{y} is the average of all the absorbances across the time points of the individual run.

In similar manner, an overall mean trajectory was created by taking the averages of the absorbances at each time point across all the runs for a given reaction system (N=24), and residuals for each run were obtained and used to calculate the root mean square deviation with respect to the overall average.

2.3 Results

Representative data for perchlorate quantification are given in Figures 2 and 3, where Figure 2 shows the potential versus volume graph for the standardization of CETAB against 1000 ppm perchlorate standard along with its associated second derivative graph, and Figure 3 shows the titration curve and corresponding second derivative for a sodium perchlorate stock solution. Both procedures were carried out in triplicate (N=3).

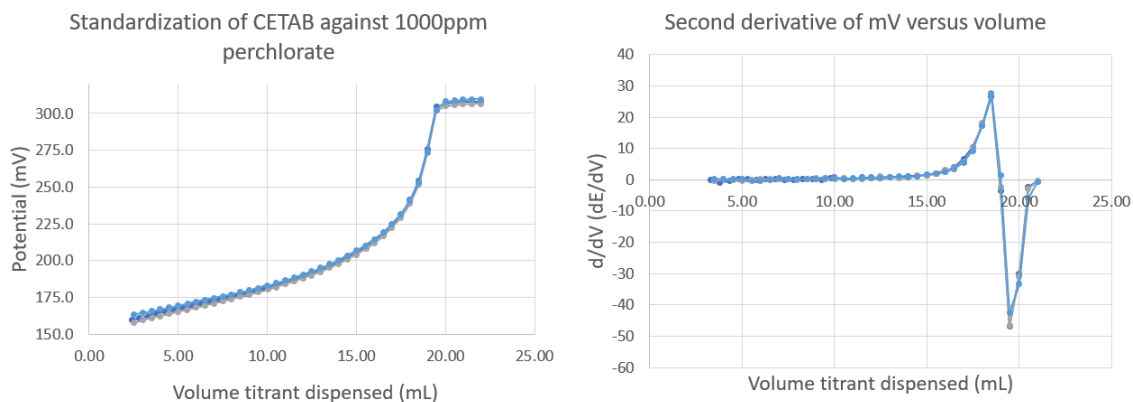


Figure 2. Potential versus volume and second derivative curves for the standardization of CETAB with 1000 ppm perchlorate standard. Molarity of CETAB was calculated to be 0.02650 ± 0.00013 M at the 95% confidence interval.

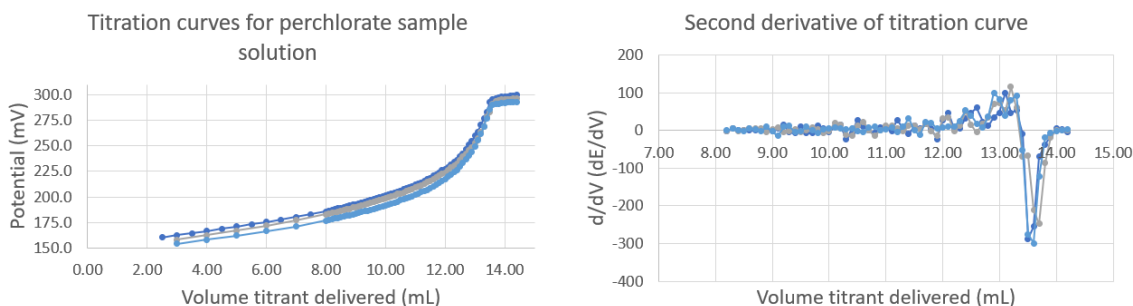


Figure 3. Potential versus volume and second derivative curves for the titration of sodium perchlorate sample solution with standardized CETAB. Molarity of sodium perchlorate stock solution was calculated to be 8.853 ± 0.044 M at 95% CI. Mass percent NaClO_4 was calculated to be 66.13 ± 0.33 %w/w at the same confidence level.

Absorbance-time plots for separate batches of each reaction system are given in Figures 4 to 7. The offset of the data points from time $t=0$ is due to the delay between reaction initiation and first absorbance reading, caused by the need to homogenize each replicate in a given batch after the final addition of potassium permanganate solution.

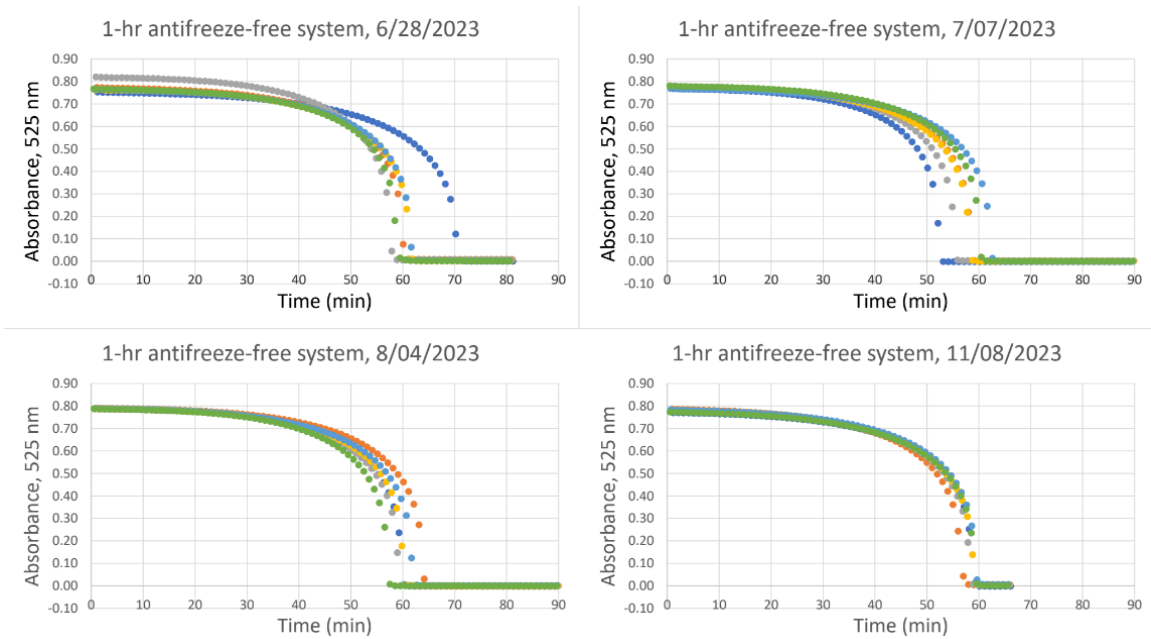


Figure 4. Absorbance versus time plots for antifreeze-free TTI system with target run time 1 hour at 25°C. N=6 for each day, for a total N=24.

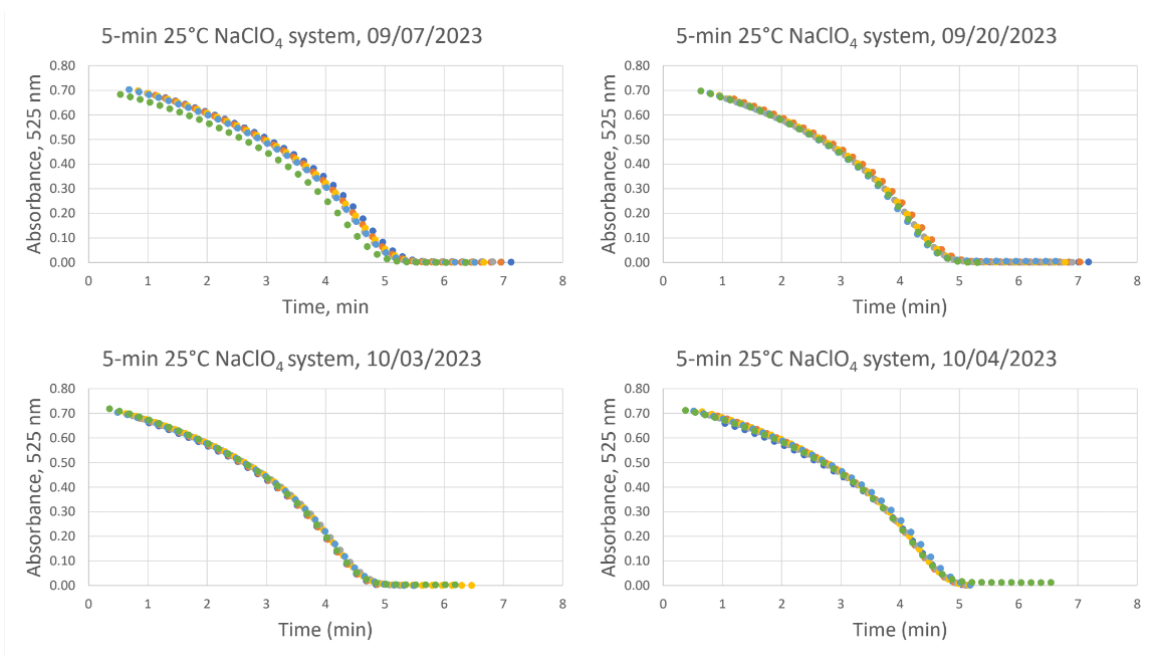


Figure 5. Absorbance versus time plots for eutectic (52% w/w) sodium perchlorate TTI system with target run time 5 minutes at 25°C. N=6 for each day, for a total N=24.

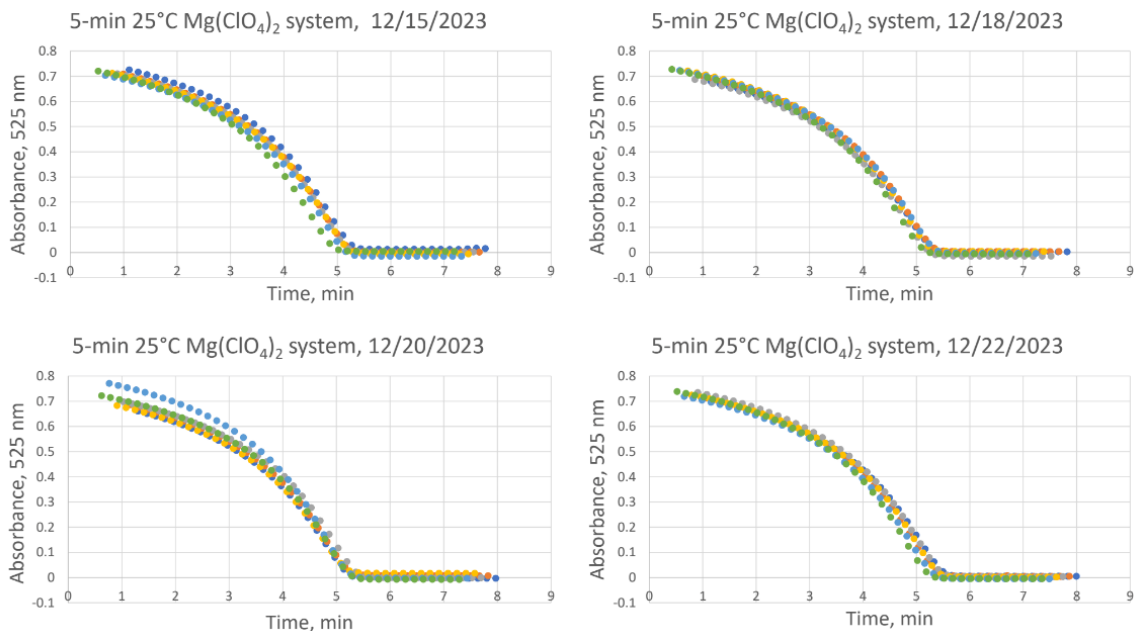


Figure 6. Absorbance versus time plots for eutectic (44.0% w/w) magnesium perchlorate TTI system with target run time 5 minutes at 25°C. N=6 for each day, for a total N=24.

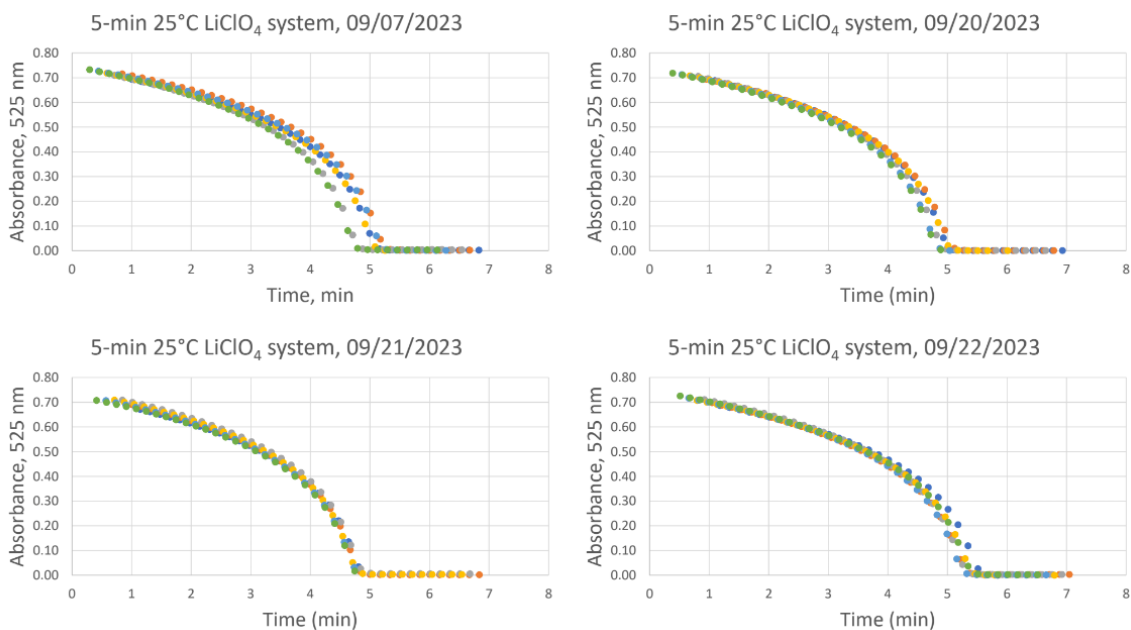


Figure 7. Absorbance versus time plots for eutectic (25.1% w/w) lithium perchlorate TTI system with target run time 5 minutes at 25°C. N=6 for each day, for a total N=24.

Because the transition from descending absorbance to a static value around and after the endpoint is sometimes more gradual than sudden, and because of variability in post-endpoint absorbance values, a cutoff absorbance value is necessary as an arbitrary

benchmark below which the absorbance is considered negligible enough to judge the reaction complete. For the purposes of trajectory analysis then, an absorbance value of 0.02 was chosen as the cutoff beyond which the reaction was considered to be complete, based on the absorbance value below which the reaction systems were judged as visually colorless. Using these criteria, the average run times for each batch of each reaction system is given in Tables 1 to 4, as well as their respective coefficients of variation and percent accuracy. Overall averages for each system, calculated as the average of the run times of all 24 runs for a given system, are also given in the respective tables, along with their corresponding coefficients of variation and percent accuracy.

Table 1: Average run times for antifreeze-free system, with coefficient of variation and percent accuracy. N=6 for each day, for a total N=24 for overall average.

	Daily average (min)	CV	% accuracy
Day 1	62.50	7.1	104.2%
Day 2	58.50	6.1	97.5%
Day 3	61.17	4.1	101.9%
Day 4	59.50	1.8	99.2%
Overall average	60.42	5.5	100.7%

Table 2: Average run times for eutectic (52% w/w) sodium perchlorate system, with coefficient of variation and percent accuracy. N=6 for each day, for a total N=24 for overall average.

	Daily average (min)	CV	% accuracy
Day 1	5.28	3.3	105.6%
Day 2	4.92	1.9	98.3%
Day 3	4.78	1.8	95.6%
Day 4	4.94	1.7	98.9%
Overall average	4.98	4.3	99.6%

Table 3: Average run times for eutectic (44.0% w/w) magnesium perchlorate system, with coefficient of variation and percent accuracy. N=6 for each day, for a total N=24 for overall average.

	Daily average (min)	CV	% accuracy
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Day 1	5.25	3.3	105.0%
Day 2	5.31	1.3	106.1%
Day 3	5.36	1.3	107.2%
Day 4	5.47	2.3	109.4%
Overall average	5.35	2.6	106.9%

Table 4: Average run times for eutectic (25.1% w/w) lithium perchlorate system, with coefficient of variation and percent accuracy. N=6 for each day, for a total N=24 for overall average.

	Daily average (min)	CV	% accuracy
Day 1	5.11	4.5	102.2%
Day 2	5.03	3.3	100.6%
Day 3	4.86	2.6	97.2%
Day 4	5.47	2.3	109.4%
Overall average	5.12	5.4	102.4%

Average root-mean-square deviations for each batch in the chosen reaction systems are given in Tables 5 to 8. These tables give average RMSD values based on the deviations of each replicate run from the mean trajectory of the batch it belongs to (intra-day comparison), as well as average RMSD values based on the deviations of each run from the overall mean trajectory across all 24 runs for a given reaction system (inter-day comparison). Overall averages for both intra-day and inter-day comparisons, obtained by calculating the average of the corresponding RMSD values for all 24 runs of a given system, are also given in the same tables.

Table 5: Average root-mean-square deviations for antifreeze-free system, with respect to the mean trajectory of each day (intra-day comparison) as well as with respect to the overall mean trajectory (inter-day comparison). N=6 for each day, for a total N=24 for inter-day comparison.

	Average RMSD, intra-day comparison	Average RMSD, inter-day comparison
Day 1	11.3	9.9
Day 2	12.9	12.1
Day 3	8.0	7.9

Day 4	4.1	5.8
Overall average	9.1	9.0

Table 6: Average root-mean-square deviations for eutectic (55% w/w) sodium perchlorate system. N=6 for each day, for a total N=24 for inter-day comparison.

	Average RMSD, intra-day comparison	Average RMSD, inter-day comparison
Day 1	5.5	12.5
Day 2	2.6	3.7
Day 3	2.5	9.0
Day 4	3.5	3.9
Overall average	3.6	7.3

Table 7: Average root-mean-square deviations for eutectic (42.0% w/w) magnesium perchlorate system. N=6 for each day, for a total N=24 for inter-day comparison.

	Average RMSD, intra-day comparison	Average RMSD, inter-day comparison
Day 1	6.3	7.6
Day 2	3.0	3.8
Day 3	6.2	6.2
Day 4	4.1	6.6
Overall average	4.9	6.0

Table 8: Average root-mean-square deviations for eutectic (25.1% w/w) lithium perchlorate system. N=6 for each day, for a total N=24 for inter-day comparison.

	Average RMSD, intra-day comparison	Average RMSD, inter-day comparison
Day 1	8.5	9.5
Day 2	6.2	7.4
Day 3	4.5	13.5
Day 4	3.6	13.5
Overall average	5.7	11.0

2.4 Discussion

In general, trajectory analysis shows a fairly high degree of precision between replicate runs on a given day, and to a lesser extent between replicate runs across different days. This trend appears to hold across all four reaction media (antifreeze-free, eutectic sodium perchlorate, magnesium perchlorate, and lithium perchlorate), though the scatter is

most pronounced in antifreeze-free systems. Even with one run each for two of the antifreeze-free batches (as well as one in one of the eutectic LiClO₄ batches) for which the reaction run time falls outside the +/-10% margin of accuracy, the mean run time on individual days remains within the acceptable margin for the target run time.

Average root-mean-square deviations tend to increase when comparing average RMSD based on the batch mean to average RMSD based on the overall mean, due to the larger sample size involved in the calculation of the latter. The first two batches of the antifreeze-free system do not follow this trend (that is, their batch-based average RMSD is larger, not smaller, than their overall average RMSD), but this is likely due to the fact that these two batches contain one run each whose run times fall outside the acceptable $\pm 10\%$ margin, and RMSD values are sensitive to larger deviations from the mean.

CHAPTER 3

FREEZE-THAW EXPERIMENTS

3.1 Introduction

The purpose of a time-temperature indicator is to track whether the item it is attached to has been held above a certain temperature threshold for a certain period of time. This responsivity is dependent on how much the time-temperature-dependent process has progressed at the time of examination, and this degree of reaction progress is dependent on the amount of time spent in storage as well as the range of temperatures it has been exposed to thus far. To this end it was deemed necessary to study the effect of preliminary freeze time as well as that of repeated freeze-thaw cycles on TTI systems, as the reaction is not expected to progress while in the solid state. This would also ascertain whether the eutectic composition of the reaction system has any sort of effect on system behavior under conditions of repeated freezing and thawing out, as part of the rationale behind using eutectic compositions is to avoid the formation of 2-phase systems that might cause the reaction to behave differently when the system is frozen and thawed.

3.2 Methods

All materials were rinsed with 2% nitric acid and dried as described in chapter 2.2. All stock solutions were prepared as described in the same section.

3.2.1 Preliminary freeze time experiments

Reaction systems were prepared in Eppendorf tubes as described in chapter 2.2. Immediately after reaction was initiated by vortexing, the tubes were flash frozen in an ethanol-dry ice bath and transferred into a freezer at -80°C , where they were incubated for periods of 2 weeks, 1 month, 2 months, and 4 months. At the end of the incubation period,

the tubes were taken out of the freezer and thawed in a stirred beaker of room-temperature water until fully liquid, or for 1 minute in the case of eutectic magnesium perchlorate systems, after which their contents were transferred into 96-well plates and their absorbance at 525 nm was monitored over time. Non-parametric one-way analysis of variance (the Kruskal-Wallis test) was performed to determine whether there was any significant difference among all run times in the system, with Dunn's multiple comparisons test performed afterwards to compare the run times of each test system (those which were preliminarily frozen) to those of the control systems (which were not) and determine the presence of any significant difference. Dunn's test was repeated a second time to compare "adjacent" sets (i.e. the control with the 2-week, the 2-week with the 1-month, the 1-month with the 2-month, and the 2-month with the 4-month) in like manner.

Reaction system compositions for these experiments were identical to those for reproducibility experiments described in chapter 2.2, with the only differences in final concentrations of components being for that of $\text{Mn}(\text{ClO}_4)_2$. For antifreeze-free aqueous systems, target run time 1 hour at 25°C: all final concentrations as given in 2.2. For sodium perchlorate-based systems, target run time 5 minutes at 25°C: all final concentrations as given in 2.2, except that of $\text{Mn}(\text{ClO}_4)_2$, which was 0.23 mM. For magnesium perchlorate-based systems, target run time 5 minutes at 25°C: all final concentrations as given in 2.2, except that of $\text{Mn}(\text{ClO}_4)_2$, which was 27.5 μM . For lithium perchlorate-based systems, target run time 5 minutes: all final concentrations as given in 2.2, except that of $\text{Mn}(\text{ClO}_4)_2$, which was 0.06 mM. All reaction systems were prepared to a final volume of 200 μL .

3.2.2 Freeze-thaw cycle experiments

Reaction systems were prepared in Eppendorf tubes and flash-frozen in an ethanol-dry ice bath as described in the previous section, following which they were transferred into a freezer at -80°C to be subjected to repeated cycles of freezing and thawing. Freeze-thaw cycles consisted of 24 hours frozen at -80°C followed by thawing in a stirred beaker of room-temperature water until the reaction systems had spent a full minute in the fully liquefied state, after which they were either flash-frozen in ethanol-dry ice in preparation for another freeze-thaw cycle, or else their contents were transferred into 96-well plates and their absorbance at 525 nm was monitored over time. Non-parametric one-way analysis of variance was performed as in chapter 2.2.1 to determine whether there was any significant difference among all run times in the system, and Dunn's multiple comparisons test was performed afterwards to compare average reaction run times of sets exposed to different numbers of freeze-thaw cycles with those of the control systems which had not been. As in the previous section, Dunn's test was performed a second time to compare adjacent systems (i.e. control and 1 cycle, 1 cycle and 2 cycles, 2 cycles and 3 cycles) to determine any significant difference in run times between each pair of sets.

Reaction system compositions for these experiments were identical to those for reproducibility experiments described in chapter 2.2. All reaction systems were prepared to a final volume of 200 μL .

3.3 Results

3.3.1 Preliminary freeze time experiments

A representative plot of absorbance versus time for one reaction system, in this case the eutectic sodium perchlorate system, is given in Figure 8 as an example of the kind of behavior seen in this experiment. As was the case with Figures 4 to 7, the offset between

time $t=0$ and the first absorbance reading for each set is due to the time needed to transfer the reaction systems into 96-well plates and load them into the spectrophotometer after complete thawing was achieved (full liquefaction for antifreeze-free, eutectic NaClO_4 , and LiClO_4 ; 1 full minute for eutectic $\text{Mg}(\text{ClO}_4)_2$), which was taken as the starting point for measuring reaction run time. Figure 9 shows the reaction run times for each reaction system, with each dot indicating a single replicate, and the error bars having a width of 1 standard deviation about the mean. Brackets connect pairs of systems for which Dunn's test gave a p value below 0.05, with one asterisk (*) indicating $p < 0.05$, two (**) indicating $p < 0.01$, and three (***) indicating $p < 0.001$.

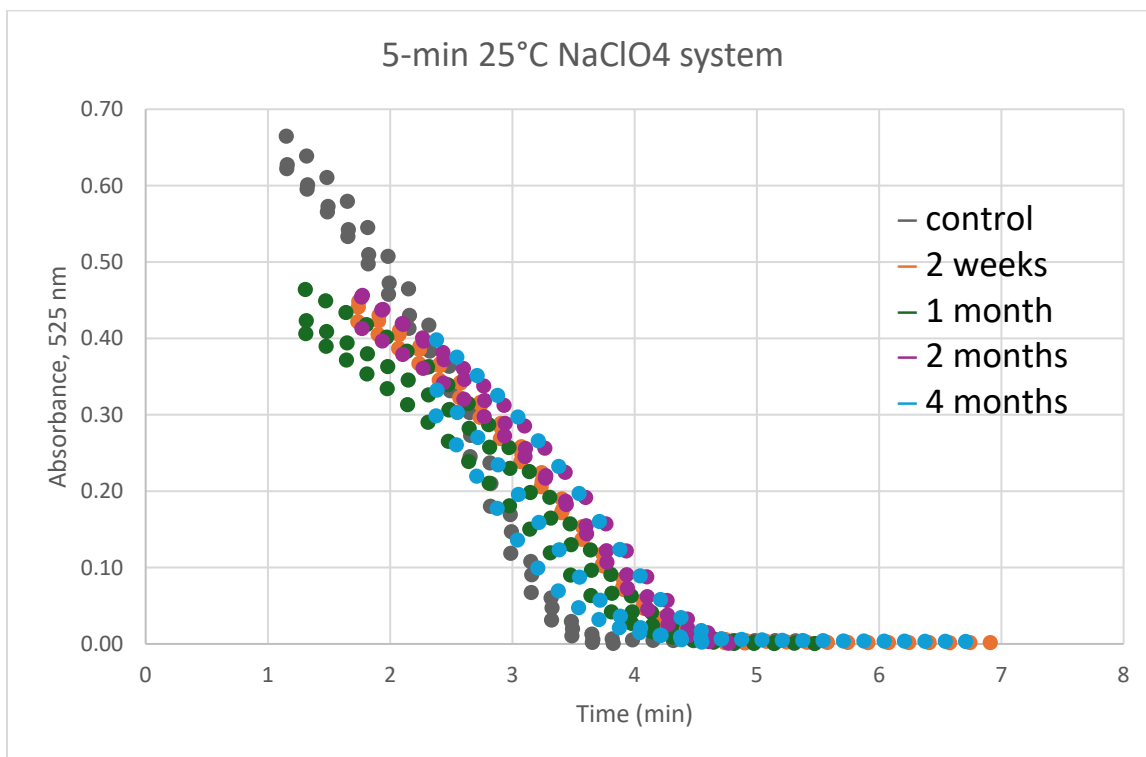


Figure 8: Absorbance versus time plot of eutectic (52% w/w) sodium perchlorate systems subjected to varying lengths of preliminary freeze time. $N=3$ for each set.

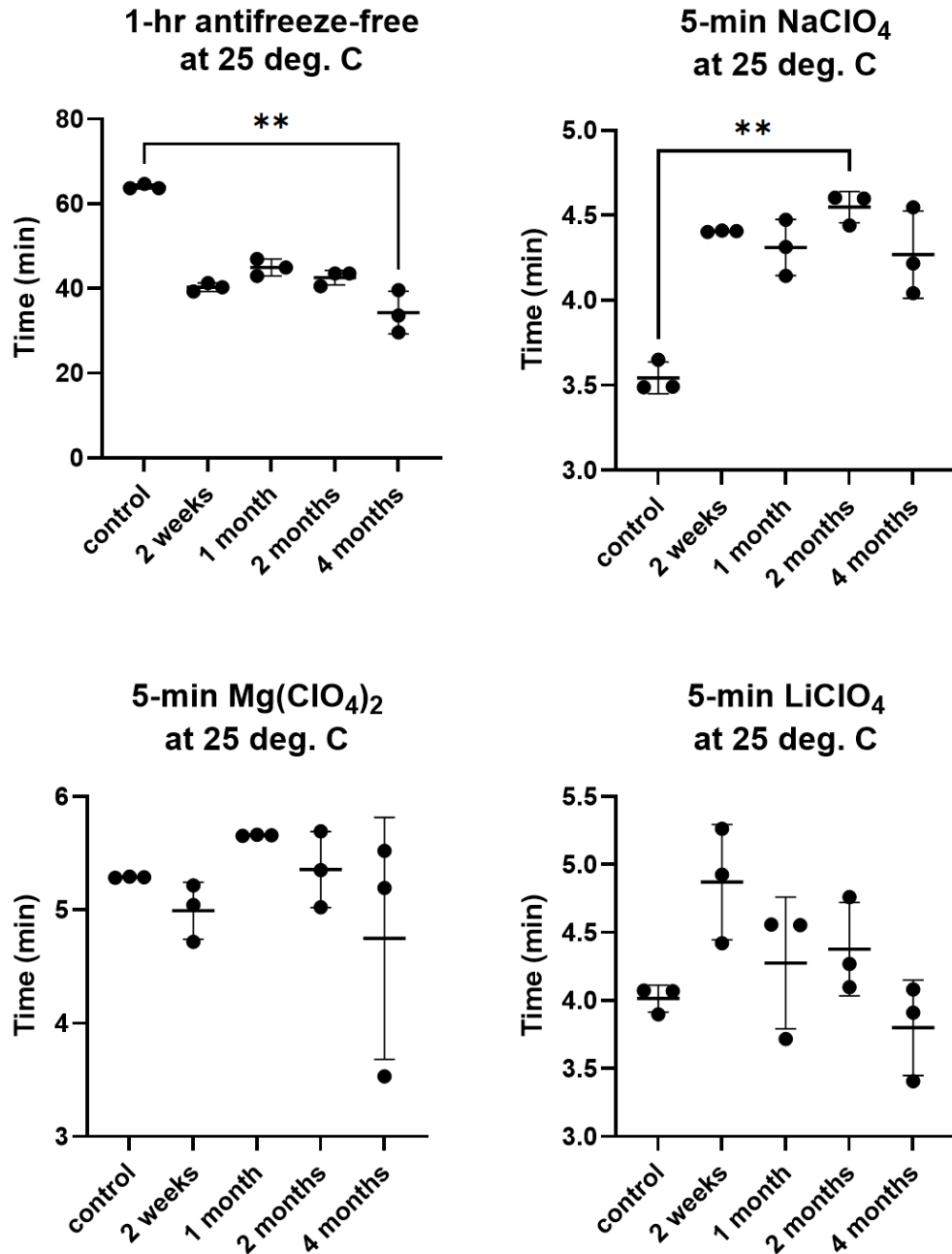


Figure 9: Reaction run times for TTI systems subjected to preliminary freezing at -80°C for 2 weeks, 1 month, 2 months, or 4 months, compared to run times for TTI systems run without freezing. $N=3$ for each set. $p < 0.05$ is indicated as *, $p < 0.01$ as **, and $p < 0.001$ as ***.

The results of the Kruskal-Wallis test and the Dunn's tests performed after it are given in Table 9, with the pairs of sets tested indicated in the leftmost column and

significant p values marked in accordance with the significance levels mentioned previously.

Table 9: Summary of results for Kruskal-Wallis test with Dunn's multiple comparison post-test. N=15 for each system, with N=3 for each set (control, 2 weeks, 1 month, 2 months, 4 months). K (number of sets tested) = 5.

	Antifreeze-free	Sodium perchlorate	Magnesium perchlorate	Lithium perchlorate
Kruskal-Wallis test (non-parametric one-way ANOVA)				
Kruskal-Wallis statistic, <i>H</i>	12.40	9.833	7.233	8.433
p value	0.0002 (***)	0.0143 (*)	0.1058	0.0464 (*)
Multiplicity-adjusted p values, Dunn's multiple comparisons test (K=5)				
control vs 2 weeks	0.0548	0.2209	>0.9999	0.0899
control vs 1 month	>0.9999	0.4014	0.6836	>0.9999
control vs 2 month	0.4828	0.0076 (**)	>0.9999	0.5765
control vs 4 months	0.0056 (**)	0.4828	>0.9999	>0.9999
2 weeks vs 1 month	0.5765	>0.9999	0.0548	0.8050
1 month vs 2 months	>0.9999	0.5765	>0.9999	>0.9999
2 months vs 4 months	0.4014	0.4828	>0.9999	0.4014

3.3.2 Freeze-thaw cycle experiments

Only two systems were investigated for freeze-thaw cycle experiments, namely the 5-minute sodium perchlorate system and the 5-minute magnesium perchlorate system. The graph of absorbance over time for the former is given in Figure 10, with the offset of the absorbance readings in each set from t=0 being due to the same cause as for Figure 8.

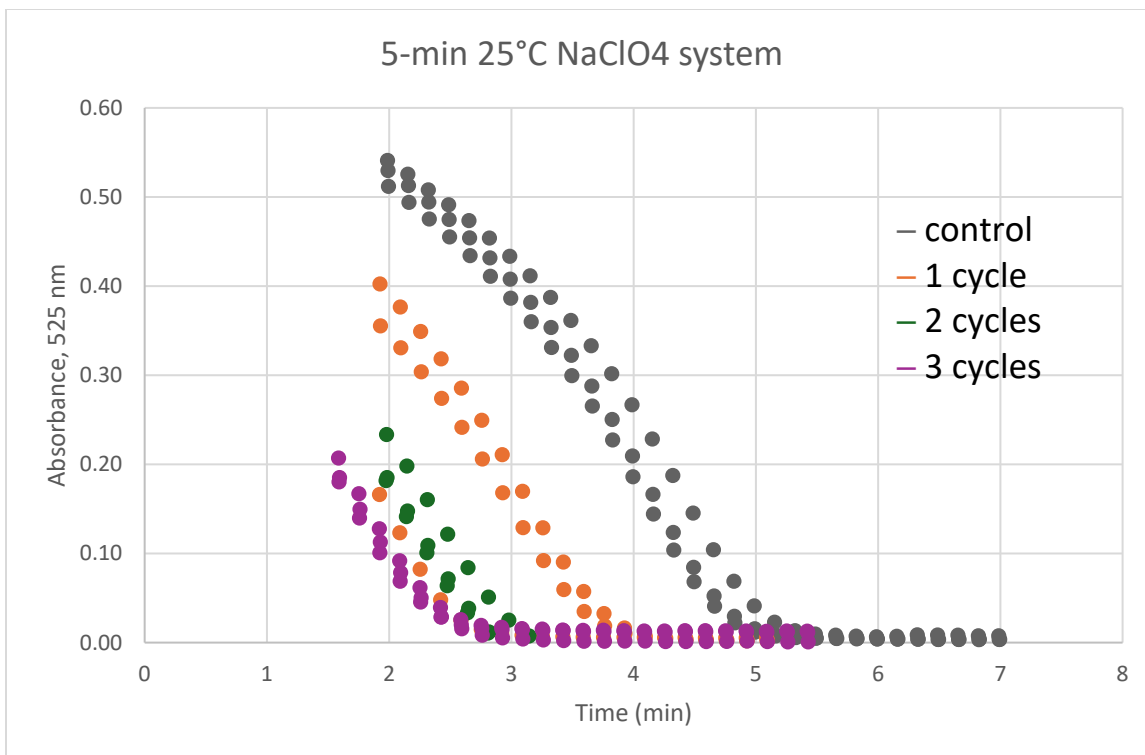


Figure 10: Absorbance versus time plot for eutectic (52% w/w) sodium perchlorate reaction systems subjected to repeated freeze-thaw cycles. N=3 for each set.

A graphical summary of the run times for each system after a given number of freeze-thaw cycles is given in Figure 11, where, as in Figure 9, each dot is one replicate, and the error bars have a width of one standard deviation around the mean. Brackets join sets with a significant difference in means as described in the previous section.

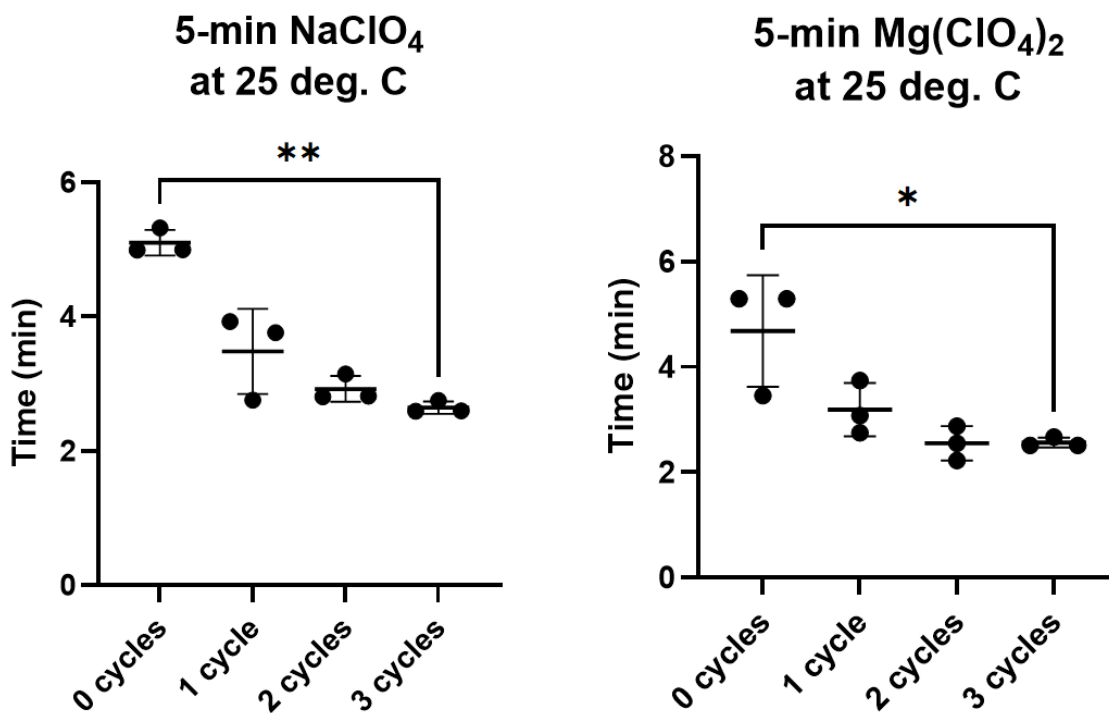


Figure 11: Reaction run times for TTI systems subjected to repeated freeze-thaw cycles. N=3 for each set. $p < 0.05$ indicated by *, $p < 0.01$ by **, $p < 0.001$ by ***.

As was done in chapter 3.3.1, the results of the non-parametric one-way ANOVA and its post test are given in Table 10, with pairs of sets tested indicated and p values less than 0.05 marked according to their level of significance.

Table 10: Summary of Kruskal-Wallis test with Dunn's multiple comparison post-test, for reaction systems subjected to multiple freeze-thaw cycles. N=12 for each system, N=3 for each set (control, 1 cycle, 2 cycles, 3 cycles). K = 4.

	Sodium perchlorate	Magnesium perchlorate
Kruskal-Wallis test (non-parametric one-way ANOVA)		
Kruskal-Wallis statistic, H	9.462	8.282
p value	0.0014 (**)	0.0121 (*)
Multiplicity-adjusted p values, Dunn's multiple comparisons test (K=4)		
control vs 1 cycle	0.5227	>0.9999
control vs 2 cycles	0.2683	0.0706
control vs 3 cycles	0.0067 (**)	0.0382 (*)
1 cycle vs 2 cycles	>0.9999	0.5227
2 cycles vs 3 cycles	0.5227	>0.9999

3.4 Discussion

3.4.1 Preliminary freeze time experiments

It is clear that there is a distinct decrease in reaction run time for the antifreeze-free system, going from the control to the 2-week incubation set. This decrease appears to become minuscule going from the 2-week to 1-month to 2-month sets, and is more discernible going from the 2-month to 4-month sets. Despite this appearance, the one-way ANOVA results suggest that while there is a significant difference among the various sets in the system ($p = 0.0002$), the differences between the 2-week, 1-month, and 2-month sets and the control are not significant ($p \geq 0.05$ in all cases), whereas there is a significant difference in means between the 4-month set and the control ($p = 0.0056$).

For eutectic-containing systems, the trends are less clear. It is sometimes the case that the control runs for a shorter length of time than the systems incubated in the freezer. This is most pronounced in the sodium perchlorate-based systems and less evident in the lithium perchlorate-based systems, and is likely due to the fact that the control systems were not flash-frozen and incubated at -80°C , whereas the systems incubated in the freezer were thawed out only to the point of full liquefaction before being transferred into 96-well plates to have their absorbances monitored. If this is done quick enough, these systems may enter the spectrophotometer at a somewhat lower temperature than the control, and so the rate is slower than expected, leading to somewhat prolonged run times.

Regardless, the general trend observed for the eutectic-containing systems, based on the one-way ANOVA results, seems to suggest a lack of significant difference in run time across sets in a given reaction system, with a p value less than 0.05 found only for the sodium perchlorate system ($p = 0.0143$). Looking at the results of the Dunn's post test

comparing individual sets, the only sets for which the test suggested a significant difference with respect to the control were the 4-month antifreeze-free system ($p = 0.0056$) and the 2-month sodium perchlorate-based system ($p = 0.0076$), both cases where the control and the sets in question represented the highest and lowest run time values overall.

However, when Dunn's test is run to compare adjacent sets (that is, comparing the control and 2-week sets, the 2-week and 1-month sets, the 1-month and 2-month sets, and the 2-month and 4-month sets), in every comparison across all four reaction systems the p value is found to be greater than 0.05, suggesting that there is no significant difference in average run time between each pair of sets thus examined. This would seem to suggest that there is no significant change in run time with increasing length of preliminary freeze time, but care must be taken not to read too much into these results, as the smallness of N in these experiments ($N=3$ for each set, total $N=15$ for each system) limits the statistical power of the analyses conducted.

3.4.2 Freeze-thaw cycle experiments

It was mentioned in the previous section that the thawing protocol for the preliminary freeze time experiments, in which the reaction systems were thawed to full liquefaction (or for 1 minute in the case of eutectic magnesium perchlorate systems), may have led to these systems entering the spectrophotometer somewhat lower in temperature than anticipated. Because of this, the thawing protocol was revised for the freeze-thaw cycle experiments, adding an additional minute of immersion in room-temperature water to allow the reaction systems to warm up and the reaction to proceed to a more significant extent with each cycle.

Both reaction systems examined for freeze-thaw cycle behavior exhibit a decrease in reaction run time with successive freeze-thaw cycles, but this decrease in reaction run times appears itself to decrease in magnitude with each consecutive cycle. It does not appear to be possible to measure a decrease in run time beyond 3 freeze-thaw cycles, as the run time may risk growing short enough as to fall within the time period allotted for transferring the reaction mixtures into 96-well plates for absorbance measurements, meaning that the reaction could be completed before the first absorbance readings are made.

Non-parametric one-way ANOVA indicates that there is at least one significant difference in one of the run times vs. the control for both systems, with the Dunn's post test identifying a difference between the control set and the 3-cycle set for both sodium perchlorate ($p = 0.0067$) and magnesium perchlorate ($p = 0.0382$) systems. Using Dunn's test to compare "adjacent" sets (control and 1 cycle, 1 and 2 cycles, 2 and 3 cycles) shows no significant difference between them in each case, with the implication for both systems that the change in run time with each additional freeze-thaw cycle is insignificant until after the 3rd consecutive cycle.

CHAPTER 4

CONCLUSION

From the outset this thesis was part of a wider project focused on developing TTI systems with a broad range of ultracold working temperatures based on the redox reaction between permanganate and oxalate in aqueous acidic media. Four reaction systems were investigated on the basis of properties conducive to commercial viability, namely reproducibility and accuracy of run times, effect of prolonged storage at ultracold temperatures on run time, and effect of repeated freezing and thawing on run time.

All reaction systems studied were shown to be able to give reproducible run times within 10% of their target run time. The overall average run time accuracy could range from 99.6% for eutectic sodium perchlorate systems to 106.9% for eutectic magnesium perchlorate systems, but daily batch average accuracies could be as low as 97.2% or as high as 109.4%. Run times were reproducible to good precision between replicates in a single day's batch, with daily coefficients of variation ranging from 1.3 to 7.1, and also reproducible across different days, with the overall average CV as low as 2.6 for eutectic magnesium perchlorate systems and as high as 5.5 for antifreeze-free aqueous systems.

All perchlorate-containing systems exhibited little to no change in run time after prolonged periods of storage at -80°C , though in some cases due to the manner in which the reaction systems were thawed their run times came out slightly longer than for systems which were not frozen at all. In general, this change was not judged to be significant. Antifreeze-free aqueous systems on the other hand showed a pronounced decrease in run time after 2 weeks frozen at -80°C , but little to no decrease beyond that for systems frozen

for up to 4 months. The decrease after 2 weeks is not judged to be significant, the decrease in run time only being considered as significant after 4 months.

The eutectic sodium and magnesium perchlorate systems were found to lose run time as the reaction progressed under conditions of repeated freezing and thawing, a change which was seen to decrease in magnitude with each successive freeze-thaw cycle. This change in run time is not judged to be significant until after 3 freeze-thaw cycles for either system. Further investigation beyond 3 cycles could not be undertaken for concern that it would not be possible to measure reaction progress on account of premature reaction completion.

Taken as a whole, all of these indicate that TTI systems based on the acidic permanganate-oxalate systems can be prepared reproducibly and consistently, and are insensitive to long periods of storage at -80°C . In addition, perchlorate-based systems can tolerate up to three short (<5 minutes) excursions into room temperature before run times are significantly shortened. Such accurate reproducibility and stability at low temperature speaks in favor of the commercial viability of these systems, opening the way for further developments in the future.

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