Evaluation of Buffer Stability for the Production of Small Molecules

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***Abstract*— There are many types of buffering agent that can be used in the process line of biopharmaceutical industry. The most common buffering agent used in the biopharmaceutical industry mainly in the production of human recombinant insulin are ammonium acetate buffer, glycine buffer, potassium dihydrogen phosphate buffer, sodium acetate buffer, and tris(hydromethyl)aminomethane buffer solutions. These buffering agents are responsible to resist the pH changes throughout the production processes according to their compatibility towards the processes. The purposes of this research are to evaluate the stability of buffer solutions for a small molecules production mainly in the production of insulin and to propose the planning and management of the buffers uses and its storage. The research included the preparation of the buffer solutions, filtration of the buffer solutions, and the analysis of the buffer stabilities were based on the appearance changes, pH changes, and determination of the buffering capacities for each of the buffer solution. All the buffer solutions were stored at vary condition which are refrigerated and room temperature conditions. The buffering capacity for the ammonium acetate buffer is high in the room temperature condition. The glycine buffer rich in the buffering capacity when stored at the refrigerated condition. For the potassium dihydrogen phosphate, the buffering capacity is better in the room temperature. The buffering capacities for tris(hydromethyl)aminomethane and the sodium acetate buffer shows good buffering capacity at the room temperature respectively.**

Keywords*— Biopharmaceuticals, Buffering agent, pH buffers, Small molecule, Stability

I. INTRODUCTION

Small molecule is a low molecular weight organic compounds which are not part of polymers [7]. It may help in a regulation of biological process with a size on the order of 1nM. Most of the small molecule has a molecular weight approximately 700 to 900 Da [4]. Its upper molecular weight limit is about 900 Da which allows the rapid possible diffusion across a cell membrane [4]. Thus, it can reach intracellular sites of action. Usually, pharmaceutical products are mostly the type of a small molecule. Small molecules may have a variety of biological functions. For instance, the small molecules can serve as cell signaling molecules, as drugs in medicine, as well as in farming that act as pesticide. Apart from that, small molecules can be natural which presence as secondary metabolites or artificially like antiviral drugs. It can give both beneficial and negative impacts against the consumers.

Small molecule products are widely produced in the pharmaceutical industries. Pharmaceutical is a medicinal product which consist of both active agents and formulated products including the therapeutics, prophylactics and in vivo diagnostics [8]. The two major subsets of pharmaceutical are drugs and biopharmaceuticals. Drug is defined as a pharmaceutical inherently chemical in nature which is not a biological and manufactured using a chemical method while the biopharmaceutical is a pharmaceutical inherently biological in nature and being manufactured through the biotechnology [4]. The biotechnology is the manufacture of products by or from the living organisms and usually involving the bioprocessing [4].

In the production of biopharmaceutical products, there are a few buffering agents were involved in the process line. Buffering agent is a part of essential excipient used in producing biopharmaceutical products. It is being used in a variety of processes in biopharmaceutical industry such as isolation and purification during the protein capture, polishing, filtration, chromatographic reaction, as well as the crystallization procedure [2]. A difference types of buffering agent will be used in a difference processes. The buffering agent must be specialized according to the compatibility of the processes. In the downstream processing, buffer is essential to protect products from the variation of pH. The resistance of pH changes is resulting from the buffering or adsorbing reaction.

Besides that, the significance of the buffering agent in the biopharmaceutical industry is to protect the preparation of product from any sudden pH changes when there is an addition of acid or base throughout the processes [2]. The biopharmaceutical products are sensitive towards the changes in pH which can lead to aggregation, denaturation, and also fragmentation [2].

According to the pharmacological perspective, it is essential to control the pH of the solution in order to minimize product degradation. Thus, it can make the improvement for the consumer comfort and compliance. Apart from that, buffer with the high – quality help to ensure the maximum recovery of biological products.

There are many types of buffering agent that can be used in the process line of biopharmaceutical industry. These buffering agents are responsible to resist the pH changes throughout the production processes according to their compatibility towards the processes. However, the presence of biological and particle contaminants in the buffer also can give impact towards the purity of final products. Therefore, the filtration of buffer is introduced in order to reduce or eliminate any contaminants that exists in the buffering agent.

The estimation of buffer volume used per year exceeding 2000 kL [2]. The huge amount of buffer used in the biopharmaceutical industry can cause negative impact towards the buffer stability when there are no appropriate planning and management of the buffer solutions.

The purpose of this research is to evaluate the stability of the buffers solution for the production of the small molecule products. This research is mainly focusing on the production of human recombinant insulin. Common buffering agent used throughout the production line of insulin are ammonium acetate, glycine,

potassium dihydrogen phosphate, sodium acetate, and tris(hydromethyl)aminomethane [13,14,15].

II. METHODOLOGY

A. Materials

Acetic acid (glacial) 100%, ammonium acetate, glycine, potassium dihydrogen phosphate, sodium acetate, and tris(hydromethyl)aminomethane were purchased from Merck. Hydrochloric acid 37% was purchased from Bendosen. Sodium Hydroxide (pellets) was purchased from R&M Chemical.

B. Preparation of ammonium acetate buffer

15.63 g of ammonium acetate, $C_2H_7NO_2$ were dissolved into 400 mL of distilled water. The desired pH was adjusted to 5.0 by the addition of acetic acid (glacial), CH_3COOH. The final volume was make up to 1000 mL by adding distilled water.

C. Preparation of glycine buffer

7.5 g of glycine, $C_2H_5NO_2$ were dissolved into 400 mL of distilled water. The desired pH was adjusted to 10.5 by the addition of sodium hydroxide, NaOH. The final volume was make up to 1000 mL by adding distilled water.

D. Preparation of potassium dihydrogen phosphate buffer

100 g of potassium dihydrogen phosphate, KH_2PO_4 were dissolved into 400 mL of distilled water. The desired pH was adjusted to 3.1 by the addition of hydrochloric acid, HCl. The final desired volume was make up to 1000 mL by adding distilled water.

E. Preparation of sodium acetate buffer

8.2 g of sodium acetate were dissolved into 400 mL of distilled water. The desired pH was adjusted to 3.5 by the addition of acetic acid (glacial), CH_3COOH. The final desired volume was make up to 1000 mL by adding distilled water.

F. Preparation of tris(hydromethyl)aminomethane buffer

12.11 g of Tris, $(HOCH_2)_3CNH_2$ base were dissolved into 400 mL of distilled water. The desired pH was adjusted to 7.5 by the addition of hydrochloric acid, HCl. The final desired volume was make up to 1000 mL by adding distilled water.

G. Filtration of buffer solutions

Ammonium acetate buffer, glycine buffer, potassium dihydrogen phosphate buffer, sodium acetate buffer, and tris(hydromethyl)aminomethane buffer were filtered by using the compressor and vacuum pump equipment in order to prevent the undesired particles presence in the buffers solution. The method of filtration is shown as **Fig. 1** below.

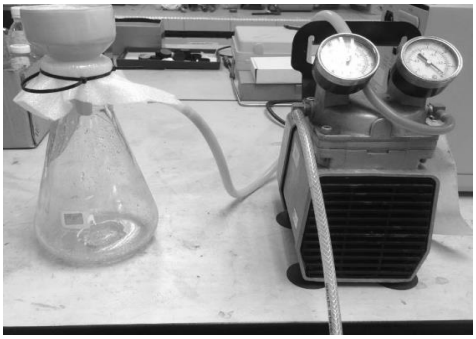


Fig. 1: The filtration of buffers solution method by using the compressor and vacuum pump

H. Analysis of buffer solutions

Ammonium acetate buffer, glycine buffer, potassium dihydrogen phosphate buffer, sodium acetate buffer, and tris(hydromethyl)aminomethane buffer were analyzed by the visual inspection and pH reading. pH of all the buffers solution were taken by using the Mettler Toledo pH probe. All the buffer solutions were stored at two different conditions which are at the room temperature and refrigerated condition. The method of pH analysis is shown as **Fig.2**.

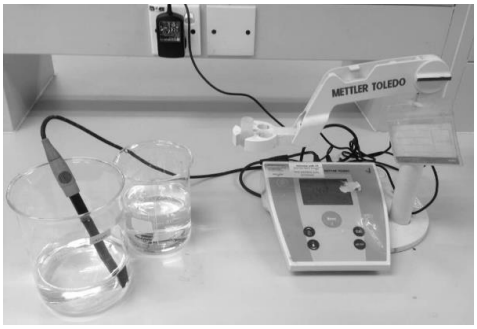


Fig. 2: The pH analysis of the buffer solutions by using pH probe

I. Determination of buffering capacity of buffer solutions

Buffering capacity of ammonium acetate buffer, glycine buffer, potassium dihydrogen phosphate buffer, sodium acetate buffer, and tris(hydromethyl)aminomethane buffer were calculated by using the equation as below:

$$\text{Buffering capacity, } \emptyset = \frac{\text{No. of moles added into 1L}}{\text{Change in pH}}$$

III. RESULTS AND DISCUSSION

A. The visual inspection on the buffer solutions at two different temperature

For the visual inspection of the ammonium acetate buffer, glycine buffer, potassium dihydrogen phosphate buffer, sodium acetate buffer, and tris(hydromethyl)aminomethane buffer solutions, there are no appearance changes were shown since initial day of the sampling. The color of this buffer solutions remained colorless started from day 0 until day 45 for both temperature conditions. There were also no unwanted particles or foreign substances were seen on these buffer solutions either for the refrigerated or the room temperature conditions.

This occurrence may be affected by the filtration method that was carried out by using the *compressor and vacuum pump* equipment. The significance of the filtration is mainly to reduce the contamination or unwanted content that may be presence in the buffer solutions [9]. All these buffer solutions can be considered did not contaminated throughout the experimental processes.

However, the definition of a good buffer to be used in processes of the production does not only depends on the visual inspection but the pH changes also need to be taken into consideration. The pH changes should not be out of specification in terms of the pH tolerance range.

B. The effects of temperature on the stability of ammonium acetate buffer

Based on the **Fig.1** shown below, the trend of the pH analysis is fluctuated. From the initial day until day 3, the pH of the ammonium acetate was decline. Then, on day 6 the pH was slightly increase and fall back on the day 9. The pH keeps increasing until day 15 and decreased until day 27. Next, the pH was inclined back until day 33. Finally, the pH is dropping from day 33 until day 45.

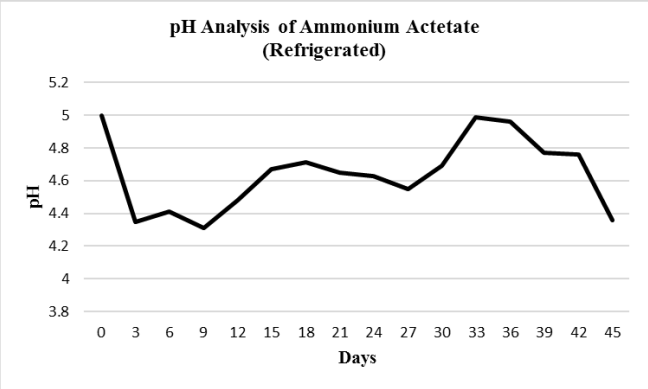


Fig. 1: pH trend of refrigerated ammonium acetate buffer

Based on the **Fig. 2**, the pH analysis trend shown also fluctuated. The trend of the pH changes from the initial day until the third day is decreasing and extremely fluctuated between day 3 until day 45. Based on these two pH analysis trend for the ammonium acetate buffer, the trend for pH buffer at the room temperature showed that the pH will easily changed compared to the refrigerated condition. However, the trend of analysis for the room temperature and refrigerated conditions still within the range of 5 to 4.2 which is still on the tolerance range.

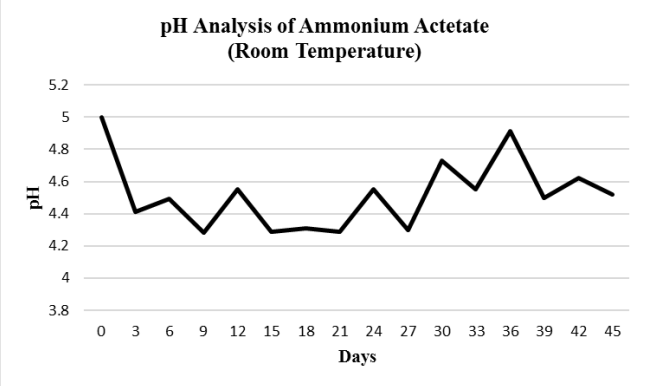


Fig. 2: pH trend of ammonium acetate buffer in room temperature

C. The effects of temperature on the stability of glycine buffer

Based on **Fig. 3** shown below, the pH analysis trend for the refrigerated glycine buffer is extremely decreases from the initial day to the third day. Then, from day 3 until day 12 the pH trend shown is slightly increases. The constant pH analysis trend was shown within day 15 until day 18 and then fluctuated within day 18 to day 27. At day 45, the pH analysis showed that the pH is dropping from the initial pH value.

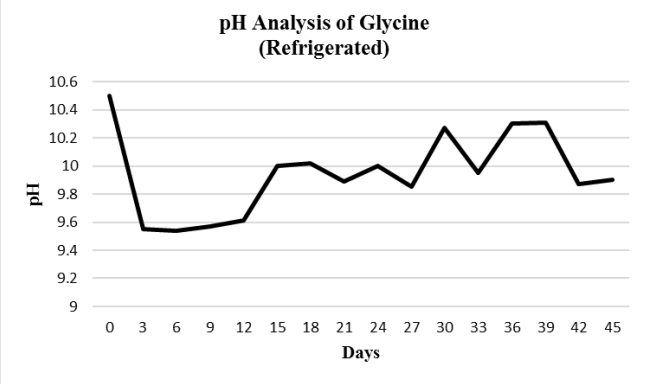


Fig. 3: pH trend of refrigerated glycine buffer

Based on **Fig. 4** shown below, the pH analysis trend for the glycine buffer at the room temperature also extremely decreases from the initial day to the third day. From day 3 until day 27, the trend shown is fluctuating and increases at day 30. On the day 45, the pH showed decreasing from the initial pH value.

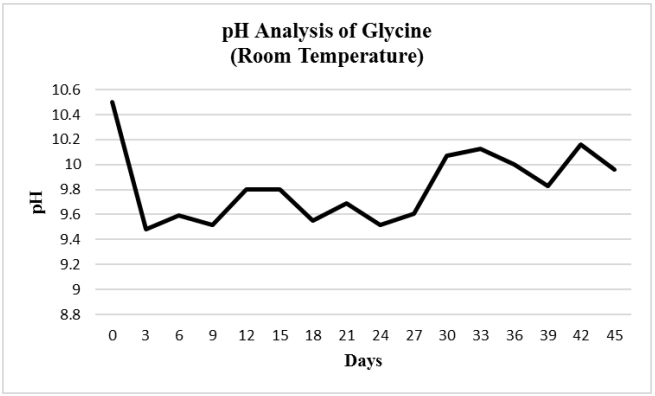


Fig. 4: pH trend of glycine buffer in room temperature

The pH analysis trend for the refrigerated glycine and the room temperature condition showed the same trend but different at the fluctuated part. The range of the pH changes for both conditions still lies within the pH tolerance range for the glycine. The behaviour of glycine buffer in this research shown that this buffer can be stored at both conditions.

D. The effects of temperature on the stability of potassium dihydrogen phosphate buffer

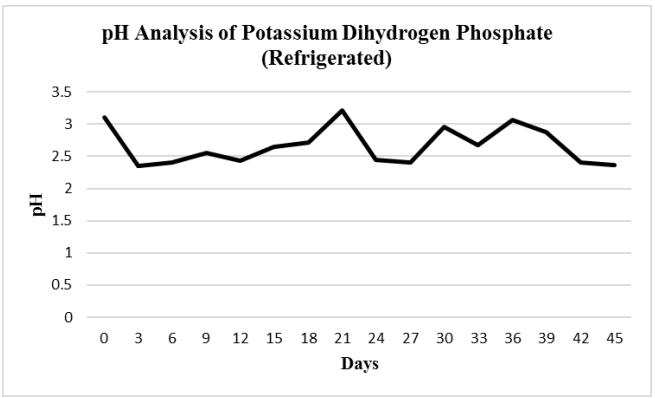


Fig. 5: pH trend of refrigerated potassium dihydrogen phosphate buffer

Based on **Fig. 5** and **Fig. 6**, the trend showed for both conditions are fluctuated between the same range which is 3.1 until 2.0 which is still within the tolerance range of the potassium dihydrogen phosphate. However, the trend shown in the pH analysis of refrigerated conditions is quite fluctuated compared to the room temperature condition between day 3 to day 27. It is shown that, at the refrigerated condition, the potassium dihydrogen phosphate buffer can be easily fluctuate compared to the room temperature conditions.

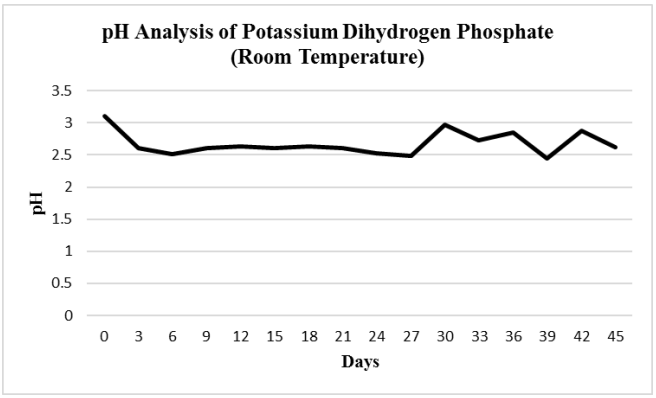
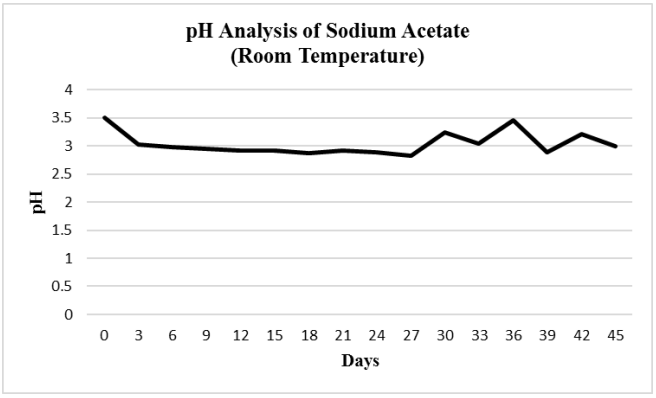


Fig. 6: pH trend of potassium dihydrogen phosphate buffer in room temperature

E. The effects of temperature on the stability of sodium acetate buffer

Fig. 7: pH trend of refrigerated sodium acetate buffer



For the pH analysis of sodium acetate buffer, based on **Fig. 7** and **Fig. 8** the trends also indicated the fluctuate mode for both refrigerated and room temperature condition. From the initial day until the third day, the pH trends for both condition showed that decline of the pH from the initial pH. Then, from the third day until the day 27, the trend for refrigerated sodium acetate based on **Fig. 7** shown below is fluctuating as compared to the sodium acetate pH analysis at the room temperature. However, the range of pH changes for both condition showed that, the range is out of the tolerance range of the buffer. It is unidentified whether the buffer should be stored at the refrigerated condition or room temperature. This cause may due to the disturbance from surrounding while preparing the buffer solutions. The pH meter may be having a problem that lead to no detection of actual pH values of the buffer.

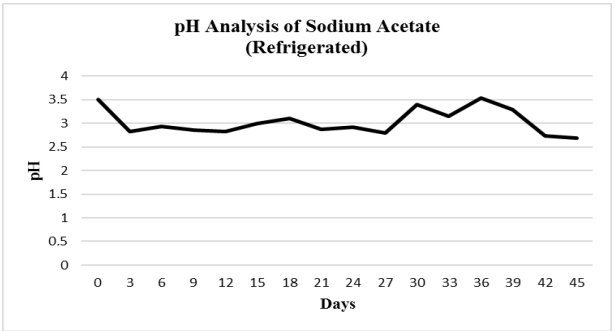


Fig. 8: pH trend of sodium acetate buffer in room temperature

F. The effects of temperature on the stability of tris(hydromethyl)aminomethane buffer

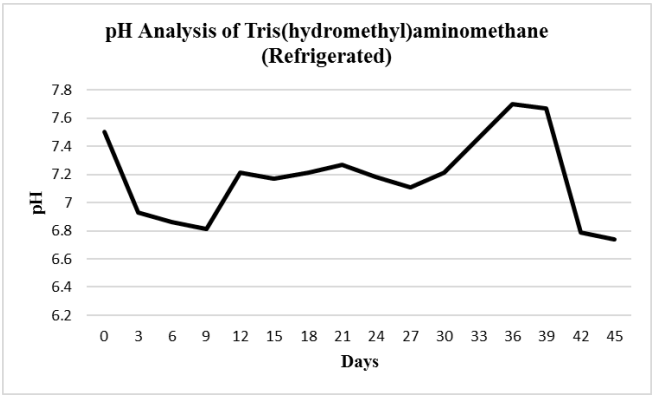


Fig. 9: pH trend of refrigerated tris(hydromethyl)aminomethane buffer

Based on the **Fig. 9** and **Fig. 10**, the pH changes range is between 7.5 to 6.6 which showed the fluctuated trend of pH analysis. The pH of refrigerated tris(hydromethyl)aminomethane buffer and at room temperature condition are decreases within the initial day to day 3 out from the tolerance range. For the refrigerated tris(hydromethyl)aminomethane, the range of fluctuating trend from day 12 until day 39 are still in the tolerance range compared to the tris(hydromethyl)aminomethane in the room temperature. This shown that the tris(hydromethyl)aminomethane buffer is better to store in the refrigerated condition compared to the room temperature. However, at the day 45 the pH of tris(hydromethyl)aminomethane for refrigerated condition was out of the tolerance range too. This occurrence may be affected by the pH meter problem while took the reading for tris(hydromethyl)aminomethane pH values or the contamination of the buffer from surrounding that could change the pH value. Thus, it cannot detect the actual pH at that time.

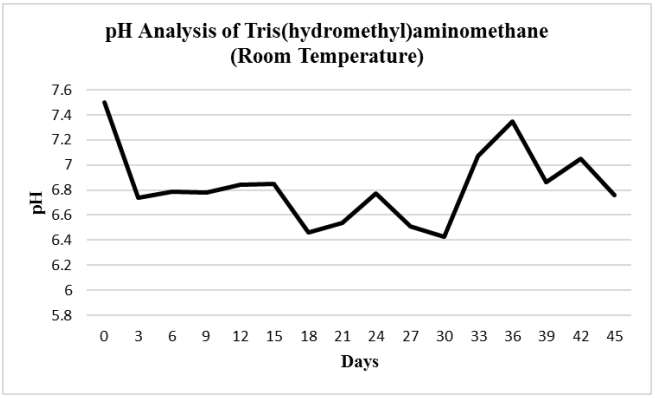


Fig. 10: pH trend of tris(hydromethyl)aminomethane buffer in room temperature

Table 1: The record of pH changes for each buffer solutions at refrigerated condition

Days	pH				
	Ammonium Acetate	Glycine	Potassium Dihydrogen Phosphate	Sodium Acetate	Tris(hydromethyl)aminomethane
0	5.00	10.50	3.10	3.50	7.50
3	4.35	9.55	2.35	2.82	6.93
6	4.41	9.54	2.41	2.93	6.86
9	4.31	9.57	2.55	2.86	6.81
12	4.48	9.61	2.43	2.83	7.21
15	4.67	10.00	2.65	3.00	7.17
18	4.71	10.02	2.71	3.10	7.21
21	4.65	9.89	3.21	2.87	7.27
24	4.63	10.00	2.44	2.91	7.18
27	4.55	9.85	2.41	2.80	7.11
30	4.69	10.27	2.95	3.39	7.21
33	4.99	9.95	2.68	3.15	7.45
36	4.96	10.30	3.06	3.54	7.70
39	4.77	10.31	2.88	3.28	7.67
42	4.76	9.87	2.41	2.74	6.79
45	4.36	9.90	2.37	2.68	6.74

Table 2: The record of pH changes for each buffer solutions at room temperature

Days	pH				
	Ammonium Acetate	Glycine	Potassium Dihydrogen Phosphate	Sodium Acetate	Tris(hydromethyl)aminomethane
0	5.00	10.50	3.10	3.50	7.50
3	4.41	9.48	2.60	3.02	6.74
6	4.49	9.59	2.51	2.98	6.79
9	4.28	9.52	2.60	2.95	6.78
12	4.55	9.80	2.63	2.92	6.84
15	4.29	9.80	2.61	2.91	6.85
18	4.31	9.55	2.63	2.87	6.46
21	4.29	9.69	2.60	2.91	6.54
24	4.55	9.52	2.53	2.89	6.77
27	4.3	9.61	2.49	2.82	6.51
30	4.73	10.07	2.97	3.24	6.43
33	4.55	10.13	2.73	3.04	7.07
36	4.91	10.00	2.85	3.45	7.35
39	4.50	9.83	2.45	2.88	6.86
42	4.62	10.16	2.87	3.21	7.05
45	4.52	9.96	2.62	3.00	6.76

A. The effects of temperature on the buffering capacity of ammonium acetate buffer

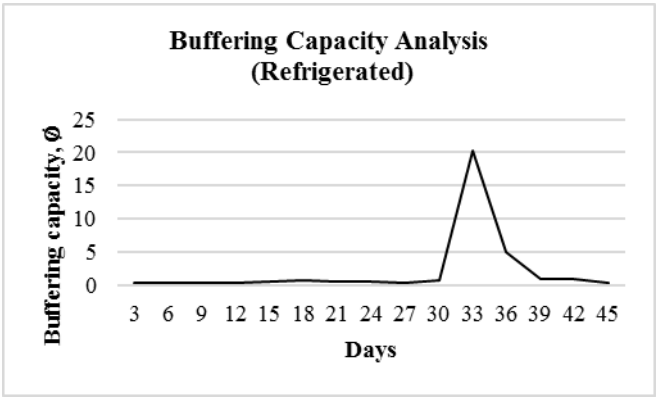


Fig. 11: Ammonium acetate buffering capacity trend at refrigerated condition

Fig. 11 and **Fig. 12** shows the buffering capacity analysis trends for refrigerated ammonium acetate and at room temperature conditions respectively. Based on **Fig. 11**, the buffering capacity shown within day 3 until day 30 is constant and at the value of zero. This occurrence means that the buffer did no had the ability to resist the changes of temperature within that time interval. However, the buffering capacity is increase drastically at day 33 and decline back at day 36 to day 45. The highest ability to resist the pH changes is at day 33.

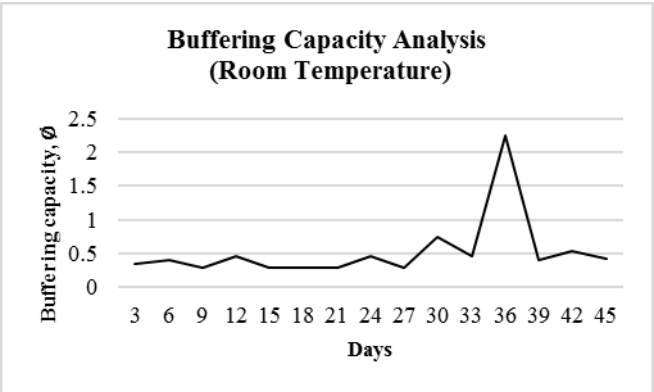


Fig. 12: Ammonium acetate buffering capacity trend at room temperature

Based on **Fig. 12**, the buffering capacity shown within day 3 until day 33 is fluctuating at less than 0.5 buffering capacity. It is shows that the ability of the ammonium acetate buffer at the room temperature to resist the changes of pH is at least value within day 3 to day 33. Then, the buffering capacity is increases drastically and decreses drastically at day 36 and day 39 respectively.

Based on the trend for both conditions, the ammonium acetate buffer is having the most ability to resist the pH changes at the room temperature storage.

B. The effects of temperature on the buffering capacity of glycine buffer

Based on **Fig. 13**, the buffering capacity on day 3 until day 12 shows the constant value and rise to 0.2 on day 15. The buffering capacity within day 3 until day 27 shows below 0.2. After day 27, the trend of buffering capacity of the glycine shows fluctuation but in the high values range. The higher buffering capacity is on day 39. After the day of 39, the ability to resist the pH changes is declining.

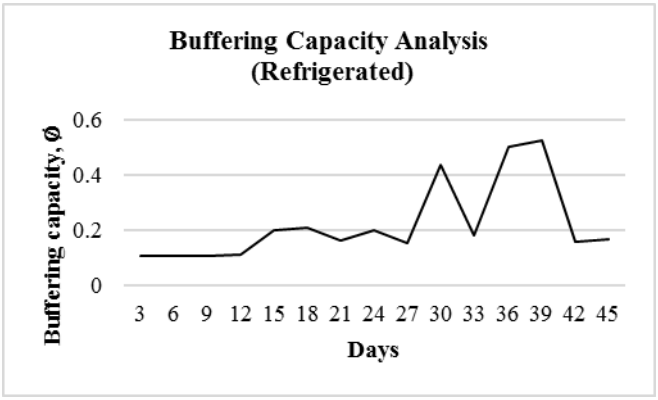


Fig. 13: Glycine buffering capacity trend at refrigerated condition

Based on **Fig. 14** the buffering capacity trend at room temperature is fluctuating within day 3 until day 27 in between the buffering range capacity of 0.1 to 0.2. then, the buffering capacity is increases at day 30 to day 33. The highest buffering range capacity is on day 42 before its fall back on day 45 at the buffering range within 0.1 to 0.2. Based on the result obtained for the buffering capacity of glycine buffer, the rich buffering capacity is shown at the refrigerated glycine stored condition.

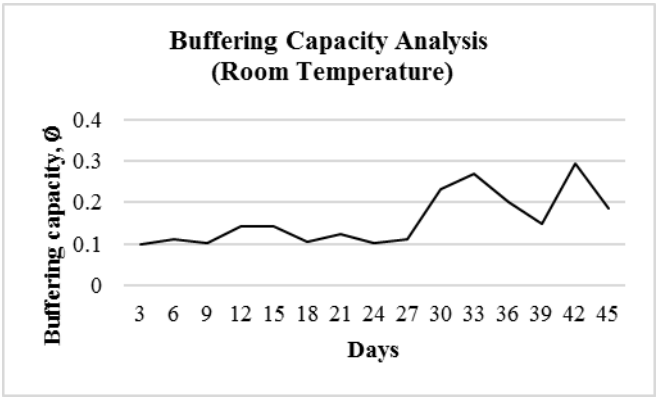


Fig. 14: Glycine buffering capacity trend at room temperature

C. The effects of temperature on the buffering capacity of potassium dihydrogen phosphate buffer

Based on **Fig. 15**, the potassium dihydrogen phosphate buffering capacity for the refrigerated condition is fluctuating at the lowest range within day 3 to day 33. The ability to resist the pH changes is inclining to the highest value on day 36 and then the capacity fall back to low values within day 39 to day 45.

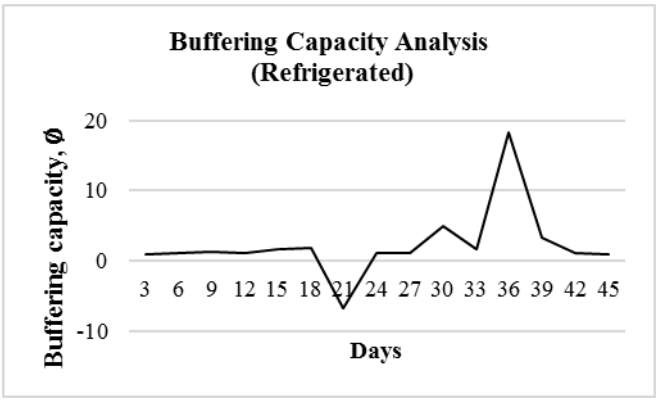


Fig. 15: Potassium dihydrogen phosphate buffering capacity trend at refrigerated condition

Based on **Fig. 16** the room temperature storage condition of potassium dihydrogen phosphate is below then 2.0 within day 3 until day 27. Then, the capacity is increases at highest value on the day 30 and decreases on day 33. The trend from day 33 to day 45 keep fluctuating at the average values.

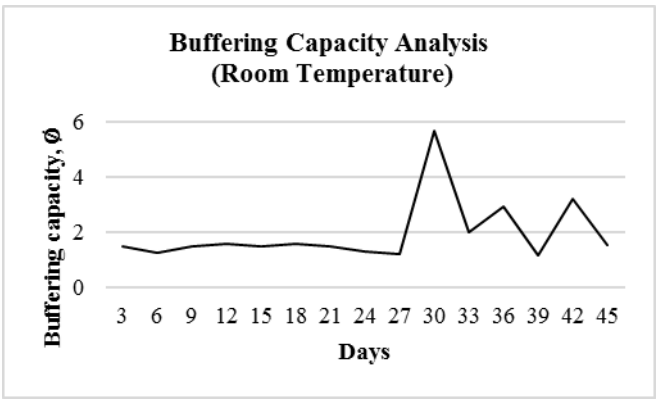


Fig. 16: Potassium dihydrogen phosphate buffering capacity trend at room temperature

D. The effects of temperature on the buffering capacity of sodium acetate buffer

The buffering capacity based on the **Fig. 17** shows poor ability to resist the pH changes while stored at the refrigerated conditions. The sodium acetate buffer does not reach good behavior of the buffering capacity within day 3 until day 27. The highest ability to resist the pH changes only on day 30. Then, the values fall until the negative value which shows poor buffering capacity.

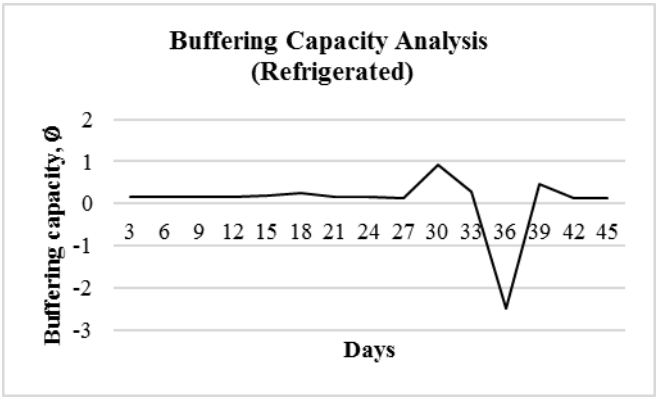


Fig. 17: Sodium acetate buffering capacity trend at refrigerated condition

Based on **Fig. 18**, the buffering capacity for the sodium acetate at the room temperature is better compared to the refrigerated condition. The ability to resist the pH changes within day 3 to day 27 shows constant trend. Then, it is slightly increses at day 30 and

fluctuated until day 33. The highest buffering capacity is on day 36 and drastically fall on day 39.

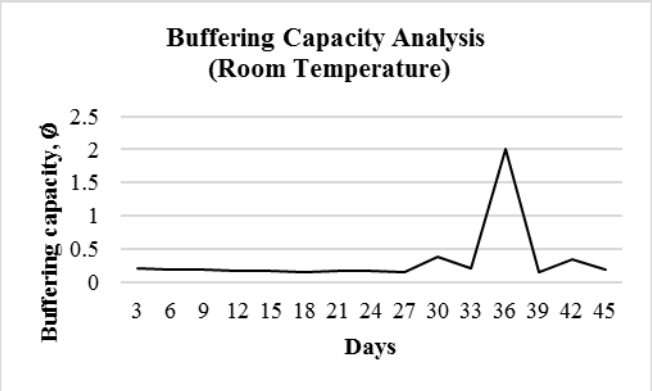


Fig. 18: Sodium acetate buffering capacity trend at room temperature

E. The effects of temperature on the buffering capacity of tris(hydromethyl)aminomethane buffer

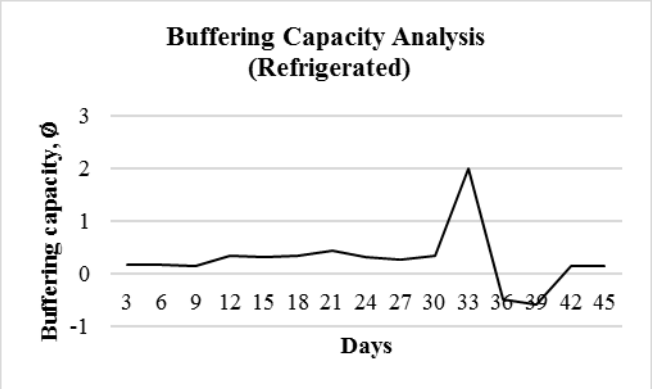


Fig. 19: Tris(hydromethyl)aminomethane buffering capacity trend at refrigerated condition

Based on **Fig. 19** and **Fig 20**, the highest buffering capacity for the refrigerated tris(hydromethyl)aminomethane buffer solutions is higher than the tris(hydromethyl)aminomethane buffer solutions in the room temperature which is on day 33 and day 36 respectively. However, the trend of the refrigerated condition does not shown good trend as it is fluctuating at the lowest buffering capacity until reached negative value. The occurrence shows that the ability to resist pH changes is poor at the refrigerated condition compared to room temperature condition.

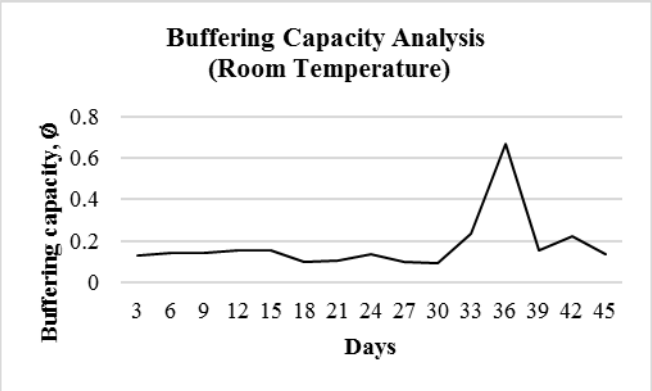


Fig. 20: Tris(hydromethyl)aminomethane buffering capacity trend at room temperature

IV. CONCLUSION

The filtration of all the buffer solution successfully make those buffers to prevent or reduce the contamination in the buffers solution. Thus, the color did not change throughout the experiment and there is no foreign material that been observed in all the buffer solutions.

However, does not only the color changes of the buffers indicates a good buffer to be used in the processes line but the pH stability also plays the roles in the consideration of choosing a suitable buffer. Apart from that, the suitable pH condition must be considered for every process. Thus, it is important to employ the stability study in order to plan the uses of the buffers solution.

For this research, it is found that the room temperature is more suitable to store the buffering agent. However, there are also some of the buffer could change it pH when stored in the room temperature. Thus, the store condition temperature should be considered the behavior of the way they will react towards surrounding for certain buffer solution.

In the next research for the evaluation of the buffer stabilities, it is recommended to do the research on the any other molecules that used the buffering agent throughout the process line of the production. Apart from vary the condition temperature, it is recommended to do the research in varying the types of vial used to store the buffer solutions.

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