INFLUENCE OF FREEZING STAGES ON
INFLUENZA VIRUS HAEMAGGLUTININ
CONFORMATION DURING LYOPHILIZATION

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อิทธิพลของขั้นตอนการทำเยือกแข็งต่อโครงรูปฮีแมกกลูตินิน ของไวรัสไข้หวัดใหญ่ระหว่างการทำแห้งเยือกแข็ง

นางสาวดวน ธาน พูออง

Thesis Title	INFLUENCE OF FREEZING STAGES ON INFLUENZA VIRUS HAEMAGGLUTININ CONFORMATION DURING LYOPHILIZATION
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ดวน ธาน พูออง : อิทธิพลของขั้นตอนการทำเยือกแข็งต่อโครงรูปฮีมแอกกลูตินิน ของไวรัสไข้หวัดใหญ่ระหว่างการทำแห้งเยือกแข็ง (INFLUENCE OF FREEZING STAGES ON INFLUENZA VIRUS HAEMAGGLUTININ CONFORMATION DURING LYOPHILIZATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภญ. ดร.นฤพร สุตัณฑวิบูลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ภญ. ดร.จิตติมา ชัชวาลย์สายสินธ์, 121 หน้า.

วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาผลกระทบของขั้นตอนการทำเยือกแข็งต่อโครงรูปฮีแมกกลูตินิน ของไวรัสไข้หวัดใหญ่และอิทธิพลของวัฏจักรการทำแห้งเยือกแข็งต่อคุณสมบัติของวัคซีนไข้หวัดใหญ่ชนิดเชื้อเป็นจากการทำแห้งแบบเยือกแข็ง รวมถึงความคงตัวของวัคซีน ผลจากอัตราเร็วการทำเยือกแข็งสามระดับ ได้แก่ การทำเยือกแข็งความเร็วสูง (0.50 องศาเซลเซียสต่อนาที) การทำเยือกแข็งความเร็วปานกลาง (1.08 องศาเซลเซียสต่อนาที) และการทำเยือกแข็งความเร็วต่ำ (2.58 องศาเซลเซียสต่อนาที) ต่อความคงตัวของโครงรูปฮีแมกกลูตินิน โดยใช้เครื่องมือ ยูวีสเปกโทรสโกปีย่านไกล ยูวีสเปกโทรสโกปีย่านไกล้ เซอร์คิวลาร์ไดโครอิซึมสเปกโทรสโกปี และฟลูออเรสเซนต์สเปกโทรสโกปี จากการศึกษาพบว่าอัตราเร็วในการทำเยือกแข็งทั้งสามระดับมีผลให้โครงสร้าง ทุติยภูมิและตติยภูมิของสารละลายฮีแมกกลูตินินเสียหาย ดังนั้นจึงควรเพิ่มสารเพิ่มความคงตัวที่เหมาะสมในตำรับฮีแมกกลูตินินเพื่อรักษาสภาพระหว่างวัฏจักรการทำแห้งเยือกแข็งให้ดียิ่งขึ้น ตำรับที่มีแมนนิทอลเป็นองค์ประกอบหลักในการศึกษานี้มี 4 ตำรับ ได้แก่ G6S0, G6S1, G0S1 และ G0S0 เมื่อ G และ S คือ ไกลซีนและซูโครส และตัวเลขในสูตรแสดงถึงร้อยละโดยน้ำหนักของสารเพิ่มความคงตัวในสารละลาย จากการศึกษา ตำรับ G6S0 มีผลให้โครงสร้างทุติยภูมิและตติยภูมิของฮีแมกกลูตินินเปลี่ยนแปลงน้อยที่สุด ภายหลังจากการทำเยือกแข็งด้วยอัตราเร็วปานกลาง (1.08 องศาเซลเซียสต่อนาที) เมื่อเปรียบเทียบกับฮีแมกกลูตินินที่ไม่ได้ใส่สารเพิ่มความคงตัว ดังนั้นการทำเยือกแข็งอัตราเร็วปานกลาง (1.08 องศาเซลเซียสต่อนาที) จึงนำมาใช้ในกระบวนการการทำแห้งเยือกแข็งของสูตรตำรับวัคซีนไข้หวัดใหญ่ชนิดเชื้อเป็น พบว่ากระบวนการทำแห้งเยือกแข็งนี้ให้ผลิตภัณฑ์วัคซีนไข้หวัดใหญ่ชนิดเชื้อเป็นที่ยอมรับได้ โดยวิเคราะห์จากลักษณะทางกายภาพ เวลาในการละลายผงยาแห้ง ความเป็นกรดด่าง ค่าออสโมลาริตี คุณสมบัติทางความร้อน ความเป็นผลึก และค่าไตเตอร์ที่สามารถระงับการติดเชื้อไวรัส อย่างไรก็ตามมีเพียงความชื้นหลงเหลือเท่านั้นที่มีค่ามากกว่าเกณฑ์ที่กำหนด ทำการทดสอบความคงตัวภายใต้สามสภาวะ ได้แก่ สภาวะที่หนึ่ง อุณหภูมิ 30 องศาเซลเซียส ความชื้นสัมพัทธ์ ร้อยละ 75 สภาวะที่สอง อุณหภูมิ -20 องศาเซลเซียส และสภาวะที่สาม อุณหภูมิ 2-8 องศาเซลเซียส จากการทดสอบ พบว่าทุกตำรับสูญเสียค่าไตเตอร์ที่สามารถระงับการติดเชื้อไวรัสภายในสัปดาห์แรกของการเก็บตัวอย่างที่สภาวะที่หนึ่ง สำหรับภาวะที่สามพบว่าเฉพาะสูตร G6S0 และ G6S1 มีความคงตัวตลอดระยะเวลา 6 เดือน โดยมีค่าไตเตอร์ที่สามารถระงับการติดเชื้อไวรัสดีในระดับเดียวกันกับการเก็บตัวอย่างที่สภาวะที่สอง ดังนั้นโดยสรุป ไกลซีนเป็นสารเพิ่มความคงตัวที่สำคัญต่อการรักษาโครงรูปของฮีแมกกลูตินินและความคงตัวของผลิตภัณฑ์วัคซีนไข้หวัดใหญ่ชนิดเชื้อเป็นที่ผ่านการทำแห้งเยือกแข็ง ดังนั้นจึงควรนำความรู้ไปพัฒนาต่อเนื่องในอนาคตเพื่อขยายขนาดการผลิตวัคซีนไข้หวัดใหญ่ชนิดเชื้อเป็นโดยการทำแห้งเยือกแข็งต่อไป

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DOAN THANH PHUONG: INFLUENCE OF FREEZING STAGES ON INFLUENZA VIRUS HAEMAGGLUTININ CONFORMATION DURING LYOPHILIZATION. ADVISOR: NARUEPORN SUTANTHAVIBUL, Ph.D., CO-ADVISOR: JITTIMA CHATCHAWALSAISIN, Ph.D., 121 pp.

The purpose of this study was to evaluate the impact of freezing stages on influenza Haemagglutinin (HA) conformation and the influence of lyophilization cycle on properties of final lyophilized live attenuated influenza virus (LAIV) vaccine as well as their stabilities. Firstly, the effect of three freezing rates, rapid freezing (0.50°C/min), moderate freezing (1.08°C/min) and slow freezing (2.58°C/min) on HA conformational stability was investigated by far-UV, near-UV circular dichroism and fluorescence spectroscopy. The three freezing rates resulted in the loss in secondary and tertiary structures when HA powder was prepared in aqueous solution. Therefore, suitable stabilizers were added in HA formulations for better preservation during freezing cycles. Four mannitol-based formulations were tested, G6S0, G6S1, G0S1 and G0S0, where G and S were glycine and sucrose and numerals expressed as concentrations (%w/v) of each stabilizer. G6S0 formulation exhibited the smallest effect in both secondary and tertiary structures of HA after frozen at moderate freezing rate (1.08°C/min) as compared to unstabilized HA. Consequently, the moderate freezing rate of 1.08°C/min was used as the freezing stage of the final lyophilization procedure applied on LAIV vaccine formulations. Initially, lyophilization procedure resulted in acceptable LAIV products when characterized on physical appearance, reconstitution time, pH, osmolality, thermal behavior, crystallinity and infectivity titer. Only residual moisture contents were greater than specified. The stability studies were carried out under three sets of conditions: 30°C/75%RH, -20°C and 2-8°C. All formulations displayed loss in infectivity titers during the first week of storage at 30°C/75%RH. Stability determination at 2-8°C showed that only G6S0 and G6S1 products were stable throughout the 6 months study period with excellent infectivity titers similar to products stored at -20°C. In summary, glycine plays an important role in stabilizing HA conformation as well as stability of lyophilized LAIV vaccine mannitol-based products. It should be used in the future development for the manufacturing of lyophilized LAIV vaccine products.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	_____
		Advisor's Signature	_____
Field of Study:	Pharmaceutical Technology	Co-Advisor's Signature	_____
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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ABBREVIATION

º	Angle
%	Percentage
º/min	Degree per minute
ºC	Degree Celsius
CD	Circular dichroism
cm	Centimeter
DSC	Dynamic scanning calorimetry
DVS	Dynamic vapor sorption
FLUVAC	Name of liquid influenza vaccine produced by GPO
G0S0	Formulation of HA with 0% glycine and 0% sucrose
G0S1	Formulation of HA with 0% glycine and 1% sucrose
G6S0	Formulation of HA with 6% glycine and 0% sucrose
G6S1	Formulation of HA with 6% glycine and 1% sucrose
GPO	Government Pharmaceutical Organization
HA	Haemagglutinin
LAIV	Live attenuated influenza virus
mdeg	Millidegree
mg	Milligram
ml	Milliliter
mosm/kg	Milliosmole per kilogram

mTorr	Millitorr
NA	Neuramidase
nm	Nanometer
pH	The negative logarithm of the hydrogent concentration
qs.	Quantum sufficit
RH	Relative humidity
SD	Standard deviation
TCID$_{50}$	Tissue cell infectivity titer
Tg'	Glass transition temperature
TGA	Thermogravimetry analysis
UV	Ultraviolet
vs.	Versus
w/v	Weight per volume
WFI	Water for injection
WHO	World Health Organization
XRPD	X-ray powder diffraction

CHAPTER I – INTRODUCTION

Seasonal influenza (seasonal flu) is a transmittable respiratory tract disease caused by influenza virus. It occurs every year and spreads throughout the world easily (Simonsen, 1999). It is mainstay of public health concern due to the severity of the disease or deaths. The morbidity, mortality and economic loss each year, which are originated from influenza infection, have a significant impact on the quality of life. The estimated number of severe illness each year is approximately 3-5 million people. The number of deaths are between 250,000 and 500,000 people annually (WHO, 2014).

On the market, some medicines are available for influenza infection treatment such as Zanamivir, Amantadin, Olseltamivir (Ison and Hayden, 2001). These medicines can reduce the illness outcome after infection. However, vaccine is considered as the most effective method for preventing this disease worldwide according to the recommendation of World Health Organization (WHO, 2014).

Currently, initial liquid live attenuated influenza virus (LAIV) vaccine formulation (FLUVAC) developed by Government Pharmaceutical Organization (GPO) contained sucrose, phosphate, glutamate requires storage at -20°C with stability of less than 6 months. This means the obligation of storage at extremely low temperature and the short stability are problematic. Consequently, stockpiling of liquid vaccine for influenza pandemics is somehow impossible. The production of lyophilized LAIV vaccine, which is capable to be stored at refrigerated temperature, 2-8°C therefore is beneficial to product stability, shipping and storage issues.

Today, most of conventional influenza vaccines composed of Haemagglutinin (HA) and/ or Neuraminidase (NA), the two main surface antigens (Jang and Seong, 2014). However, most antibodies, which are produced from the immunogenic system, matched globular head of HA (Oxford, 2013). HA peptide, with its residues from 98 to 106 was pronounced to have strong immunogenicity. This peptide was found to be specific with antibodies against influenza infection (Arya, et al., 2014; Cox, Brokstad and Ogra, 2004; Dyson, Lerner and Wright, 1988; Müller, Shapira and Arnon, 1982; Wilson and Cox, 1990; Wilson, et al., 1984).

Freeze drying, also known as lyophilization is a downstream process providing long-term stability vaccines in the solid state. Freezing step is the most critical stage during the entire lyophilization process. The size and the distribution of ice crystals are determined during freezing stages and the efficiency of subsequent drying stages are subsequently affected. Thus, the effect of freezing step on HA conformation is necessary to study the development of stable freeze-dried vaccine.

Objectives of study:

- To study the effect of freezing stages on the conformation of influenza virus haemagglutinin

- To evaluate the influence of lyophilization cycle on properties of final lyophilized live attenuated influenza virus vaccine as well as their stabilities

CHAPTER II - LITERATURE REVIEW

I. Influenza pandemics

Seasonal influenza (seasonal flu) is a transmissible respiratory tract disease caused by influenza virus. It occurs globally and usually reaches peak in the winter. The virus can transmit easily from infected people to healthy people through coughing, sneezing or direct contact. Therefore, this flu spreads worldwide easily and then becomes pandemic.

Seasonal flu affects on nose, throat, bronchi and occasionally in lung. The symptoms include high fever, cough, headache, muscle and joint aches, sore throat, runny nose, acute respiratory distress, etc. Severely, it can lead to the death (WHO, 2014).

In human history, influenza flu did claimed many lives in many countries throughout the world. The estimated number of dead people went up to 50 - 100 million peoples in 1918 pandemic (H1N1 Spanish flu), 2 million in 1957 (H2N2 Asian flu) and 1 million in 1968 (H3N2 Hong Kong flu) (Chen, Ma and Wong, 2011). The most recent pandemic flu in 2009 caused by H1N1, was initially detected in the border between the United States and Mexico before spreading around the world (Chang, et al., 2009), had killed at least 18,036 people (WHO, 2010).

II. Influenza virus

Influenza virus, RNA virus, classified in Orthomyx oviridae family, has genome containing 8 negative strand RNA segments (Bouvier and Palese, 2008). There are three types of influenza virus - A, B and C (Chen, Ma and Wong, 2011). Mainly, influenza A, B viruses are responsible for the seasonal flu. However, virus type A plays the most important role in the global outbreak. The envelope of influenza A virus consists of HA and NA (Figure 1). They have pronounced 18 HA subtypes and 9 NA subtypes (Chen, Ma and Wong, 2011). However, only H1, H2, H3 and N1, N2 are able to circulate among human (Landolt, 2006).
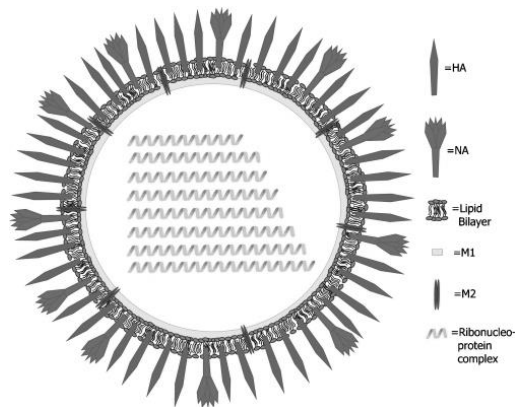
Figure 1. Influenza virus structure (Amorij, et al., 2008)

III. Haemagglutinin (HA)

HA is the principal glycoprotein on the viral surface inducing the entry process by binding the virus to the host cell and causing fusion with the viral and endosomal membrane (Hilleman, 2002). This homotrimer weighs approximately 225kD. Each HA monomer contains 2 smaller polypeptides: HA1 and HA2 subunits, which connected together by disulfide bonds. HA1 generates sialic acid binding sites and the subsequent attachment of HA to the host cell envelope (Shangguan, et al., 1998). Whereas, HA2 is responsible for the fusion activity through the amino terminal region (Gething, et al., 1986). The three monomers of HA create stem domain and globular domain (Chen, Ma and Wong, 2011).

HA peptide (98-106), which contained TYR-PRO-TYR-ASP-VAL-PRO-ASP-TYR-ALA is typically used as an epitope tag. HA peptide (98-106) was known as HA-antibodies specific sequence (Arya et al., 2014). This nine amino acid peptide is considered as the prominent sequence in immunogenic activity to produce antibodies against influenza infection (Müller, Shapira and Arnon, 1982; Muller and Regenmortel, 1999; Wilson, et al., 1984).

The HA peptide sequence with residues from 98 to 106 displayed the conformation in aqueous solution and it could lead to protein reactive antibodies (Wilson and Cox, 1990).

Residues 98-106 of HA was noticed to have a strong immunogenicity (Dyson, Lerner and Wright, 1988). Wherein, the position 101 was corresponded to

monoclonal antibodies (Dyson, Lerner and Wright, 1988).

Antibodies, which were produced against HA peptide residue from 75-110, were reported to be specific directly to the amino acid sequence from 98-106 (Wilson and Cox, 1990).
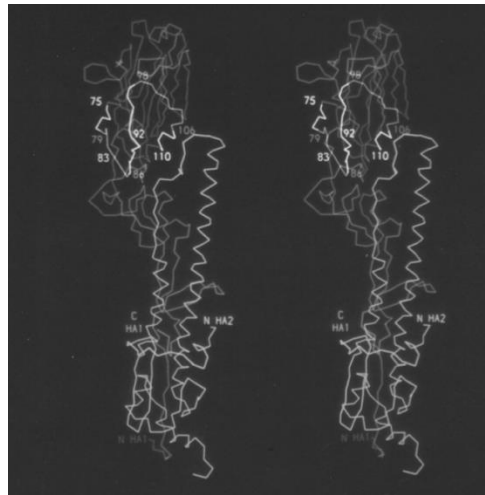
Figure 2. Heamagglutinin monomer (Wilson, et al., 1984)

IV. Influenza vaccine

Most effective strategy to prevent influenza and/or severe outcomes from the illness is vaccination (WHO, 2014). Children from 6 months to 5 years, elders from 65 years or higher, pregnant women, chronically ill individuals, health-care workers are high-risk groups whom World Health Organization recommends to receive flu vaccination annually (WHO, 2014).

There are many types of influenza vaccines on the market. The whole inactivated influenza vaccines (WIV), split, subunit and virosomal influenza vaccines are representives of inactivated influenza vaccines. While another group is known as live attenuated influenza virus (LAIV) vaccine in liquid and solid dosage forms (Amorij, et al., 2008).
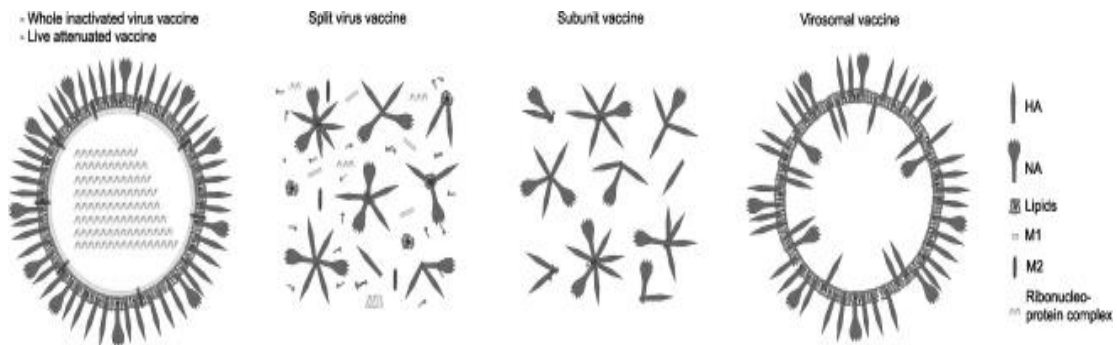
Figure 3. Four types of influenza virus vaccine (Amorij, et al., 2008)

LAIV vaccine is available commercially. It is administered intranasally instead of delivering through intramuscular or subcutaneous injection as the inactivated vaccine administration. LAIV vaccine stimulates both mucosal and systemic immunogenic response. In contrast, the inactivated vaccines only induce systemic immunogenic response. LAIV vaccine properties were reported to be more immunogenic in younger children than inactivated or killed virus vaccines (Cox, Brokstad and Ogra, 2004).

Most of conventional influenza vaccines are produced in liquid form, which are administered parenterally. However, liquid formulations do not exhibit good stability. The physical and chemical modifications of the protein in solution are the causes of instability (Dexiang and Kristensen, 2009). In addition, unexpected chemical reactions such as hydrolysis, oxidation, de-amidation induces the loss of vaccine potency due to unfolding, aggregation and alteration of protein structure. Therefore, dry powder vaccine is more preferable than liquid dosage forms.

For influenza, applying dry powder influenza vaccine to human by nasal spray (intranasally) is much more attractive (Huang, et al., 2004). The intranasal administration presents more advantage over parenteral injection: easy administration, large surface area of absorption, rapid and high absorption (Berens and Sullivan, 2007).

V. Lyophilization

Lyophilization or freeze-drying is a downstream process widely on labile

pharmaceutical products. The purpose is to obtain a solid final product with desired moisture content that will eventually prolong its shelf life (Liu, 2006). This process is characterized by two main stages: freezing and drying. Drying step consists of two other phases: primary drying and secondary drying. Water frozen during the freezing step, is removed from product through vapor state without passing through liquid state during primary drying (Tang and Pikal, 2004). This is known as sublimation. The concentration gradient of water vapor between condenser and drying front induces the removal of water during freeze drying (Baheti, Kumar and Bansal, 2010). During the primary drying, the vapor pressure is decreased and the temperature is increased simultaneously. Finally, the non-frozen water is removed through the secondary drying in increased temperature and vacuum pressure condition.

5.1. Advantages of lyophilization process

- **Retain the activity of active ingredient** (Cameron, 1997)

Low temperatures during freezing help to protect active ingredient, which is heat labile. When freezing, the immobilization avoids the chemical reaction between the components of freeze-dried formulation. Additionally, the degradation reaction cannot occur at low temperatures. On the other hand, the absence of oxygen while drying at vaccum pressure condition prevents the oxidation reactions.

- **Retain the form of active pharmaceutical ingredient** (Cameron, 1997)

The liquid formulation converts to solid state after freezing step. This state prevents the migration of nonvolatile molecules, which leads to the change of active ingredient form.

- **Ease of reconstitution** (Cameron, 1997)

The freeze-dried product composes of lattice, which is formed by removing the surrounded frozen water. Therefore, the surface area of formula increases. Then, the reconstitution with water becomes easier.

- **Long shelf life** (Cameron, 1997)

Typically, the final lyophilized products show low moisture contents of approximately 2-3%. Therefore, freeze-drying process produces long shelf life

products. This also helps to improve the manufacturing, transportation and storage of product.

5.2. Disadvantages of lyophilization process

- High capital cost
- Long processing time
- High energy cost

5.3. Lyophilizer

A typical lyophilizer contains five main parts: control system, product chamber, ice condenser, vacuum pump and refrigerator (Figure 4). All parts of the system are adjusted by control system. The vials of product are placed in the product chamber, which is connected with a refrigerator for cooling purpose. Condenser is helped to remove the frozen water from products during drying. Vacuum pump is used to remove air and water vapor out of the chamber.
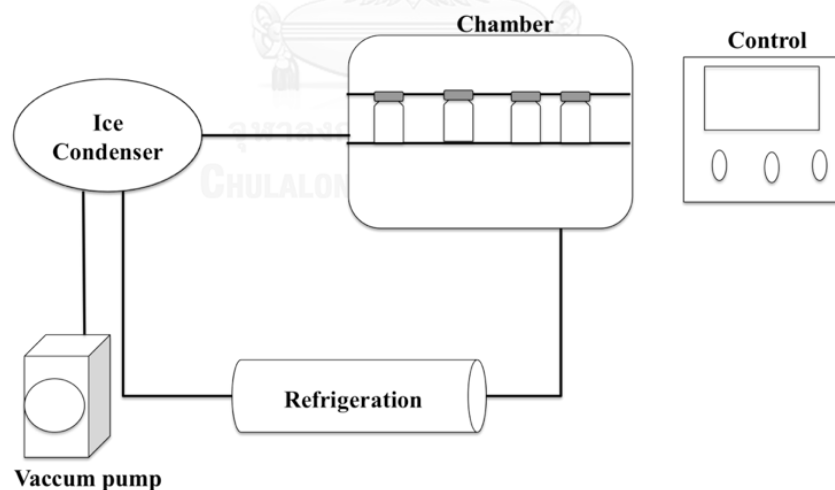
Figure 4. Components of a lyophilizer

5.4. Triple point

The main mechanism of lyophilization process is based on the transition of water between its phases. The relationship of different stages of water is illustrated

in the diagram shown in Figure 5. Water can exist at solid, liquid or vapor states. There is a point that these three stages co-exist, called triple point at pressure of 4.58mmHg and temperature of 0.0098°C (Fetterolf, 2010).

Ice is melted to water when the temperature is greater than 0°C at atmospheric pressure (760mmHg). Afterward, water converts to vapor state when the temperature is reached to 100°C. At vaccum pressure (pressure below triple point), ice crystals sublimate directly to vapor phase without passing through liquid phase at the temperature lower than 0°C.

When active ingredient is diluted in aqueous solution, the freezing point of the solution is below the triple point of pure water. The complete solidification of lyophilized solution only occurs when the temperature during freezing stage is conducted below the freezing point of the solution.
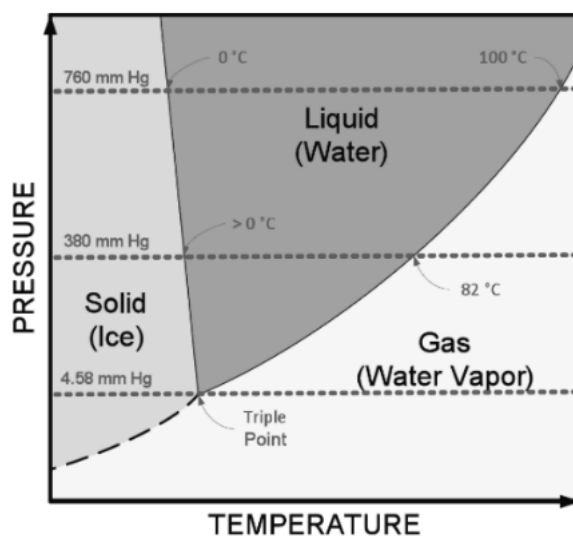
Figure 5. Phase diagram of water (Fetterolf, 2010)

5.5. Freezing

The freezing stage is considered as the critical step in freeze-drying process. It influences on the active pharmaceutical ingredient stability, physical and ice crystal morphology (Schwegman, Hardwick and Akers, 2005). It is also associated with the efficiency of primary drying and secondary drying (Kasper and Friess, 2011). Thus,

the freezing step plays an important role to optimize not only the quality but also the stability of lyophilized products.
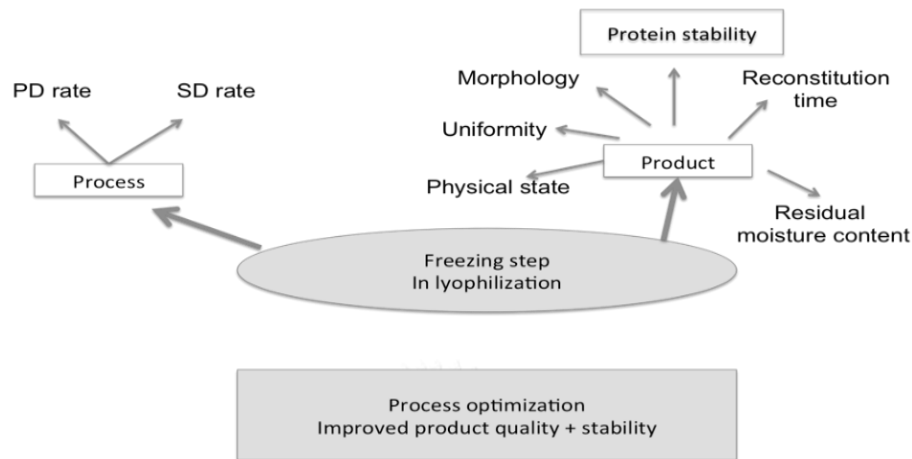
Figure 6. The importance of freezing stage in lyophilization process (Kasper and Friess, 2011)

Initially, sample solution is cooled when freezing is applied. There are two main processes occurred when temperature is reduced, ice nucleation and ice crystallization. The phenomenon that sample still maintains in liquid state at temperature below its equilibrium freezing point is called supercooling. The difference between equilibrium freezing temperature and ice nucleation temperature is known as the supercooling degree, which is related to freezing rate (or cooling rate). Supercooling degree is typically in the range of 10-15°C (Kasper and Friess, 2011). High degree of supercooling is correspondent to rapid freezing rate, while slow supercooling degree is involved with slow freezing rate. The first ice nuclei appears as the temperature of freezing stage is reached to ice nucleation temperature. The amount as well as size of ice nuclei is increased over the time when the temperature is continuously reduced. It is hard to control the ice nucleation temperature during freezing. It occurs randomly (Patel, Bhugra and Pikal, 2009). When the ice nuclei grow and reach to their critical masses, ice crystallization initiates. Finally, it takes time for the growth of ice crystals.

Freezing rate, freezing temperature and freezing time are important parameters of freezing stage. Freezing rate is involved in the subsequent stages of lyophilization

process. Slow freezing rate results in large ice crystal, correspondent to large pore size in dried layer. This means that slow freezing rate reduces the time-consuming primary drying and therefore improves the efficacy of entire lyophilization process (Nireesha, et al., 2013). Rapid freezing rate that creates large number of small ice crystals makes the ice sublimation time longer (Franks and Auffret, 2007). Final freezing temperature is typically conducted at -40°C or below (Fetterolf, 2010). Moreover, the time maintained at final temperature should be sufficient for the solidification of the entire solution. It was reported that samples are usually held at final temperature for 1 hour or more (Tang and Pikal, 2004).

5.6. Primary drying

Primary drying is known as the most time-consuming stage in lyophilization process. The majority of water amount in sample is removed during this stage. Increasing temperature and reducing pressure initiate primary drying. Vaccum pressure must be applied for the sublimation occurrence. Solid ice crystals transform directly to water vapor without passing through liquid phase. The difference of water vapor pressure between ice interface of product and condenser surface generates the driving force for sublimation phenomenon.

5.7. Secondary drying

Secondary drying is the final stage of lyophilization process. At the end of primary drying, the sample still contains unfrozen water. In secondary drying, the removal of unfrozen water is occurred in elevated temperature and vaccum pressure condition. Usually, the shelf temperature of secondary drying is started at 25-30°C for protein formulations. A lower temperature induces the longer duration. The chamber pressure is at least 200mTorr. When the residual moisture reaches to the desire level, the secondary drying state is ended (Chang and Patro, 2004).

5.8. Excipients used in lyophilized formulation

A lyophilized formulation may contain active ingredient, buffer, bulking agents, stabilizers, tonicity adjusters, surface-active agents (Schwegman, Hardwick and

Akers, 2005). There are two kinds of stabilizers: cryoprotectants prevent the stress during freezing and lyoprotectants reduce the drying damage (Abdelwahed, et al., 2006).

Active pharmaceutical ingredients and the route of administration are the most important factors that decide the selection of excipients (Nireesha, et al., 2013). Bulking agents, buffer, tonicity modifiers and stabilizers are main ingredients required in freeze-dried formulations.

- **Buffer** (Nireesha, et al., 2013)

Buffers, such as phosphate buffer, citrate buffers are used for the pH stabilization purpose. They help to adjust and pH of the formulation during freeze-drying process. It is highly recommended to use a low concentration buffer that presents a minimal pH shift during freezing for example histidine buffer and citrate buffer.

- **Bulking agent** (Nireesha, et al., 2013)

This excipient is usually applied in the low dose of active ingredient formula. It provides the bulk of the cake resulted in an elegant appearance and a good physical stability product. Mannitol, sucrose, glycine, trehalose, lactose are the most common bulking agents. This type of agent could be in crystalline or amorphous state.

- **Stabilizers**

The purpose of stabilizers is to protect active ingredients from freezing stress (cryoprotectants) and drying stress (lyoprotectants). The crystallization of ice, the high freeze concentration may pose a threat to the active ingredients such as protein, peptide, liposomes leading the destabilization of product (Abdelwahed, et al., 2006). The presence of cryoprotectants is to prevent the aggregation, and other undesirable action during freezing phase. Whereas, lyoprotectants ensure the stability of protein or liposome based on the water replacement hypothesis. Through the hydrogen bond formation between lyoprotectant and protein, the native structure of protein could be preserved (Abdelwahed et al., 2006). Example of stabilizers: sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, etc.

- **Tonicity adjusters** (Nireesha, et al., 2013)

Tonicity adjusters are needed for an isotonic solution and an osmotic pressure.

Excipients such as sodium chloride, mannitol, sucrose and glycine are popular tonicity adjusters.

Glycine was reported to have good impact on the stability of lyophilized human growth hormone when combine with mannitol (Pikal, et al., 1991). Sucrose could be used to protect the conformation of recombinant human serum albumin during lyophilization process and storage (Han, 2007). The combination between sucrose and glycine was demonstrated to preserve the activity recovery of the model proteins (lactate dehydrogenase and glucose 6-phosphate dehydrogenase) better than single glycine formulation (Liu, Wang and Nail, 2005). The mixture of glycine and sucrose provided the final lyophilized cake without collapse (Kasraian, et al., 1998).

5.9. Protein stability during lyophilization process

Although the main purpose of lyophilization process is to enhance the stability of protein, a number of stresses that always occurred during the process may contribute to losses in biological activities of protein. In particular, the dramatic physical changes during freezing stage may increase the majority risk on the protein stability.

5.9.1. Freezing stresses

- **Cold denaturation**

Cold denaturation is known as an event that protein unfolded when the temperature is decreased, typically below than 0°C (Bhatnagar, Bogner and Pikal, 2007). Cold denaturation is probably a reason of destabilization of protein native conformation since the thermodynamical stability of protein only maintained in determined range of temperatures (Tang and Pikal, 2005).

- **Freeze-concentration**

During freezing stage, the appearance of ice crystals is accompanied with an increase in concentration of solutes. Also, the viscosity of the solution increased. This fact could stimulate the reaction between protein molecules, resulted in the denaturation of protein (Bhatnagar, Bogner and Pikal, 2007).

On the one hand, freeze-concentration induces the crystallization of excipients in lyophilized formulations. The crystallization of buffer components leads to the alteration of pH during freezing. In fact, protein presents its stability depending on pH change. Additionally, the crystallization of non-buffer excipients that reduces the stability of protein is well-documented (Bhatnagar, Bogner and Pikal, 2007). On the other hand, freeze-concentration probably leads to the phase separation. This phenomenon occurs possibly between different excipients or between protein and excipients. In consequence, the molecular interaction affects directly the protein conformational stability (Bhatnagar, Bogner and Pikal, 2007).

- **Ice formation**

The formation and growth of ice are indirect factors that destabilize protein during freezing since it causes the following increase of solute concentration and ice-water interface (Bhatnagar, Bogner and Pikal, 2007). The formation of ice is related to the freezing rate and freezing temperature.

5.9.2. Primary drying and secondary drying stresses

The alteration of protein structure is come from unstable thermodynamical properties of native conformation due to the loss of water during primary drying and especially secondary drying (Jiang and Nail, 1998).

5.10. Mechanism of stabilizer

5.10.1. Cryoprotectant

- **Glass matrix mechanism**

When temperature is reduced and reaches glass transition temperature Tg', cryoprotectants vitrify. The viscosity of freeze-concentration reduces the mobility of protein molecules and therefore prevents their destabilization due to the glassy matrix (Pikal, 1999).

- **Particle isolation hypothesis**

The cryoprotectants, especially sugars separate particles in unfrozen state during freezing stage at temperature higher than glass transition temperature (Tg') (Allison, Molina and Anchordoguy, 2000).

5.10.2. Lyoprotectant

- **Water replacement hypothesis**

The generation of hydrogen bond between protectant molecule and protein molecule during drying stages could protect protein from the instability in structure (Abdelwahed, et al., 2006).

- **Amorphous state hypothesis**

The existence of protein and stabilizers in amorphous state maximize the stability of protein due to the hydrogen bond between molecules (Abdelwahed, et al., 2006). The crystallization of excipients induces the loss of activity of protein.

VI. Protein

6.1. Primary structure

Protein primary structure is defined as the sequence of amino acid that is started by N-terminus and ended by C-terminus. In protein molecule, two amino acids are combined together by peptide bond (Buxbaum, 2007).

6.2. Secondary structure

Protein secondary structure reveals some local conformation of amino acids. It is formed by the hydrogen bond between backbone atoms. There are some types of secondary structure: α-helix, β-sheet, β-turn or random coil (Buxbaum, 2007).

- **α-helix**

Typically, α-helix is a right-handed chain with 3.6 amino acid per turn. The length of each turn is around 5.4Å. The hydrogen bonds between C=O group of amino acid residues and N-H group of four amino acid further in the chain stabilize the secondary structure of protein (Buxbaum, 2007).

- **β-sheet**

Beta-sheet is stabilized by hydrogen bond between N-H and C=O. There are two kinds of β-sheet: parallel β-sheet and anti- parallel β-sheet. All the N-terminus of the parallel sheets are oriented to the same directions. The anti-parallel β-sheet consists of N-terminus which always change the direction alternately (Buxbaum, 2007).

- **β-turn**

The polypeptide direction turns 180°. The hydrogen bond is formed between C=O group and NH group of three amino acids further (Buxbaum, 2007).

- **Random coil**

A protein that does not have secondary structure is called random coil.
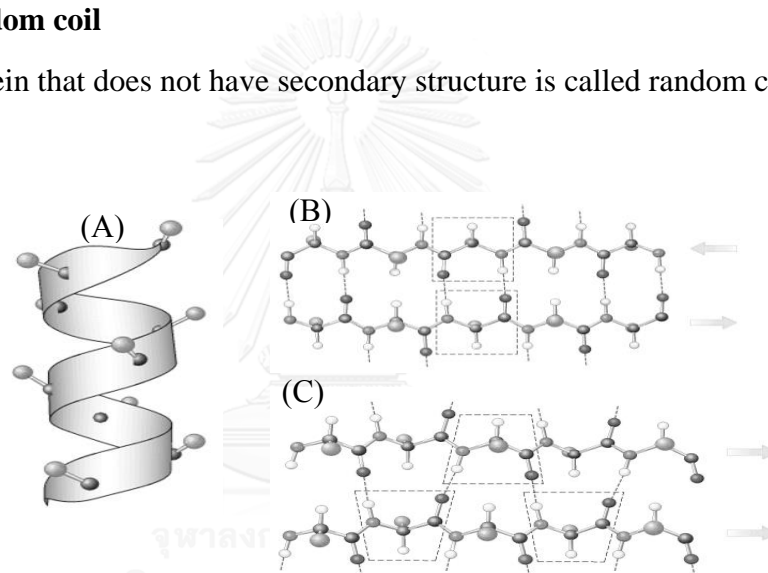
Figure 7. Secondary structure of protein (A): Alpha-helix structure, (B): Antiparallel β-sheet, (C): Parallel β-sheet (Berg Tymoczko and Stryer, 2000)

6.3. Tertiary structure

Tertiary structure represents the arrangement of secondary structure in space. This structure is stabilized through ionic interaction, hydrophobic interaction, hydrogen bond and van der Waal forces. Additionally, some proteins, which compose cysteine residues, have also disulfide bonds (Buxbaum, 2007).

6.4. Quaternary structure

Quaternary structure is determined by several polypeptide chains that bind together to form a single protein. The interactions of quaternary structure are similar to those of tertiary structure (Buxbaum, 2007).

VII. Protein structure conformation evaluation

7.1. Circular dichroism spectroscopy (CD)

Circular dichroism spectroscopy is a valuable technique used to characterize protein structure. The mechanism is due to the extent to different degree of a chromophore substance absorbed the polarized light of incident beam. The difference in absorbance in left-hand and right-hand of circularly polarized light ($\Delta A = A_L - A_R$) is measured and then expressed under the ellipticity ($\theta = 32.98\ \Delta A$).

The diagram of a spectroscopy is displayed in Figure 8. The circular polarizer is produced from unpolarized source, which passed through a plane polarizer. Then, the circular polarized light is run over the sample compartment. Finally, the ellipticity is detected.
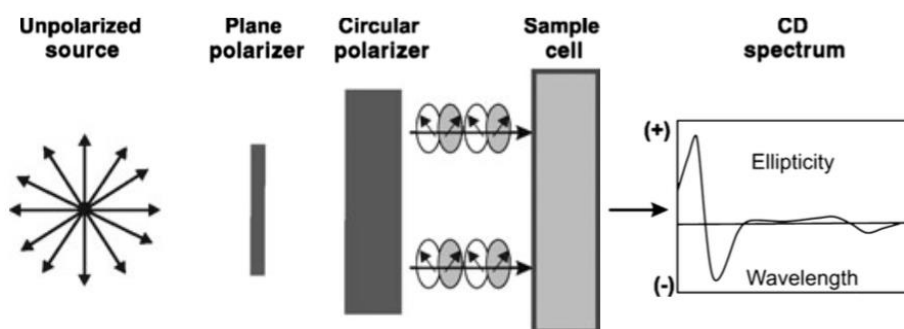
Figure 8. Anatomic structure of a CD spectroscopy (Ranjbar and Gill, 2009)

- Protein secondary structure

CD determines the secondary structure conformation of protein such as α-helix and β-sheet, random coil based on the peptide bonds, which are absorbed principally in far UV region (240nm and below) (Greenfield, 2006). Peptide chromophores show

the weakest transition n -> π* at 210-220nm due to the carbonyl group with non bonding electrons of O and the strongest transition π ->π* at 190nm due to the carbonyl with π electrons. Each type of secondary structure possesses the identified CD spectroscopic pattern (Ranjbar and Gill, 2009).

Alpha-helix proteins show a positive peak at 193nm and two negative peaks at 222 nm and 208 nm (Greenfield, 2006). Whereas, the conformation of β-sheet protein produces positive bands at 190nm, while negative bands observed at 210-220nm (Sreerama and Woody, 2003).

The detection of secondary structure of short peptide sequences (9 amino acids or shorter) by CD spectroscopy at far-UV wavelength was already reported by Sommese and colleagues (Sommese, et al., 2010).
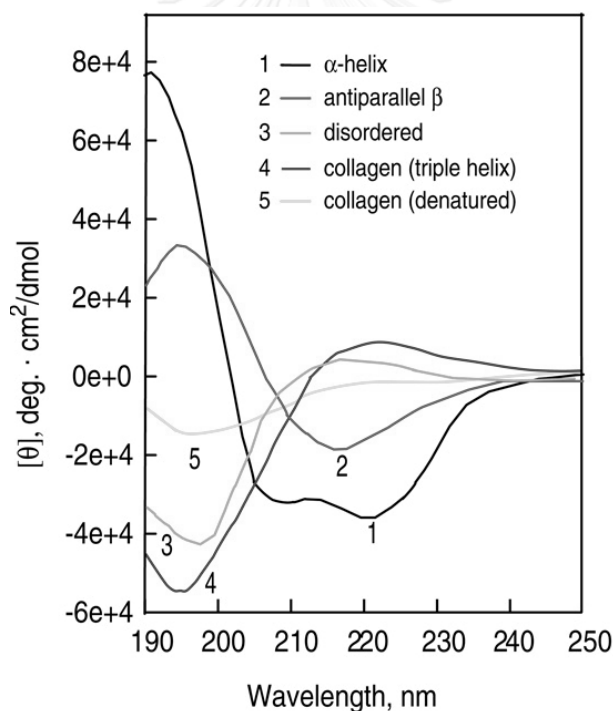
Figure 9. Circular dichroism patterns of some proteins or peptide in far-UV wavelength, characterized for their secondary structures (Greenfield; 2006)

- Protein tertiary structure

CD spectra in near UV region (320-260nm) are sensitive to the tertiary conformation of protein. In this range of wavelength, the aromatic amino acids play as

the main absorbed groups, affecting directly to structural changes of proteins. The display of aromatic group is due to the transition $\pi \rightarrow \pi^*$ (Ranjbar and Gill, 2009). Peaks at 250-270nm, 270-290nm and near 280-300nm characterize phenylalanine, tyrosine and tryptophan, respectively (Johnson et al., 2012).

7.2. Fluorescence spectroscopy

Fluorescence spectroscopy is sensitive to the stability of protein conformation. The data obtained from fluorescence spectroscopy provides the information of changes in protein structural conformation (Weichel, Bassarab and Garidel, 2008).

Aromatic amino acids, such as phenylalanine, tyrosine, tryptophan are fluorophores in protein molecules. Typically, tryptophan is the principal intrinsic fluorophore used to study the stability of protein. In protein molecule, which does not contain tryptophan group, tyrosine is the dominant chromophore showing fluorescence absorption (Bekard and Dunstan, 2009). Fluorescence spectroscopy detects the structure of protein based on the emission energy after exposing proteins into an excited state. Aromatic amino acid residues are contributed to the emission. It was found that the emission of tyrosine occurred at 303 nm when applying an excitation at 280 nm (Figure 10).
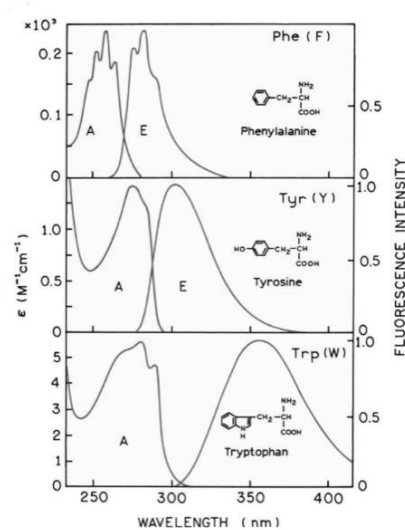
Figure 10. The absorption and emission spectra of phenylalanine, tyrosine and tryptophan at neutral pH (Lakowicz, 2006)

In fact, GPO liquid LAIV vaccine, known as FLUVAC (with sucrose, glutamate and phosphate), retained a good stability, which is shorter than 24 weeks at -20°C. Then, the storage at low temperature makes the distribution process inconvenient and costly. The production of dry powder influenza vaccines may increase the storage temperature to 2-8°C, which will be very promising to facilitate logistics procedure and stockpiling issues in a flu pandemic situation.

According to Madhu Khanna and colleagues, most antibodies responded to influenza infection are produced against globular head of HA (Khanna, et al., 2014).

Amorij et al research reported that HA maintained antigenic properties after freeze-thawing and freeze-drying process regarding to the protectant activities of carbohydrates such as inulin, dextran and trehalose (Amorij, et al., 2007). Glycine and sucrose combination was demonstrated to give good stability for some types of proteins such as recombinant factor IX, lactate dehydrogenase and glucose 6-phosphate dehydrogenase (Bush, et al., 1998). However, there is no data on the effect of glycine and sucrose mixtures on HA conformation. On the other hand, freezing stage plays an important role during lyophilization process. Its main effects are on the physical properties, biological activities and stabilities of lyophilized products (Kasper and Friess, 2011). Hence, the effect of freezing stages on the conformation of pure and formulated HA, the effect of freeze-drying on the properties and stability of final lyophilized LAIV vaccine products will be investigated in this study.

CHAPTER III - METHODS AND MATERIALS

PART 1: MATERIALS

I. Materials

- Haemagglutinin influenza: molecular weight: 1102.15 Da (Genscript, USA)
- Glycine (Merck, USA)
- Sucrose (Supplied by Government Pharmaceutical Organization, Thailand)
- Mannitol (Supplied by Government Pharmaceutical Organization, Thailand)
- Sodium dihydrogen phosphate (Fisher Scientific, USA)
- Disodium hydrogen phosphate (Fisher Scientific, USA)
- Sodium Chloride (Ajax Finechem, USA)
- Sodium Hydroxide (Merck KGaA, Germany)
- Sodium-L-glutamate monohydrate (Merck, USA)
- Gelatin (Gelita, USA)
- L-arginine monohydrochloride (Merck, USA)
- Apura Combotitrant 5 (Merck, USA)

II. Equipments

- Lyophilizer (Model Lyolab LT3S, USA)
- Circular dichroism spectropolarimeter (Model J-715, Jasco, Kyoto, Japan)
- Spectrofluorometer (Model FP-8200, Jasco, Kyoto, Japan)
- Differential Scanning Calorimeter (Model DSC822e, Zurich, Switzerland)
- Thermogravimetric analyzer (Model TGA/SDTA851e, Switzerland)
- X-ray powder diffractometer (Model Miniflex II, Rigaku, Japan)
- Analytical Balance (Mettler Toledo, USA)
- Osmometer (Model Osmomat 030, Gonotec Gmb, Germany)
- pH meter (Mettler Toledo S-47, USA)
- Karl Fisher titrator (Model Metrohm 720, USA)
- Pipette 10-100 µl (Rainnin, USA)
- Pipette 100-1000 µl (Rainnin, USA)

PART 2: METHODS

I. Effect of freezing cycles on HA conformation

1.1. Haemagglutinin concentration screening

Native structure of HA peptide (residues 98-106) was investigated by CD spectroscopy and fluorescence spectroscopy. Pure HA powder was dissolved in water for injection (WFI) to achieve various concentrations of 0.09, 0.25, 0.3, 0.4 and 0.5mg/ml.

a. CD spectroscopy

Far-UV (200-250nm) and near-UV (250-320nm) wavelengths were used to determine the secondary structure and tertiary structure of HA, respectively (Luykx, et al., 2004). Quartz sample cells used for far-UV and near-UV wavelengths were at 0.1 and 1.0cm pathlengths, respectively. The optimum instrumental condition was held constant at the sensitivity of 20mdeg, bandwidth of 1nm and the response time of 2s, accumulation of 3 repetitions.

b. Fluorescence spectroscopy

The tyrosine fluorescence emission was detected by fluorescence spectroscopy. Solution of HA was added in a 1cm quartz cell for fluorescence scanning. An excitation wavelength was set at 280nm. A band pass of 2.5nm and 5nm were set for the excitation and emission slits, respectively. Fluorescent spectra were collected at 1 nm intervals from 290-400nm with a scan speed of 250nm/min and accumulation of 3 repetitions for each sample run. The conditions for this experiment were modified from previous studies by Luykx and his colleagues, and Amorij (Luykx et al.; 2004 and Amorij; 2007)

Suitable HA concentration for the analysis was selected from these CD and fluorescence results.

1.2. Effect of freezing cycles on pure HA conformation

Pure HA solutions (0.55 mg HA in 100 µl of WFI) were frozen with various freezing rates (rapid freezing, moderate freezing and slow freezing as shown in Table

1 and Figure 11) by Differential Scanning Calorimeter (DSC). The frozen HA solutions were defrosted to room temperature at 25°C at a rate of 15°C/min and then diluted with 2.1 ml of WFI to obtain final HA concentration as determined in Section 1.1. The solutions were examined by CD and fluorescence spectroscopy. CD and fluorescence patterns of HA conformation were analyzed.

Table 1. Freezing cycles for the study of freezing rates on pure HA conformation

Slow freezing		Moderate freezing		Fast freezing	
Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
25	0	25	0	25	0
0	25	0	20	0	10
-20	75	0	20	-40	15
-20	40	-20	20	-40	80
-40	85	-20	40		
-40	100	-40	20		
		-40	70		
Average freezing rate					
0.50 (°C/min)		1.08 (°C/min)		2.58 (°C/min)	
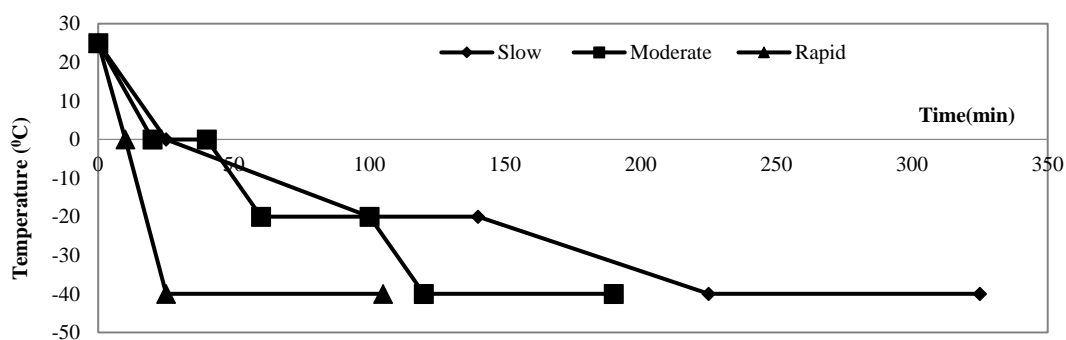
Figure 11. Freezing cycles for the study of freezing effect on pure HA conformation

1.3. Effect of freezing cycles on HA-stabilizer formulations

              1.3.1.   **Freezing**   **point**   **determination**   **of**   **HA-stabilizer**
formulations

Mixtures of HA with glycine, sucrose in phosphate buffer saline (PBS, pH 7.4, qs. 100%) at various ratios were prepared as shown in Table 2. PBS consisted of 0.03%w/v of monosodium dihydrogen phosphate, 0.26%w/v of disodium hydrogen phosphate, and 0.45%w/v of sodium chloride. The freezing point of samples were examined by DSC. Approximately 20µl of sample solution was filled into 40µl aluminium pan. Another sealed empty aluminium pan was placed at the reference position. Samples were cooled from room temperature of 25°C to -40°C at a rate of 2°C/min. Then, samples were held at -40°C for another 5 minutes before heating back to 25°C at a heating rate of 5°C/min. Resulting DSC thermograms presented freezing and melting temperatures of these formulations.

Table 2. HA-stabilizer formulations

Formulations	G6S0	G6S1	G0S1	G0S0
Glycine (%w/v)	6	6	-	-
Sucrose (%w/v)	-	1	1	-
HA	0.55 mg			
PBS	qs. 100 µl			

        1.3.2. Effect of stabilizers on HA conformation upon various freezing rates

Approximately 100µl HA - stabilizer formulations with various stabilizer concentrations as shown in Table 2 were prepared. The samples were primarily frozen by DSC with various freezing rates according to Table 1. The frozen-samples were then defrosted to room temperature of 25°C and diluted with 2.1ml of WFI (to a suitable final concentration of HA in Section 1.1). Finally, the secondary and tertiary structures of HA were evaluated by CD and fluorescence spectroscopy according to

the method described in Section 1.1. Finally, the CD and fluorescence patterns were recorded and evaluated (Equations 1 and 2).

Equation 1. Percentage of preserved HA conformation

$$\% \text{ Preserved HA conformation} = \left[\frac{(CD \text{ ellipticity/ Fluorescence intensity}) \text{ of frozen HA}}{(CD \text{ ellipticity/ Fluorescence intensity}) \text{ of native HA}} \right] \times 100$$

Equation 2. Percentage change in maximum (minimum) CD/Fluorescence value

$$\% \text{ Change} = 100 - \% \text{ Preserved HA conformation}$$

II. Lyophilization of live attenuated influenza virus (LAIV) vaccine formulations

The freezing rate and the formulations obtained from previous studies in Sections 1.2 and 1.3 were subjected to be included in the final selected formulations for lyophilization. The preparation and lyophilization process were performed by GPO personnel under controlled environment at GPO Research and Development Institute due to the use of live attenuated influenza virus.

2.1. Preparation of LAIV vaccine formulations

Sample preparation with the incorporation of LAIV was conducted by GPO specialists at GPO Research and Development Institute. The formulation compositions could be summarized as shown in Table 3.

Table 3. Formulation components of lyophilized LAIV vaccine

Formulations	G6S0	G6S1	G0S1	G0S0
Glycine (%w/v)	6	6	-	-
Sucrose (%w/v)	-	1	1	-
Mannitol				
Monosodium Glutamate		Included in the formulations		
Gelatin		(amounts are not disclosed due to		
L-arginine.HCl		confidentiality issue)		
Virus (% v/v)				
PBS		qs. 100%		

To improve the quality of lyophilized LAIV vaccine products, mannitol, monosodium glutamate, L-arginine hydrochloride and gelatin were added in the formulations of HA. Mannitol was utilized as bulking agent and stabilizer. Gelatin was used as cryoprotectant. Monosodium glutamate, L-Arginine hydrochloride were introduced to increase the shelf life and infectivity titer of products.

2.2. Lyophilization of LAIV vaccine formulations

Appropriate freezing rate for initial lyophilization process was in accordance with our previous finding in Sections 1.2. The details of primary drying and secondary drying stages were decided by GPO specialists according to their lyophilizer capability.

Table 4. Lyophilization process for LAIV vaccine formulations

Stages	Temperature (°C)	Min	Pressure (mTorr)
Samples were frozen from 25°C to -50°C at a freezing rate of 1.08°C/min		70	3000
Freezing step	-50	300	3000
Primary drying step	-50	1680	100
	-45	240	100
	-40	240	100
	-30	240	100
	-20	120	100
	-10	120	100
	10	120	100
	20	120	100
Secondary drying step	30	360	100

2.3. Characterization of lyophilized LAIV vaccine products

2.3.1. Physicochemical characterization of lyophilized LAIV vaccine products

a. Physical appearance

The lyophilized cakes obtained were inspected visually. The cakes should be in a uniform lattice without collapse or shrinkage (Carpenter, et al., 2002).

b. Reconstitution time

The lyophilized products were reconstituted with 1ml of WFI. The time of complete dissolution was evaluated visually and recorded. It should not be longer than

60 seconds. This acceptance criterion is based on the reconstitution time of some lyophilized proteins reported earlier (Schersch, et al., 2010).

c. pH and osmolality

Lyophilized samples, which were reconstituted with 1ml of WFI, were subjected to pH value determination by a calibrated pH meter and osmolality value determination by an osmometer. Acceptance criterion of pH should range between 6.5 and 7.5 (Washington, 2000) while the osmolality should be within 250-700mOsm/kg (Kulkarni and Shaw, 2012).

d. Thermal behavior

Thermal behavior was evaluated by DSC. Approximately 2-5mg of lyophilized sample is hermetically sealed in a 40µl aluminum crucible with one pin hole. An identical empty sealed aluminium pan was chosen as the reference. Products were heated from 25°C to 175°C at a rate of 10°C/min. DSC thermograms obtained were analyzed.

e. Crystallinity

The solid-state crystallinity of lyophilized products was determined by X-Ray powder diffractometry (XRPD). The condition was performed with 5-40° 2θ angle at a scan speed of 1°C/min. That method was modified from the study of Lang and Winter (Lang and Winter, 2009). The instrument is operated with CuKα radiation at a wavelength of 1.5418Å.

f. Residual moisture content

Residual moisture content was determined using two comparative methods, thermogravimetric analysis (TGA) and Karl Fisher titrimetry.

▪ Thermogravimetric analysis (TGA)

The moisture content of freeze-dried products were evaluated by using thermogravimetric analyzer (TGA). Approximately 2-10mg of lyophilized powder was placed in a 70 µl aluminium crucible and heated from 25°C to 175°C at a heating rate of 10°C/min under purge nitrogen gas. The percentage of moisture content was calculated based on the TGA thermograms obtained.

- **Karl Fisher titrimetry**

The residual moisture content of products were also measured by Karl Fisher titrator. Approximately 60-80mg of lyophilized samples were placed into the titration vessel. Apura Combotitrant 5 was used as reagent. Moisture content should be less than 3% (Grist, 1996; WHO, 1994; WHO, 1995).

g. Moisture stability determination

Dynamic vapor sorption (DVS) was used to characterize the moisture stability of lyophilized products. Sample weighing approximately 10-20mg was placed on an aluminium pan on the microbalance. Temperature of chamber was controlled at 30°C. The relative humidity (RH) was varied within the range of 0 to 90% RH at 10% RH increment. The dm/dt criterion was set at 0.05%/min. This method was modified from a previous report by Young (Young, 2012).

2.3.2. Infectivity titer measurement

The 50 % tissue culture infectious dose ($TCID_{50}$) and HA assay were performed by GPO personnel at GPO Research and Development Institute.

TCID$_{50}$ was performed with Madin-Darby Canine Kidney (MDCK) cells. Reconstituted lyophilized samples were diluted serially to 10^{-8} dilutions. Each of diluted samples at 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} was inoculated in quadruplicates into the 96-well plate containing MDCK cells. The plates were then incubated at 33°C for 5 days. The harvested supernatant (50µl) were placed into a round bottom 96-well plate and subjected to HA assay. Subsequently, 50µl of 0.5% goose red blood cell was added to each well. The plate was mixed gently. The time for incubation was approximately 30minutes. The assay was performed based on the mechanism of HA agglutination to red blood cell. The results were inspected visually. Dots in the center of wells were presented as negative results. Positive well produced reddish color across the wells. Finally, Reed Muench method (Equations 3 and 4) was used for calculation (Reed and Muench, 1938). The results were expressed as mean value of two trials (n=2).

Equation 3. Proportion distance

Proportion	(%Positive value above 50% - 50%) x Correction factor
distance $=$	(%Positive value above 50% - %Positive value below 50%)

Equation 4. Log$_{10}$ TCID$_{50}$/0.2ml

log $_{10}$ TCID$_{50}$ $=$	Σ (-*log$_{10}$* % positive value above 50% + Proportion distance)

Acceptance criteria: log$_{10}$ TCID$_{50}$/0.2ml should not be less than 6.5 (similar to the acceptance criteria of GPO liquid LAIV vaccine).

III. Stability evaluation of lyophilized LAIV vaccine products

The final lyophilized products were kept at three different conditions: -20°C, 2-8°C and 30°C/75%RH for stability evaluation. Storage periods for stability evaluation were proposed for 12 months, except 30°C/75%RH where it was proposed for only 28 days. The time intervals for sample evaluation were as follow:

- -20°C for 0, 1, 2, 3, 6, 12 months
- 2-8°C for 0, 1, 2, 3, 6, 12 months
- 30°C/75%RH for 0, 7, 14, 21, 28 days (holding time evaluation)

At each time point, the samples were tested for physical appearance, pH, osmolality, reconstitution time, thermal analysis, crystallinity, residual moisture content, TCID$_{50}$ with methodology details in Sections 2.3.1 and 2.3.2.

In this thesis, product stability evaluation at -20°C and 2-8°C were analyzed only during the first six months due to time limitation. GPO personnel will evaluate continual stability monitoring from 6-12 months.

IV. Statistical analysis

The significance of the difference in mean values between 2 storage conditions was calculated based on t-test. The difference is statistically significant at p-value < 0.05.

CHAPTER IV - RESULTS AND DISCUSSION

I. The effect of freezing cycles on HA conformation

1.1. Haemagglutinin concentration screening

   ▪ **Circular dichroism**

Far-UV wavelength displays the behavior of HA secondary structure in aqueous solution at various concentrations as shown in Figure 12. The concentrations of HA were varied from 0.09mg/ml to 0.5 mg/ml. As inspected visually, CD spectra in far-UV wavelength reveals that HA secondary structure contains mainly β-sheet with only minor part of α-helix. Negative bands are found between wavelength range of 210-220nm, indicating β-sheet of secondary structure as reported in other studies (Diego, et al., 2004; Tomczynska-Mleko, et al., 2014). Moreover, these results are in good agreement with previous study of secondary structure of HA residues from 98-106 (Wilson and Cox; 1990).
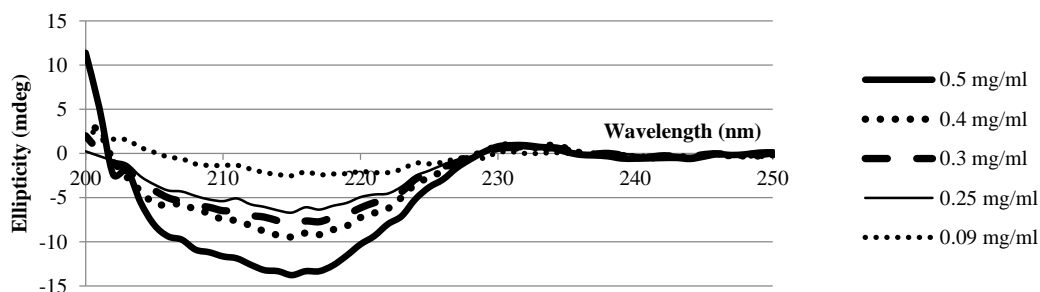
Figure 12. Far-UV CD spectra of pure HA dissolved in water at various concentrations (0.5mg/ml, 0.4mg/ml, 0.3mg/ml, 0.25mg/ml and 0.09mg/ml)

CD spectra of tertiary structure of pure HA in aqueous solution at various concentrations are presented in Figure 13. The tertiary structure of HA was determined due to CD signal of tyrosine group in the region of 270-290nm, which is in good agreement with other reports (Johnson, et al., 2012; Kelly and Price, 2000).
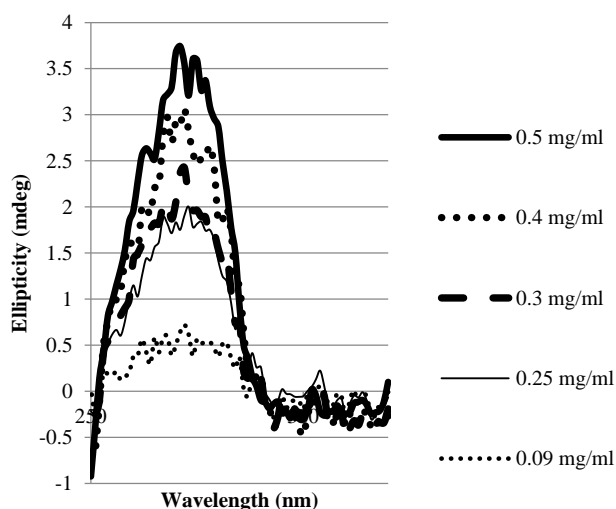
Figure 13. Near-UV CD spectra of pure HA dissolved in water at various concentrations (0.5mg/ml, 0.4mg/ml, 0.3mg/ml, 0.25mg/ml and 0.09mg/ml)

At the concentration of 0.09mg/ml, it is difficult to evaluate the conformation of HA at this concentration. On the other hand, using the concentration of HA greater than 0.25mg/ml is unnecessary. Therefore, the proper HA concentration for future studies on secondary and tertiary structures should be 0.25mg/ml.

b. Fluorescence spectroscopy

Fluorescence spectra of pure HA in water at various concentrations are presented in Figure 14. Tyrosine group in HA peptide exhibits emission maxima at approximately 303nm, in accordance with previous results (Jordano, et al., 1983). The concentration at 0.25mg/ml is also found to be suitable for fluorescence spectroscopy experiments.
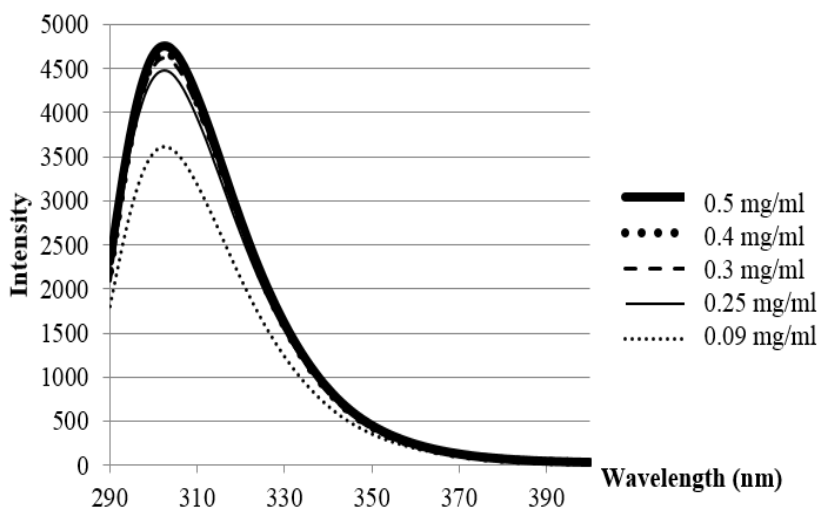
Figure 14. Fluorescence spectra of pure HA dissolved in water at various concentrations (0.5mg/ml, 0.4mg/ml, 0.3mg/ml, 0.25mg/ml and 0.09mg/ml)

In conclusion, the HA concentration of 0.25mg/ml would be used as reference in further CD and fluorescence spectroscopy studies. This concentration is the concentrated-frozen HA after diluting with WFI. This means that the initial concentration used is 5.5mg/ml, which is still in the acceptable protein concentration range of vaccine formulations reported by Sachdeva and Dutta (Sachdeva and Dutta, 2007).

✚ Far-UV, near-UV CD spectra and Fluorescence spectra properties of native HA in aqueous solution

As previous studies has reported that HA had native conformation at neutral pH (7.0-7.4) (Wiley; 1983, Bottchera; 1999), and in aqueous solution. This sample is considered as untreated HA for further comparison.

Table 5. Spectral properties of native HA dissolved in aqueous solution at the concentration of 0.25mg/ml

Spectra	Peak position (nm)	Intensity of peak (mdeg)
Far-UV CD	215	-6.70
Near-UV CD	273	2.00
Fluorescence	303	4476

1.2. Effect of freezing cycles on pure HA conformation

1.2.1. Circular dichroism results

▪ **Secondary structure**

As seen in Figure 15, secondary structure of HA in aqueous solution differs after freezing in term of peak positions and ellipticity values. Basically, the shape as well as the intensity of CD spectra are characteristics of secondary and tertiary protein structure (Meyer, 2012). This means that freezing somehow modified secondary structure of HA peptide. Ellipticity is decreased dramatically at the wavelength region below 202nm when sample was frozen by slow freezing rate. Evidently, slow freezing rate strongly induced the instability of HA secondary structure. In other words, HA in aqueous solution lost the shape of β-sheet secondary structure when frozen at slow freezing. The long duration of slow freezing cycle increased the damage caused by freezing on secondary conformation. At rapid and moderate freezing rate, minimum troughs are still in the wavelength region of β-sheet characteristic at 210-220nm. However, rapid and moderate freezing rates reduced the intensity value of minimum ellipticity dramatically. The percentages of CD minimum ellipticity preserved are 68.06 and 80.45% after rapid and moderate freezing, respectively (Table 6).

Figure 15. Secondary structure of pure HA in aqueous solution after freezing at various freezing rates (rapid, moderate and slow freezing rates)

Table 6. Summary of far-UV CD spectra of pure HA in aqueous solution in after freezing at various freezing rates (rapid, moderate and slow freezing rates)

Freezing rate	Peak position (nm)	Ellipticity minima	% Preserved	% Change
Untreated HA	215	-6.70	100.00	
Rapid	213	-4.56	68.06	-31.94
Moderate	212	-5.39	80.45	-19.55
Slow	Reduction of ellipticity at below of 202 nm	-	-	-

▪ **Tertiary structure**

Figure 16 displays HA tertiary structures in aqueous solution after freezing. Both the ellipticity intensity and the position of critical peaks are changed under the effect of freezing cycles. The variation of all the identified peaks are still in the range of tyrosine group between 270 - 290nm with the exception of HA frozen at moderate freezing rate (Table 7). The shape of HA frozen at moderate freezing rate is found to be the most similar to native HA with only 7.5% change in intensity value. The maximum ellipticity intensity changed by 20 and 21% as compared to untreated HA when rapid freezing rate and slow freezing rate were used respectively (Table 7).
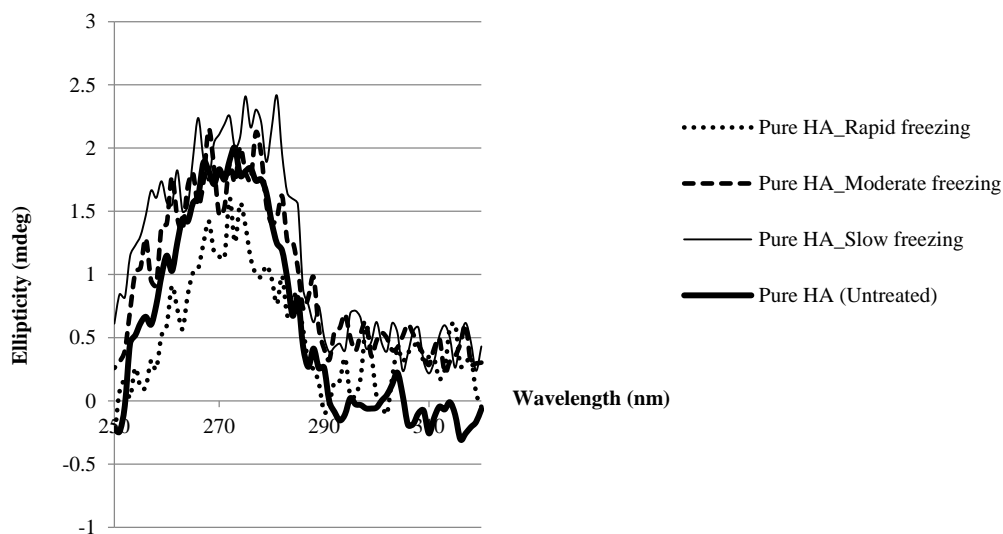
Figure 16. Tertiary structure of pure HA in aqueous solutions after freezing at various freezing rates (rapid, moderate and slow freezing rates)

Table 7. Summary of near-UV CD spectra of pure HA in aqueous solution after freezing at various freezing rates (rapid, moderate and slow freezing rates)

Freezing rate	Peak position (nm)	Ellipticity maxima (mdeg)	% Preserved	% Change
Native HA	273	2.00	100.00	
Rapid	273	1.60	80.00	-20.00
Moderate	270	2.15	107.50	+7.50
Slow	281	2.42	121.00	+21.00

1.2.2. Fluorescence spectroscopy

According to fluorescence spectra in Figure 17, the freezing processes affected structural conformation of HA. Significant changes in HA tertiary structures are observed. There is a variation in intensity with no change in the position of maximum emission. The tyrosine emission peaks remain at approximately 303nm.
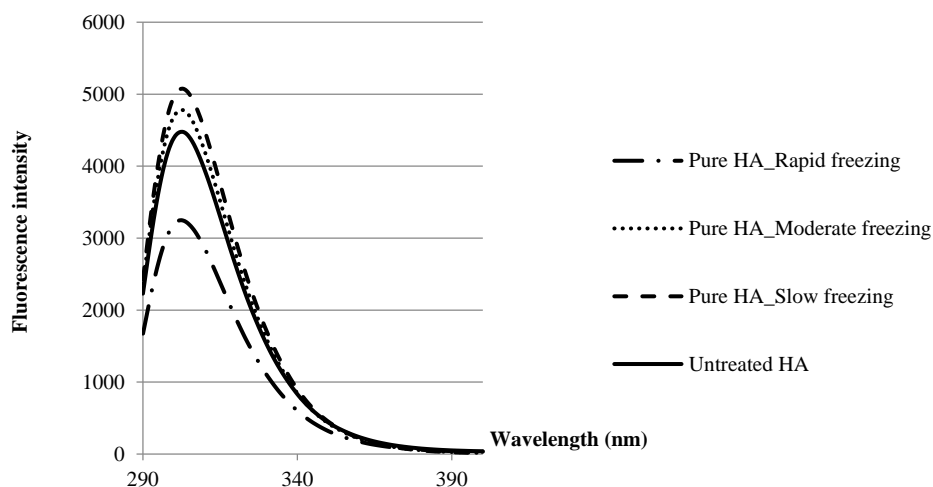
Figure 17. Fluorescence spectroscopic patterns of pure HA in aqueous solution after freezing at various freezing rates (rapid, moderate and slow freezing rates)

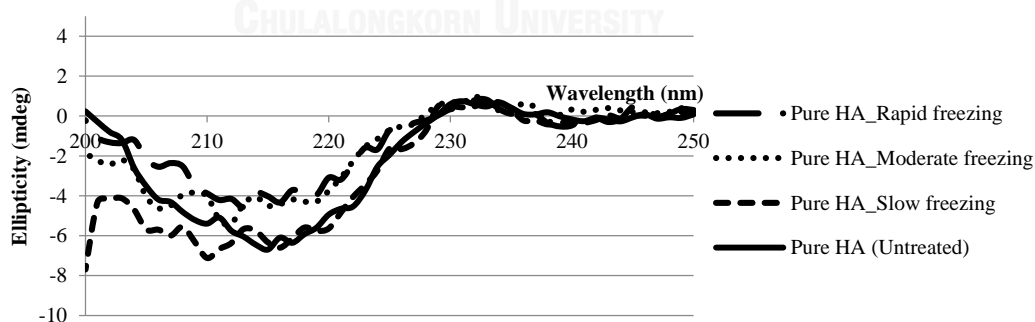*Table 8. Summary of fluorescence spectroscopic patterns of pure HA in aqueous solutions after freezing at various freezing rates (rapid, moderate and slow freezing rates)*

Freezing rate	Fluorescence intensity (peak at 303 nm)	% Preserved	% Change
Native HA	4476	100.00	
Rapid	3243	72.45	-27.55
Moderate	4779	106.77	+6.77
Slow	5075	113.38	+13.38

The secondary and tertiary structure of HA when frozen at different rates are affected to a different extent when detected by CD spectroscopy in far-UV region, near-UV region and fluorimeter. Ice crystal formation, low temperature exposure, increase in protein concentration, and pH shift are considered as reasons causing the instability of protein structural conformation (Amorij, 2007, Luykx, 2004, Pikal-Clevand, 2000). Seemingly, the moderate freezing rate had the spectrum most similar

to that of untreated HA with only 6.77% change in intensity (Table 8). According to fluorescence, the changes in intensity are 27.55 and 13.38% for rapid and slow freezing, respectively (Table 8). Taken together, it could be stated that the secondary and tertiary conformations of pure HA in aqueous solution can be disrupted to a different extent at every freezing rates used with moderate freezing rate being the most preferable. Apparently, the addition of protective agents against freezing stress is necessary to improve the conformational stability of HA.

1.3. Effect of freezing cycles on HA-stabilizer formulations

1.3.1. Freezing point determination of HA - stabilizer formulations

Figure 18 illustrates thermal behavior of G6S0, G6S1, G0S1 and G0S0 formulations when frozen from controlled room temperature 25°C to -40°C at a cooling rate of 2°C/min and heated back to 25°C at a heating rate of 5°C/min. When the temperature reached the freezing point, the formulations transformed from liquid state to solid state. Exothermic peaks due to the released energies presented this phenomenon. It was found that the freezing points of G6S0, G6S1, G0S1 and G0S0 formulations respectively are at -15, -20, -18 and -19°C. From this study, the final temperature for the freezing cycles, conducted at -40°C is suitable for the complete solidification of all formulations.
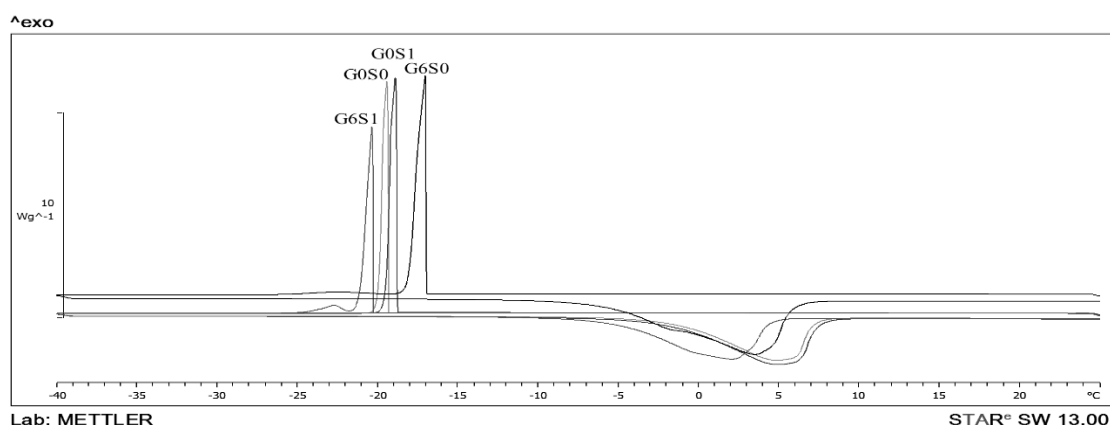
Figure 18. DSC thermograms of G6S1, G0S0, G0S1 and G6S0 formulations frozen to -40°C at a cooling rate of 2°C/min and heated back to 25°C at a heating rate of 5°C/min

1.3.2. Effect of stabilizers on HA conformation upon various freezing rates

HA, one of the most important part of the influenza virus, is subjected to be freeze-dried for LAIV vaccine manufacturing. In lyophilized formulations, cryoprotectants and lyoprotectants are essential. Glycine and sucrose are well-known and popular cryoprotectants and lyoprotectants. They are multifunctional excipients. They not only prevent the damage of freeze-drying process on protein structure but also act as bulking agents and tonicity adjusters (Baheti, 2010; Carpenter, et al.; 2002; Chang, 1996). In addition, PBS buffer is a common choice in lyophilized protein formulations. Thus, the effects of glycine to sucrose ratios in PBS on HA conformation are selected to be studied.

  ▪ **Secondary structure**

As evidence shown in Figure 19, freezing rates influenced the secondary structure of HA. Despite the fact that the characteristic peaks of β-sheet of HA are shifted to shorter or longer wavelength, they are varied in the acceptable interval of β-sheet character of 210-220nm for the majority of samples (Table 9). In terms of ellipticity minima, G6S1 formulation at moderate freezing and G0S1 formulation at rapid and moderate freezing present out-of-range negative peaks at approximately 208-209nm (Table 9). Probably, HA lost its native secondary structure severely in the cases mentioned above. G6S0 and G0S0 at moderate freezing rate show smallest changes in CD minimum intensity with only 5.52 and 3.58%, respectively (Table 9). However, only G6S0 formulation which was frozen at moderate freezing possesses the spectrum shape similar to that of initial native secondary HA. Thus, G6S0 with moderate freezing rate is considered as the most desirable formulation which preserves the original secondary structure of HA the most.

Figure 19. Secondary conformation of HA in (A): G6S0, (B): G6S1, (C): G0S1 and (D): G0S0 formulations after freezing at various freezing rates (rapid, moderate and slow freezing rates) obtained from CD spectroscopy

Table 9. Summary of far-UV CD spectra of HA in G6S0, G6S1, G0S1 and G0S0 formulations after freezing at various freezing rates (rapid, moderate and slow freezing rates)

Formulation	Freezing rate	Peak position (nm)	Ellipticity minimum	% Preserved	% Change
Untreated HA		215	-6.70	100.00	
G6S0	Rapid	216	-5.14	76.72	-23.28
	Moderate	215	-6.33	94.48	-5.52
	Slow	210	-7.48	111.64	+11.64
G6S1	Rapid	214	-5.96	88.96	-11.04
	Moderate	208	Peak in unacceptable range of wavelength		
	Slow	212	-5.33	79.55	-20.45
G0S1	Rapid	209	Peak in unacceptable range of wavelength		
	Moderate	208			
	Slow	211	-5.43	81.04	-18.96
G0S0	Rapid	211	-8.90	132.84	+32.84
	Moderate	213	-6.94	103.58	+3.58
	Slow	210	-4.04	60.30	-39.70

▪ **Tertiary structure**
- **Circular dichroism**

According to CD spectra of near-UV region, the tertiary structure of HA in various formulations are altered after freezing at various freezing rates (Figure 20). There are changes in both the ellipticity intensity values and peak positions. As observed visually, the troughs of HA frozen at moderate freezing rate are the closest to that of untreated HA in most cases. In fact, G6S0, G6S1, G0S1 and G0S0 at both

rapid and slow freezing rates lost their HA native tertiary structure severely by significant reduction in elipticity values or by shifting their positive peaks to the wavelength range below 270nm. The others retained critical peaks in the range of tyrosine group from 270 to 290nm. Among the cases that show positive peaks in appropiate wavelength range, HA in G6S0 formulation and G6S1 formulation frozen at moderate freezing rate show smallest differences in ellipticity maxima as compared to untreated tertiary HA spectrum. The ellipticity maxima intensity of G6S0 is reduced by 6.00% and G6S1 is increased by 9.00% when frozen at moderate freezing rate (Table 10).
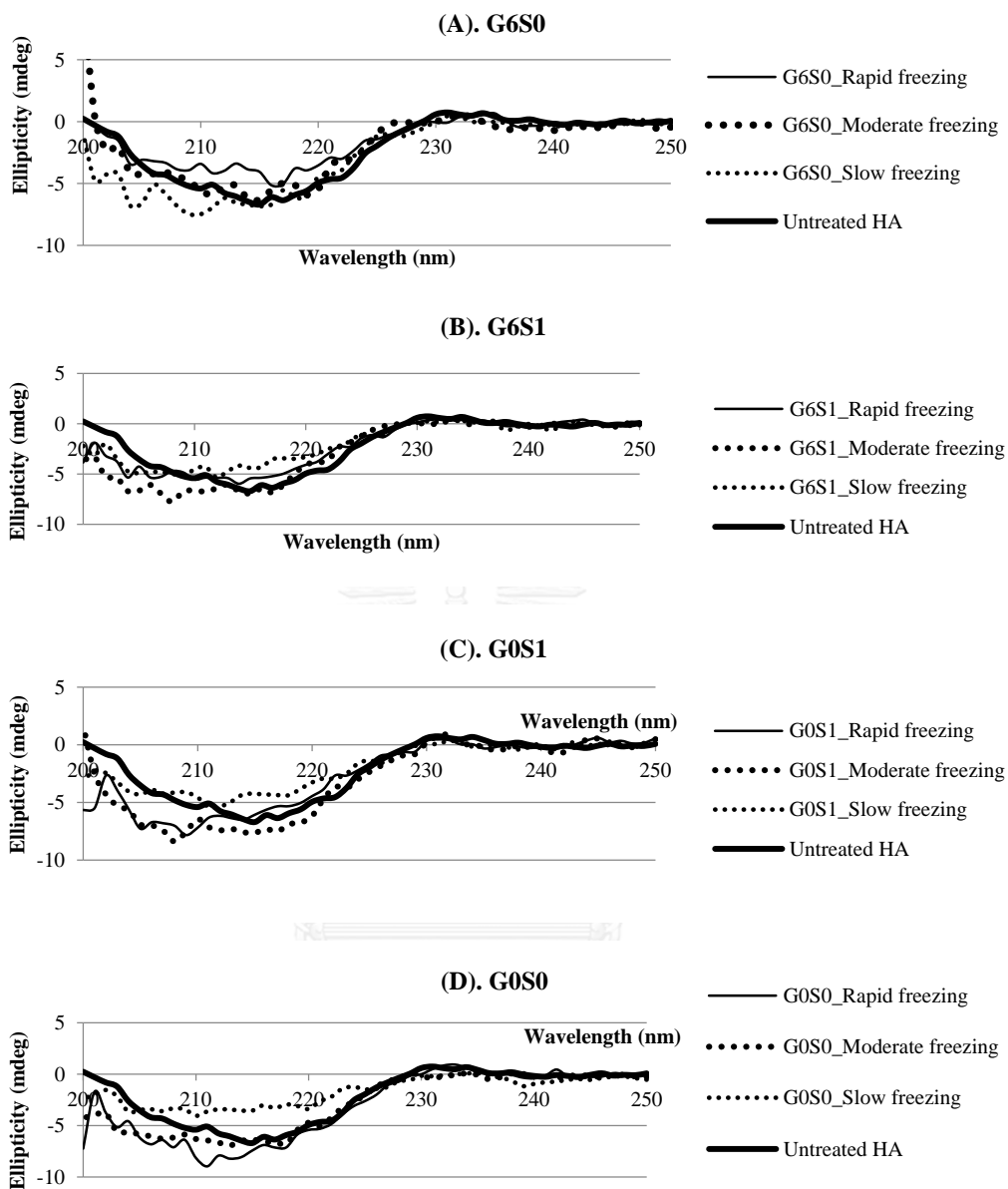

(A). G6S0

(B). G6S1

Figure 20. Tertiary conformation of HA in (A): G6S0, (B): G6S1, (C): G0S1 and (D): G0S0 formulations after freezing at various freezing rates (rapid, moderate and slow freezing rates) obtained from CD spectroscopy
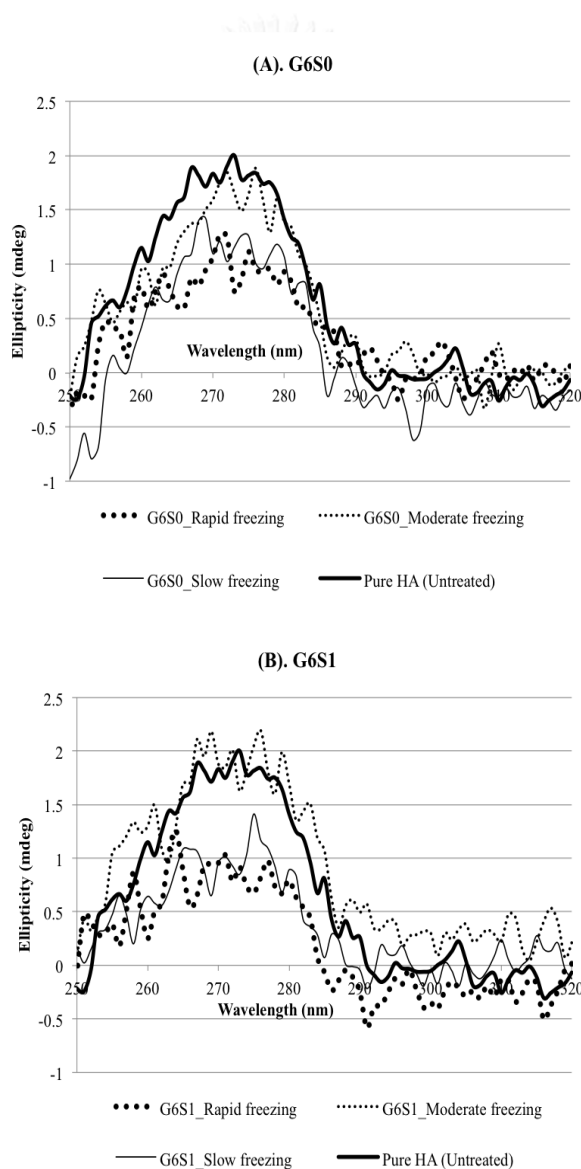
Table 10. Summary of near-UV CD spectra of HA in G6S0, G6S1, G0S1 and G0S0 formulations after freezing at various freezing rates (rapid, moderate and slow freezing rates)

Formulation	Freezing rate	Peak position (nm)	Ellipticity maxima	% Preserved	% Change
Untreated HA		273	2.00	100.00	
G6S0	Rapid	271	1.28	64.00	-36.00
	Moderate	276	1.88	94.00	-6.00
	Slow	269	Peak in unacceptable range of wavelength		
G6S1	Rapid	264			
	Moderate	270	2.18	109.00	+9.00
	Slow	275	1.41	70.50	-29.50
G0S1	Rapid	270	1.60	80.00	-20.00
	Moderate	273	2.30	115.00	+15.00
	Slow	270	1.22	61.00	-39.00
G0S0	Rapid	266	Peak in unacceptable range of wavelength		
	Moderate	270	1.56	78.00	-22.00
	Slow	270	1.11	55.50	-44.50

- **Fluorescence spectroscopy**

Figure 21 reveals that there is no difference in positions of positive peaks. Maximum emission of all formulations after frozen are still situated at approximately 303nm. The spectra of HA frozen at moderate freezing rate is considered as the closest trough to native HA. Especially, G6S0 and G0S0 formulation (moderate freezing) show spectra consistent to untreated HA spectrum with a variation of 1.85 and 2.26%, respectively (Table 11). Combined with results obtained from near-UV

CD, it could be concluded that moderate freezing rate is preferable and G6S0 displays strongest protective activity than other formulas on the tertiary structure of HA.

(A). G6S0
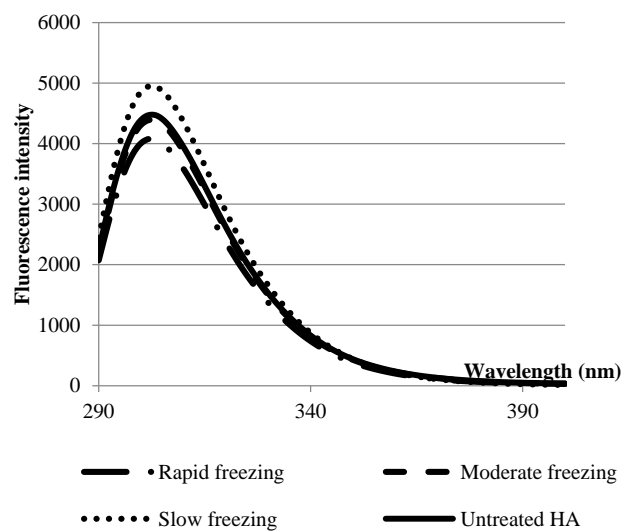
(B). G6S1
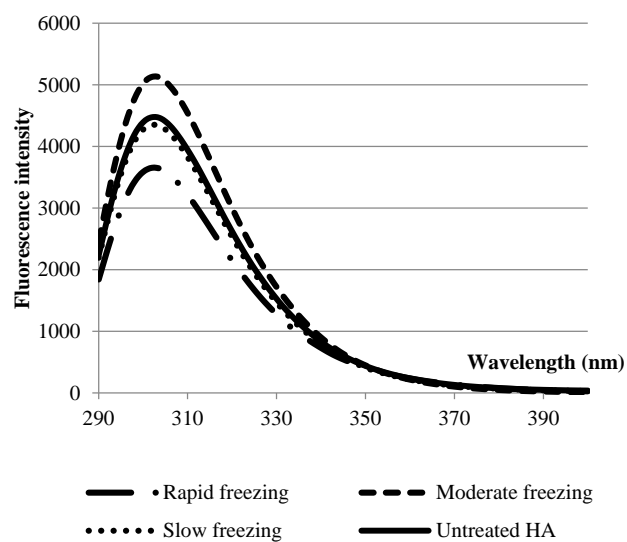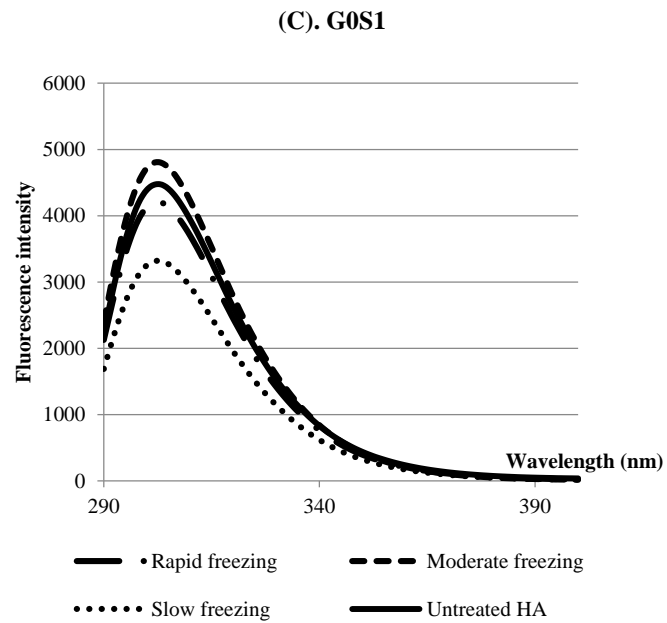
(C). G0S1

(D). G0S0

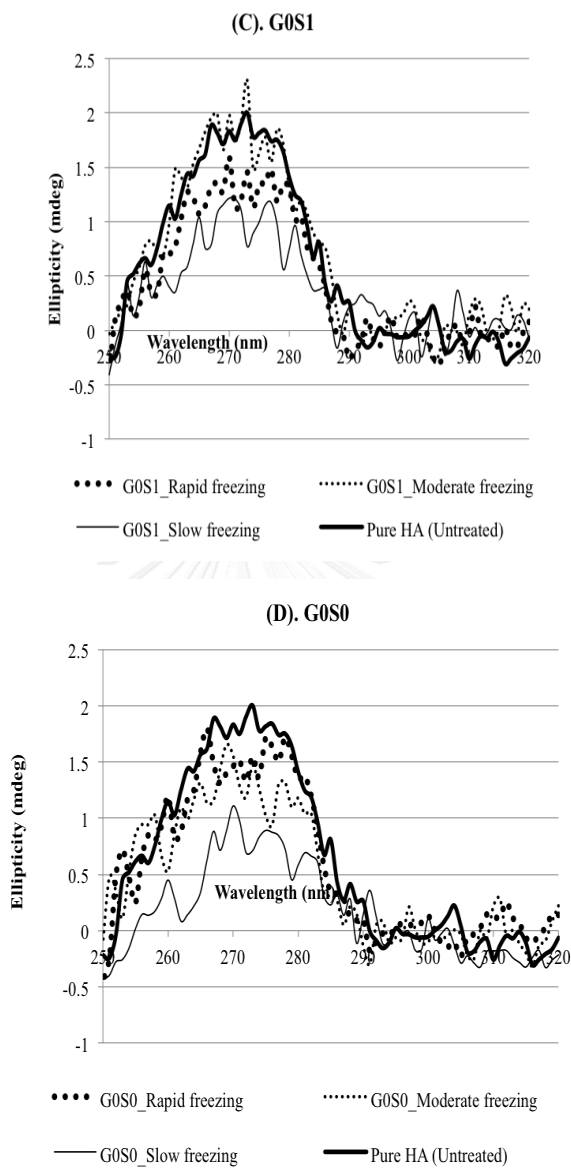*Figure 21. Fluorescence spectroscopic patterns of HA in (A): G6S0, (B): G6S1, (C): G0S1 and (D): G0S0 formulations after freezing at various freezing rates (rapid, moderate and slow freezing rates)*

Table 11. Summary of fluorescence spectroscopic patterns of HA in G6S0, G6S1,
G0S1 and G0S0 formulations after freezing at various freezing rates (rapid, moderate
and slow freezing rates)

| Formulation	Freezing rate	Emission maxima (at 303 nm)	% Preserved	% Change
Untreated HA		4476	100	
G6S0	Rapid	4079	91.13	-8.87
	Moderate	4393	98.15	-1.85
	Slow	4951	110.61	10.61
G6S1	Rapid	3653	81.61	-18.39
	Moderate	5135	114.72	14.72
	Slow	4348	97.14	-2.86
G0S1	Rapid	4205	93.95	-6.05
	Moderate	4806	107.37	7.37
	Slow	3321	74.2	-25.8
G0S0	Rapid	4266	95.31	-4.69
	Moderate	4375	97.74	-2.26
	Slow	3483	77.82	-22.18

To summarize, freezing cycles with adjusted rates (rapid, moderate and slow rates) affect HA conformation. Based on results obtained by CD and fluorescence spectroscopy, HA in G6S0 formulation consisted of 6%w/v of glycine and 0%w/v of sucrose and frozen at moderate freezing rate (1.08°C/min) shows smallest changes in secondary and tertiary conformations among the samples investigated.

In conclusion, the conformational stability of HA is dependent on the combination of formulation compositions and freezing rates. High concentration of glycine is found to have the smallest negative impact on the conformation intergrity of HA. Probably, partial amorphous state of glycine gave protective effect on HA

structure. One explanation given is that the pH shift possibly may induce incomplete crystallization of glycine during freezing (Varshney et al.; 2007). Glycine is reported to improve protein stability through water replacement mechanism, principally based on the formation of hydrogen bond between glycine (instead of water) and protein molecules. This kind of effect is maximized when glycine is partially crystallized (Pikal-Cleland, et al., 2002; Varshney, et al., 2007). On the other hand, the sucrose concentration of only 1% is probably not enough to generate a significant effect (Wang, 2000). In order to have an overall comparison, all four formulations at moderate freezing rate will be studied in lyophilization procedure with live attenuated influenza virus.

II. Characterization of lyophilized live attenuated influenza virus (LAIV) vaccine products immediately after lyophilization

2.1. Physical appearance

The physical appearance of four formulations is displayed in Figure 22. The lyophilized cakes of G6S0, G6S1 and G0S1 met acceptable criterion for physical appearance. G6S0, G6S1, G0S1 formulations had good appeances with white elegant cakes and volume similar to the initial filling volume due to the presence of mannitol, glycine as crystalline bulking agents in formulations. The insufficient crystalline bulking agents in G0S0 may induce slight shrinkage of the lyophilized product.
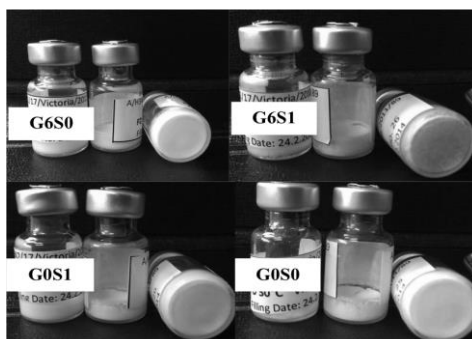

Figure 22. Physical appearances of lyophilized LAIV vaccine products of G6S0, G6S1, G0S1 and G0S0 after freeze-drying on day 0

2.2. Reconstitution time

Reconstitution time of lyophilized LAIV products are illustrated in Table 12. All formulations exhibit reconstitution time of no longer than 60seconds. From the data obtained, reconstitution times of all four formulations conform to the required specification. These results are similar to those of other lyophilized protein products reported by Schersch and colleagues (Schersch et al.; 2010).

Table 12. Reconstitution time of lyophilized LAIV vaccine products after freeze-drying on day 0

Formulations	G6S0	G6S1	G0S1	G0S0
Reconstitution time (second)	6.6 ± 1.7	8.0 ± 1.7	22.0 ± 6.2	16.4 ± 1.5

* Data are expressed as mean ± SD (n=3)

2.3. pH and osmolality

The pH and osmolality values of lyophilized products are presented in Table 13. G0S0 has the lowest osmolality at 290 ± 0mOsm/kg while G0S1, G6S0 and G6S1 has greater values of 429 ± 1mOsm/kg, 624 ± 2mOsm/kg and 688 ± 2mOsm/kg, respectively. All four formulations show the osmolality values varying within the acceptance range (250-700mOsm/kg). Only the products of G0S0 formulation are considered as isotonic, and clearly suitable for the delivery through nasal mucosa. However, it does not mean that hypertonic solution is not proper for the permeability and bioavaibility of vaccine through the nasal cavity. Salmon calcitonin and secretin are reported to have good absorption in rats and rabbits through the nasal passage with hypertonic osmolality (Dua, Zia and Needham, 1997; Ohwaki, et al., 1985)The influence of osmolality on drug absorption and nasal cells requires further clinical investigations. In addition, Zhai et al. reported that the stability of lyophilized live attenuated Herpes simplex virus-2 vaccine is superior in hypertonic environment than those in hypotonic solution (Zhai, et al., 2004). The pH of lyophilized LAIV vaccine products show slight decrease compare to the pH 7.4 of pre-lyophilized solution.

Values of pH investigated are uniform at approximately 6.776-6.953 units after freeze-drying. Normally, pH shifts are associated with the crystallization of buffer generally during freeze-drying process, especially during freezing stage (Croyle, et al., 1998; Amorij, 2007). This study, however, did not monitor pH shifts while freezing. Values in pH are detected only on the restituted lyophilized products. The values are expected to be similar to the original solutions if no additional chemical degradation occurs.

Table 13. pH and osmolality of lyophilized LAIV vaccine products after freeze-drying on day 0

Formulations	G6S0	G6S1	G0S1	G0S0
Osmolality (mOsm/kg)	624 ± 2	688 ± 2	429 ± 1	290 ± 0
pH	6.776 ± 0.008	6.792 ± 0.005	6.887 ± 0.017	6.953 ± 0.003

* Data are expressed as mean ± SD (n=3)

2.4. Thermal behavior

As shown in Figures 23 and 24, thermograms of G6S0 and G6S1 lyophilized product characterized by DSC present two events as function of temperatures. First, the evaporation of residual water contained in the final products occurred as the presence of the endothermic peak between 50-100°C. Second, result also shows major endothermic peak at approximately 120-130°C, which may be associated with the melting point of the product. No existence of exothermic event, corresponding to the transformation between polymorphic forms, is observed.

Figure 23. DSC thermogram of lyophilized LAIV vaccine product of G6S0 after freeze-drying on day 0
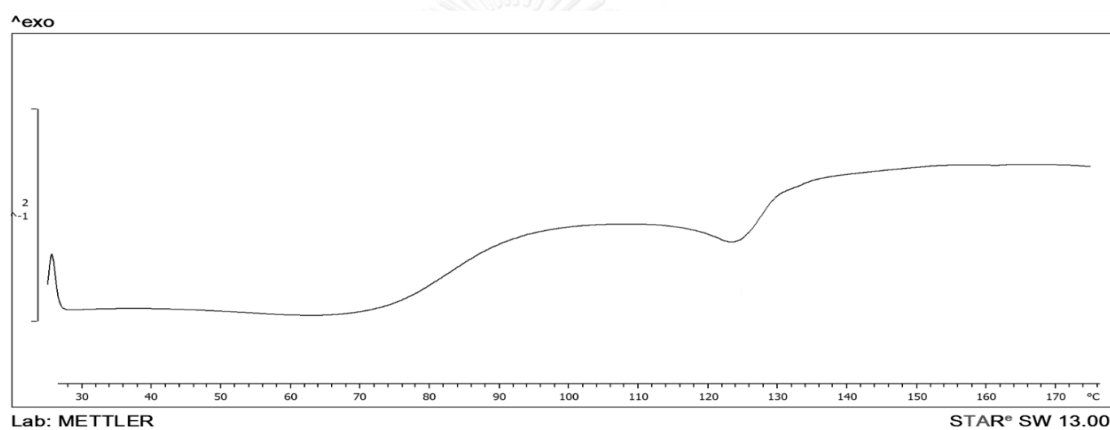
Figure 24. DSC thermogram of lyophilized LAIV vaccine product of G6S1 at after freeze-drying on day 0

According to DSC thermograms shown in Figures 25 and 26, final lyophilized product of G0S1 and G0S0 resulted in two endothermic peaks. The evaporation phenomenon of water exhibits peak at about 50-100°C. Subsequently, the event of melting is detected at approximately 125-135°C. Also, there is no signal of recrystallization in lyophilized products of G0S1 and G0S0.
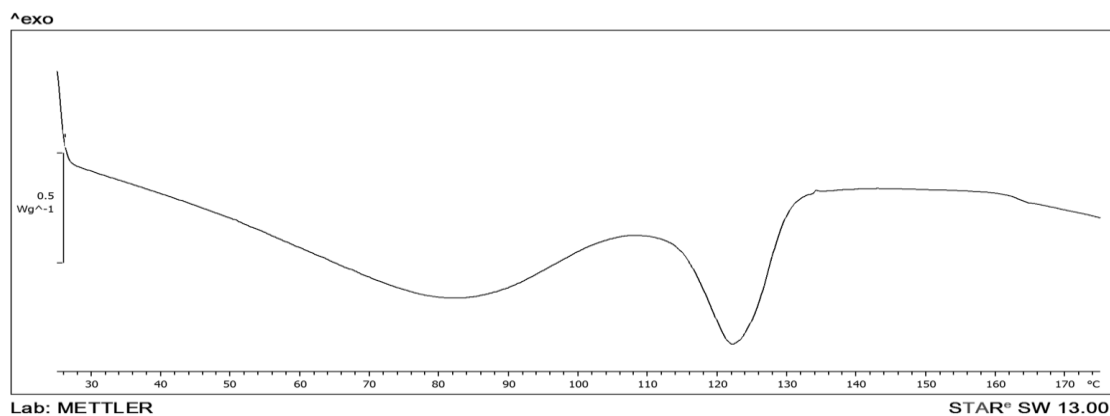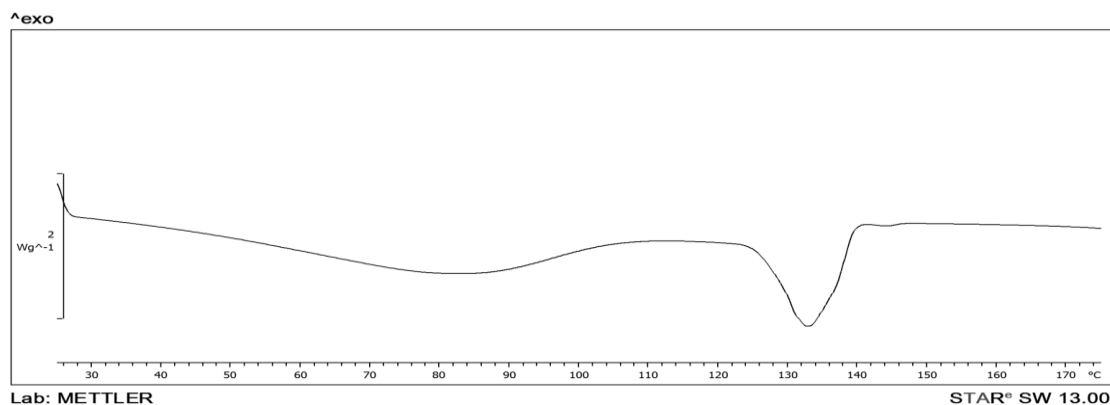
Figure 25. DSC thermogram of lyophilized LAIV vaccine product of G0S1 after freeze-drying on day 0
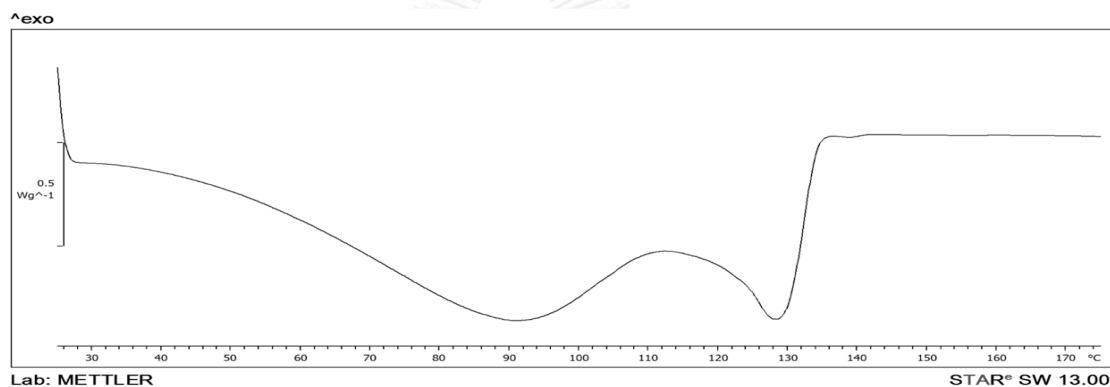
Figure 26. DSC thermogram of lyophilized LAIV vaccine product of G0S0 formulation after freeze-drying on day 0

In summary, all four formulations present good thermal behavior after freeze-drying. Only the peaks attributed to water evaporation and product melting points are observed. The evidence of crystallization/recrystallization of excipients is not found.

2.5. Crystallinity

The crystallinity patterns of mannitol and glycine (before lyophilization) are displayed in Figures 27 and 28. Initially, mannitol and glycine existed as anhydrous forms. Possibly, mannitol contains a mixture of α-, β- and δ- forms. In fact, the peaks at 19, 23 and 21° 2θ (Figure 27) are characteristic peaks of α-, β- and δ- forms, respectively (Kim, Akers and Nail; 1998). On the other hand, glycine existed as α-

and γ-glycine with identifiable peaks at 30 and 15° 2θ, respectively (Chongprasert, Knopp and Nail, 2001) (Figure 28). The other compositions contained in formulations are presented only with small concentrations. Therefore, we only focus on the crystallinity of glycine and mannitol, which are formulated as two highest concentrations.
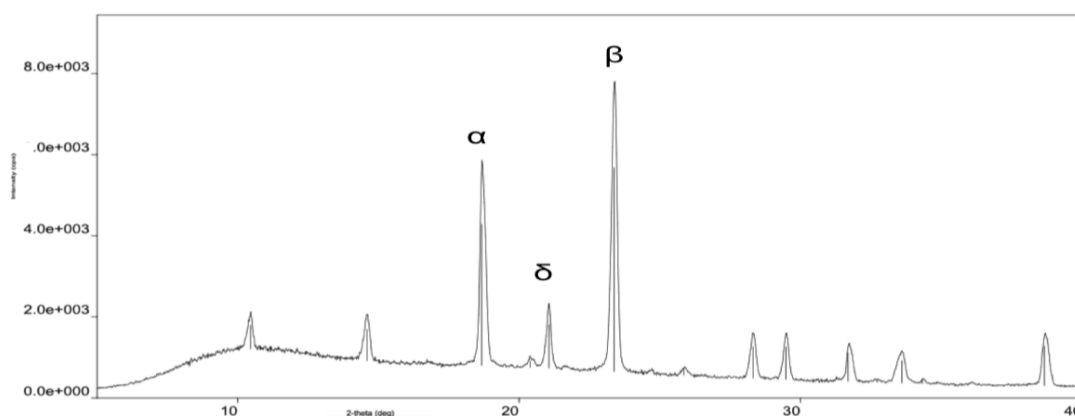
Figure 27. XRPD pattern of mannitol before lyophilization
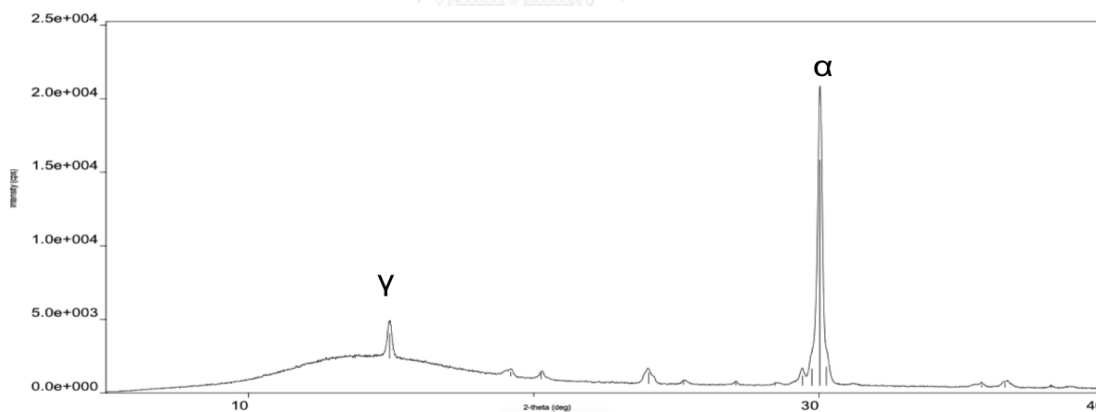
Figure 28. XRPD pattern of glycine before lyophilization

Figures 29 and 30 displays the crystalline forms of glycine and mannitol in G6S0 and G6S1 lyophilized products. The presence of two characteristic peaks at approximately 10 and 21° 2θ demonstrates that mannitol recrystallized to δ-mannitol during lyophilization process. These results are in good agreement with earlier studies (Hawe and Frieb; 2006; Pyne; Chatterjee and Suryanarayan; 2003). The absence of the peak at 16.9° 2θ suggests that mannitol hemihydrate is not present in the products.

The characteristics peaks of β-mannitol, β-glycine are known to be located at the same positions such as 18, 23, 25 and $28^{\circ} 2\theta$ (Hawe and Frieb, 2006; Kim, Akers and Nail, 1988; Pyne, Chatterjee and Suryanarayan, 2003). Therefore, it could not be concluded that lyophilized products of G6S0 and G6S1 contains β-mannitol or β-glycine or in combination. However, only peak at approximately at $25^{\circ} 2\theta$ is observed for lyophilized products of non-glycine formulations (G0S1 and G0S0) (Figures 31 and 32), meaning that this peak corresponds to β-mannitol. Thus, it could be stated that a mixture of β-mannitol and β-glycine exists in the lyophilized LAIV vaccine products of G6S0 and G6S1.

Figure 29. XRPD patterns of lyophilized LAIV vaccine product of G6S0 after freeze-drying on day 0
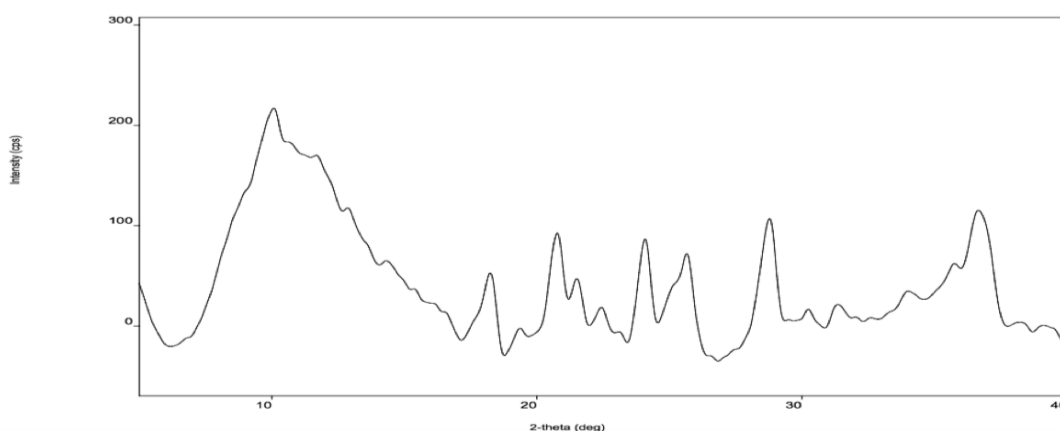
Figure 30. XPRD patterns of lyophilized LAIV vaccine product of G6S1 after freeze-drying on day 0

G0S1 and G0S0 lyophilized vaccine products resulted in XRPD pattern as shown in Figures 31 and 32. Peaks at approximately 10 and 21° 2θ are correlated to δ-mannitol peaks (Hawe and Frieb; 2006; Pyne, Chatterjee and Suryanarayan; 2003). Peaks at approximately 25° 2θ is attributed to the presence of β-mannitol (Kim, Akers and Nail; 1998; (Hawe and Frieb, 2006). Peak at 32° 2θ is contributed to the crystalline peak of mannitol. However, the form of crystalline mannitol corresponded to the peak at 32° 2θ is unknown yet. Possibly, the absence of glycine in G0S1 and G0S0 increased the sharpness of mannitol peak at 32° 2θ.
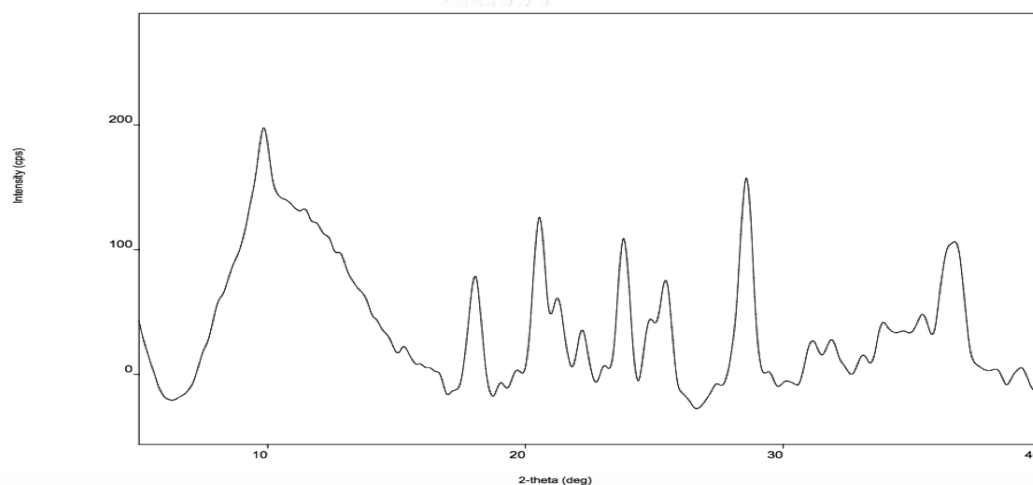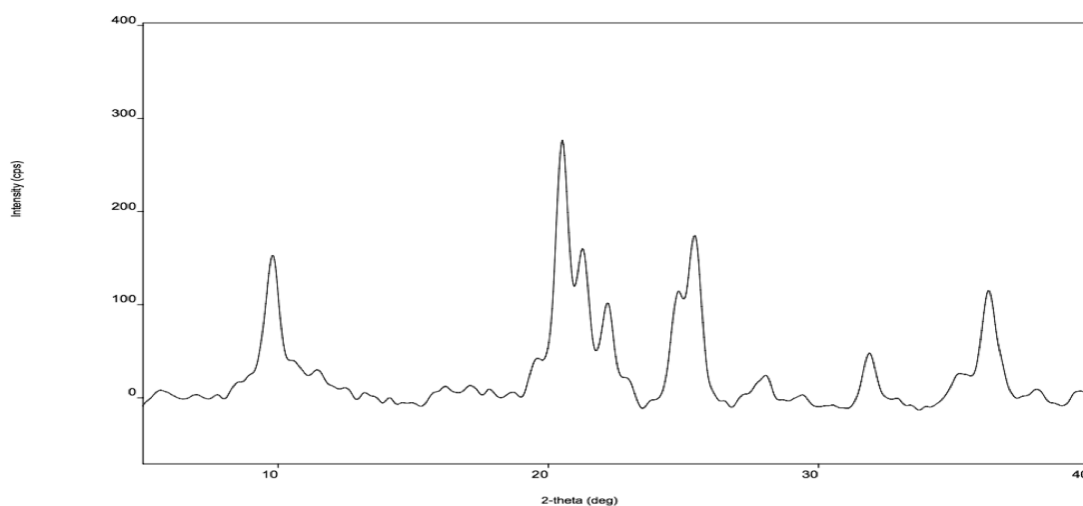
Figure 31. XRPD patterns of lyophilized LAIV vaccine product of G0S1 after freeze-drying on day 0
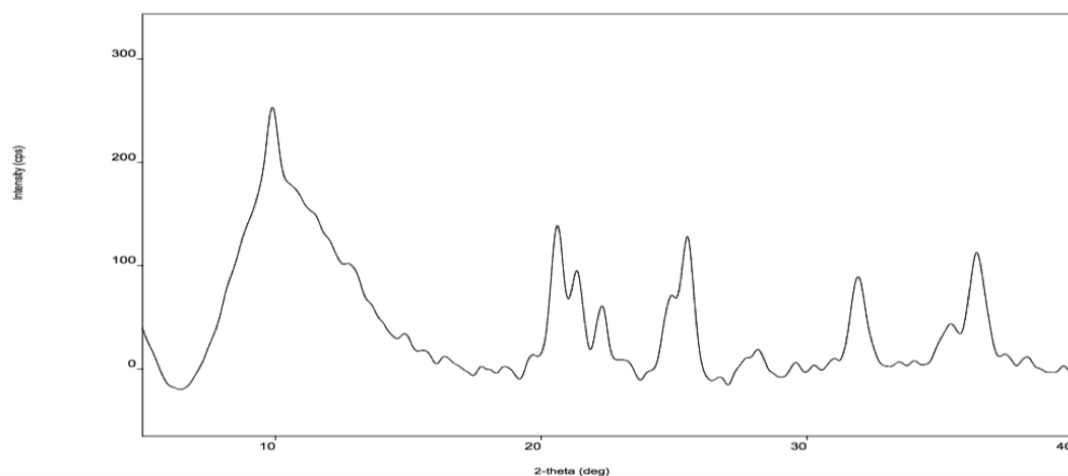
Figure 32. XRPD patterns of lyophilized LAIV vaccine product of G0S0 after freeze-drying on day 0

In general, the XRPD patterns of all four formulations reveal that mannitol was converted to δ- and β-mannitol during freeze-drying process. Glycine existed as β-glycine in lyophilized LAIV vaccine product of G6S0 and G6S1.

2.6. Residual moisture content

- ### TGA method

From TGA results in Figures 33, 34, 35 and 36 the residual moisture contents of G6S0, G6S1, G0S1, G0S0 formulations are 5.95, 3.97, 3.37 and 6.23% respectively (Table 14). All moisture content levels are found to be higher than our acceptance criteria of 3%. Insufficient secondary drying time may be the reason for these high percentages in moisture contents. Possibly, to reach desired final residual moisture content, the secondary drying time is required to be extended to more than 6 hours.
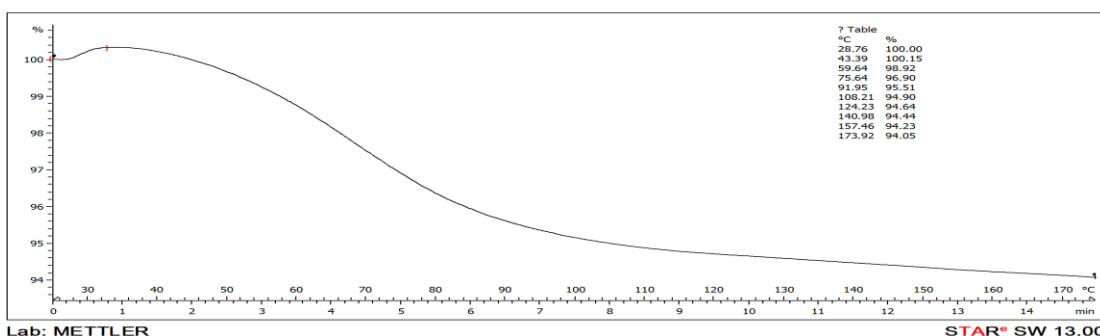
Figure 33. TGA thermogram of lyophilized LAIV vaccine product of G6S0 after freeze-drying on day 0
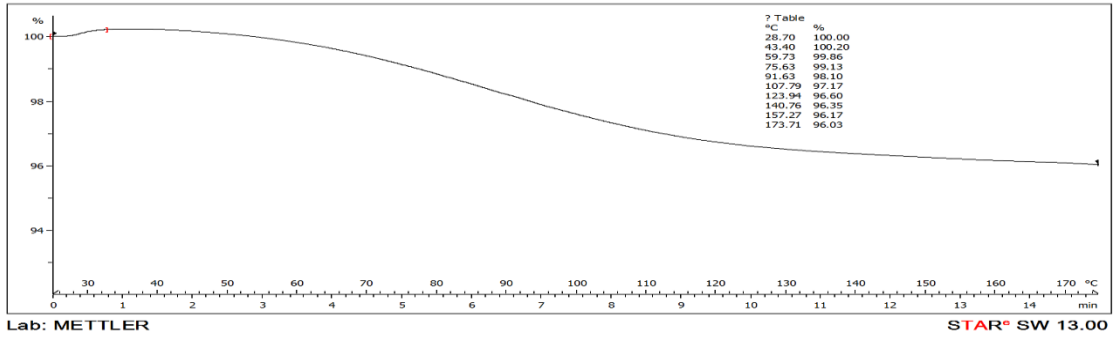
Figure 34. TGA thermogram of lyophilized LAIV vaccine product of G6S1 after freeze-drying on day 0

Figure 35. TGA thermogram of lyophilized LAIV vaccine product of G0S1 after freeze-drying on day 0

Figure 36. TGA thermogram of lyophilized LAIV vaccine product of G0S0 after freeze-drying on day 0

Table 14. Residual moisture content of lyophilized LAIV vaccine products after freeze-drying on day 0 obtained by TGA

Formulations	G6S0	G6S1	G0S1	G0S0
Residual moisture content (%)	5.95	3.97	3.37	6.23

- **Karl Fisher method**

From Karl Fisher results describe in Table 15, the moisture contents of G6S0, G6S1, G0S1, G0S0 are 3.21 ± 0.44, 4.10 ± 0.16, 2.68 ± 0.29 and 7.96 ± 1.91%, respectively. Only the water content of G0S1 is in the acceptable value, which correlates well with the results obtained by TGA where G0S1 shows the least water content.

Water percentages of the same formulations are presented differently between two methods, TGA and Karl Fisher titration. The first reason is due to the differences in techniques used in acquiring the data. The second reason may be due to the highly hygroscopic nature of the lyophilized product which can uptake water from the surrounding very rapidly causing interference with the final results. However, the following moisture stability study (Figures 37, 38, 39 and 40) shows that the second reason on hygroscopicity of the products is not the case.

Table 15. Residual moisture content of lyophilized LAIV vaccine products after freeze-drying on day 0 obtained by Karl Fisher titrimetry

Formulations	G6S0	G6S1	G0S1	G0S0
Residual moisture content (%)	3.21 ± 0.44	4.10 ± 0.16	2.68 ± 0.29	7.96 ± 1.91

* Data are expressed as mean value ± SD (n=3)

2.7. Moisture stability

According to DVS isothermal plot in Figures 37, 38, 39 and 40, the lyophilized LAIV vaccine products of four formulations take up moisture dramatically when the relative humidity is at approximately 80%RH. No hysteresis loop is observed in all four diagrams indicating that no transformation in the solid-state occurs. From literature review, if metastable forms of mannitol and glycine bulking agents are present, the transformation to stable crystalline forms under DVS condition will result in a hysteresis loop (Mehta, Bhardwaj and Suryanarayanan, 2013). Our results suggest that the crystallization of these bulking agents is already completed during the freeze-drying process. This is in a good agreement with the results obtained from XRPD, DSC and TGA. Furthermore, the rate of water adsorption and water desorption are similar, suggesting that there is only surface adsorption. No evidence of bulk water absorption is observed (Hassel and Hesse, 2007)
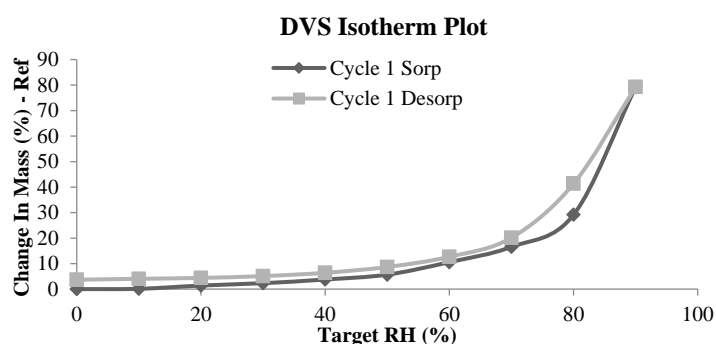
Figure 37. DVS isotherm plot of lyophilized LAIV vaccine product of G6S0 after freeze-drying on day 0
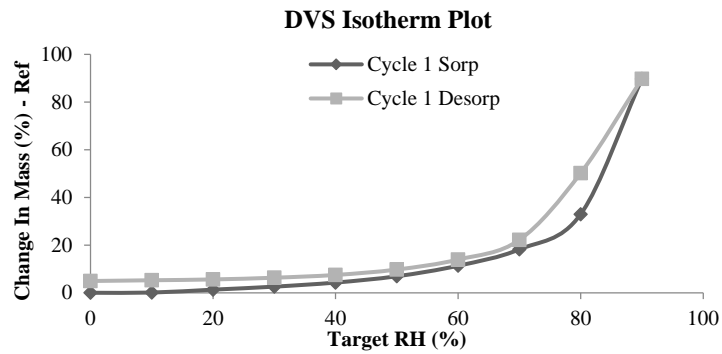
Figure 38. DVS isotherm plot of lyophilized LAIV vaccine product of G6S1 after freeze-drying on day 0
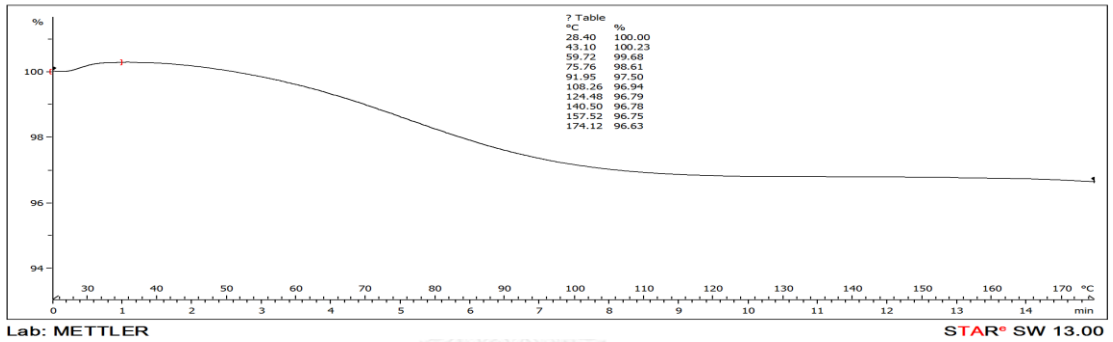

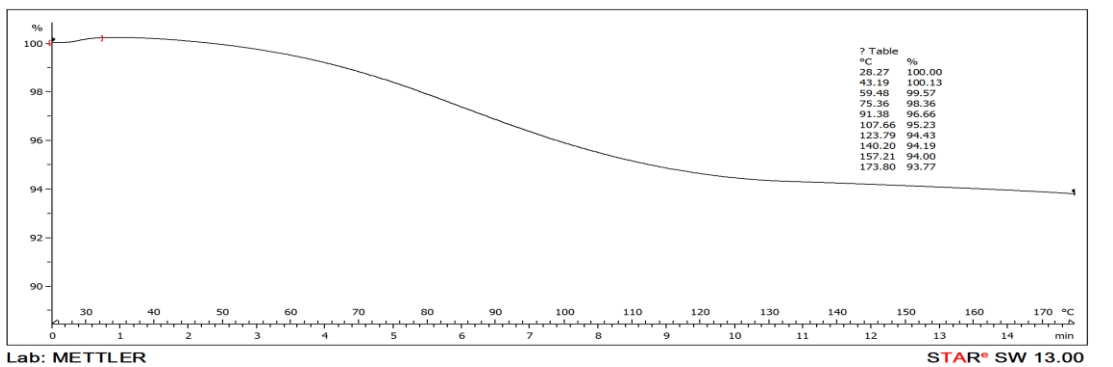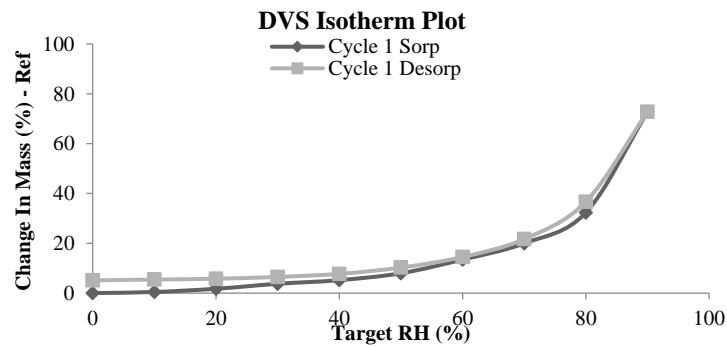*Figure 39. DVS isotherm plot of lyophilized LAIV vaccine product of G0S1 after freeze-drying on day 0*
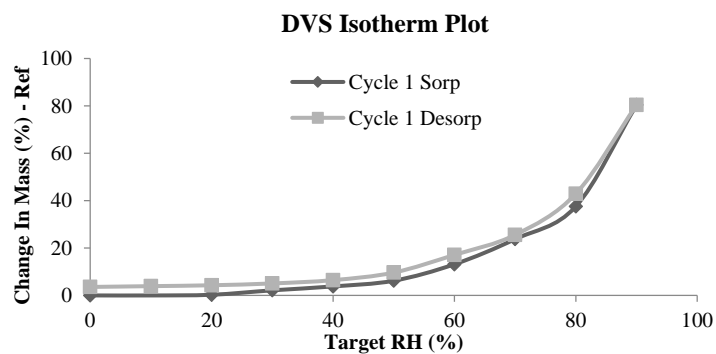
Figure 40. DVS isotherm plot of lyophilized LAIV vaccine product of G0S0 after freeze-drying on day 0

2.8. Infectivity titer measurement

The values of log $_{10}$ TCID$_{50}$/0.2 ml of lyophilized LAIV vaccine products before and after lyophilization are shown in Table 16. All formulations show decrease in infectivity titers. However, all formulations still provide satisfactory values of infectivity titers of more than 6.5 as required.

Table 16. Log$_{10}$ TCID$_{50}$/0.2 ml of lyophilized LAIV vaccine products before and immediately after lyophilization (on day 0)

Formulation	Freeze-drying	G6S0	G6S1	G0S1	G0S0
log$_{10}$ TCID$_{50}$/0.2 ml	Before	8.24 ± 0.06	8.05 ± 0.00	7.92 ± 0.12	8.26 ± 0.29
	After	7.51 ± 0.65	7.80 ± 0.00	7.33 ± 0.20	7.55 ± 0.00

* Data are expressed as mean value ± SD (n=2)

In conclusion, Table 17 summarizes the properties of final lyophilized LAIV vaccine products of four investigational formulations (G6S0, G6S1, G0S1, G0S0) obtained from moderate freezing rate of 1.08ºC/min. Lyophilized products of live attenuated influenza virus are satisfactory according to the physical requirements of lyophilized vaccine, except the moisture content requirement. Residual moisture contents are greater than expected due to the reasons discussed in Section 2.6 (Chapter IV). Moreover, it should be noted that G0S0 had slight shrinkage due to the lack of sufficient bulking agent.

Table 17. Summary of the properties for lyophilized LAIV vaccine products after freeze-drying on day 0

Parameter	Evaluation
Physical appearance	Good (except G0S0: slight shrinkage)
Reconstitution time	Acceptable
Residual moisture content	Higher than > 3%
pH and osmolality	Acceptable
Thermal behavior	Acceptable
Crystallinity	Acceptable
Moisture stability	Acceptable
Infectivity titer	Acceptable

III. Stability evaluation of lyophilized LAIV vaccine formulations (at 30°C/75% RH)

3.1. Physical appearance

The cakes of lyophilized products are examined visually after 28 days of storage at 30°C/75%RH. The physical apperances are stable over storage time at this condition (Figure 41). After 28 days, all the products maintained their lattice cake structures. The volume does not change when compare to the morphology of the products after freeze-drying.

Figure 41. Physical appearances of lyophilized LAIV vaccine products of G6S0, G6S1, G0S1 and G0S0 after 28 days of storage at 30°C/75%RH

3.2. Reconstitution time

The time for complete reconstitution of lyophilized LAIV powders are described in Table 18. The reconstitution times of all lyophilized products do not show significant changes upon 28 days of storage at 30°C/75%RH. On day 28 of the study, all the samples exhibit reconstitution times of not exceeding 60seconds, which are consistent with the initial specification. These results suggest that the transportation or handling under room temperature for a short time is possible and would not affect the reconstitution time.

Table 18. Reconstitution time of lyophilized LAIV vaccine products after 28 days of storage at 30°C/75%RH

	Reconstitution time (second)			
Formulation	**G6S0**	**G6S1**	**G0S1**	**G0S0**
Day 0	6.6 ± 1.7	8.0 ± 1.7	22.0 ± 6.2	16.4 ± 1.5
Day 7	10.7 ± 1.8	12.3 ± 0.2	20.9 ± 7.3	31.1 ± 3.2
Day 14	5.7 ± 0.5	8.6 ± 0.5	18.7 ± 0.2	26.5 ± 5.0
Day 21	7.7 ± 1.0	11.5 ± 0.9	22.1 ± 6.3	23.7 ± 1.0
Day 28	10.6 ± 3.2	11.9 ± 1.2	20.5 ± 2.8	17.5 ± 3.3

* Data are expressed as mean value ± SD (n=3)

3.3. pH and osmolality

- **pH**

The lyophilized products of all four formulations presented pH values within the expected range of 6.5-7.5 unit between the storage time at 30°C/75%RH (Table 19). After 4 weeks storage at 30°C/75%RH, pH of G6S0, G6S1, G0S1, G0S0 are varied only in the range of 6.7 to 6.9. No significant change is noticed through the timeline of stability studies. These results signify that no degradation of each

component occurred during lyophilization and through the course of stability study of 30°C/75%RH.

Table 19. pH of lyophilized LAIV vaccine products after 28 days of storage at 30°C/ 75%RH

	pH			
Formulation	**G6S0**	**G6S1**	**G0S1**	**G0S0**
Day 0	6.776 ± 0.008	6.792 ± 0.005	6.887 ± 0.017	6.953 ± 0.003
Day 7	6.775 ± 0.001	6.790 ± 0.002	6.891 ± 0.013	6.941 ± 0.009
Day 14	6.795 ± 0.002	6.782 ± 0.017	6.915 ± 0.009	6.963 ± 0.006
Day 21	6.744 ± 0.011	6.757 ± 0.006	6.871 ± 0.009	6.908 ± 0.007
Day 28	6.776 ± 0.030	6.729 ± 0.020	6.865 ± 0.010	6.908 ± 0.010

*Data are expressed as mean value $\pm$ SD (n=3)

- **Osmolality**

The reconstituted solutions of G6S0, G6S1, G0S1, G0S0 lyophilized products exhibit osmolality values within the acceptance criteria of 250-700mOsm/kg after 28 days of storage at 30°C/75%RH as describe in Table 20. The data obtained suggest that the storage condition of 30°C/75%RH did not influence on the osmolality change due to degradation of lyophilized products.

Table 20. Osmolality of lyophilized LAIV vaccine products after 28 days of storage at 30°C/75%RH

Formulation	Osmolality (mOsm/kg)			
	G6S0	G6S1	G0S1	G0S0
Day 0	624 ± 2	688 ± 2	429 ± 1	290 ± 0
Day 7	702 ± 5	679 ± 3	466 ± 1	325 ± 5
Day 14	673 ± 3	680 ± 1	424 ± 0	328 ± 0
Day 21	710 ± 4	692 ± 3	430 ± 1	287 ± 1
Day 28	572 ± 1	639 ± 2	408 ± 2	323 ± 1

* Data are expressed as mean value ± SD (n=3)

3.4. Thermal behavior

Figures 42, 43, 44 and 45 show thermal behavior of lyophilized LAIV vaccine products of G6S0, G6S1, G0S1 and G0S0, respectively when stored at 30°C/75%RH for 28 days. All four formulations exhibit proper thermal behavior as compared to lyophilized products characterized after freeze-drying on day 0. The first endothermic peaks, varying in the range of 50-100°C, contributed to dehydration. Second peaks occur at approximately 120-130°C, referring to melting point. In other words, no exothermic peaks, representative for the recrystallization of buffer components, were detected. However, DSC thermograms are not in line with XRPD detection (Figures 46, 47). Possibly, the amount of crystalline form appeared is too small for the detection of DSC.

Figure 42. DSC thermograms of lyophilized LAIV vaccine products of G6S0 over 28 days at 30°C/75%RH
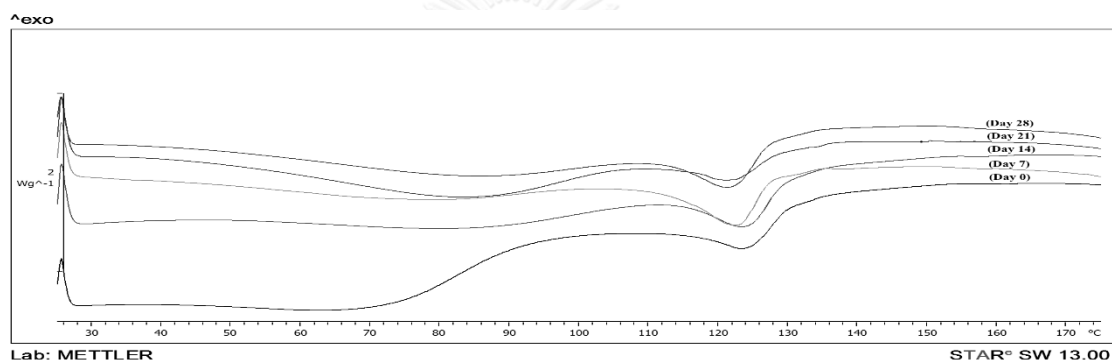
Figure 43. DSC thermograms of lyophilized LAIV vaccine products of G6S1 over 28 days storage at 30°C/75%RH
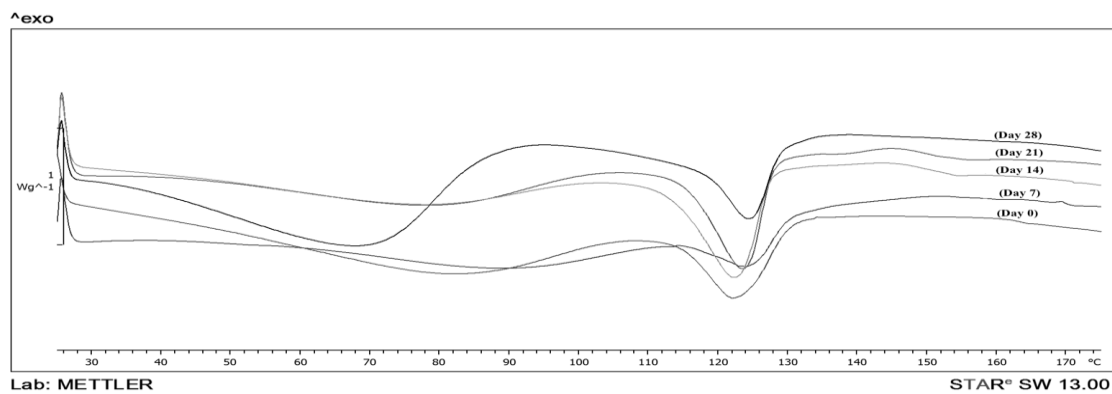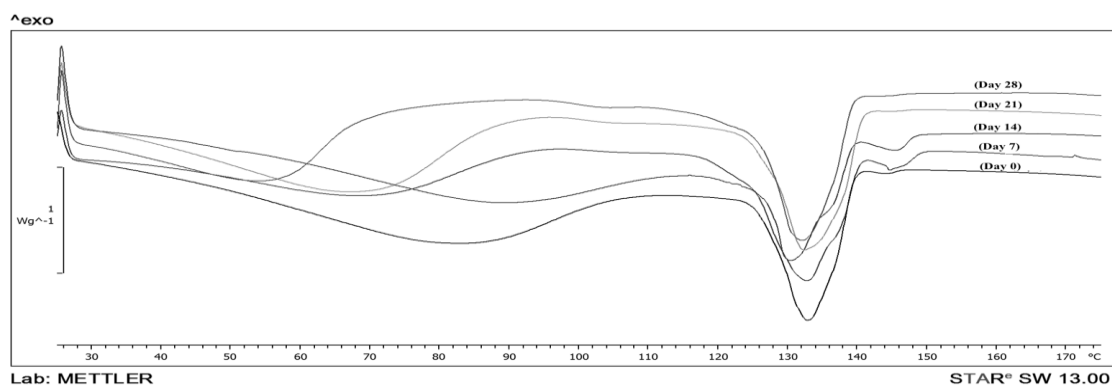

Figure 44. DSC thermograms of lyophilized LAIV vaccine products of G0S1 over 28 days at 30°C/75%RH
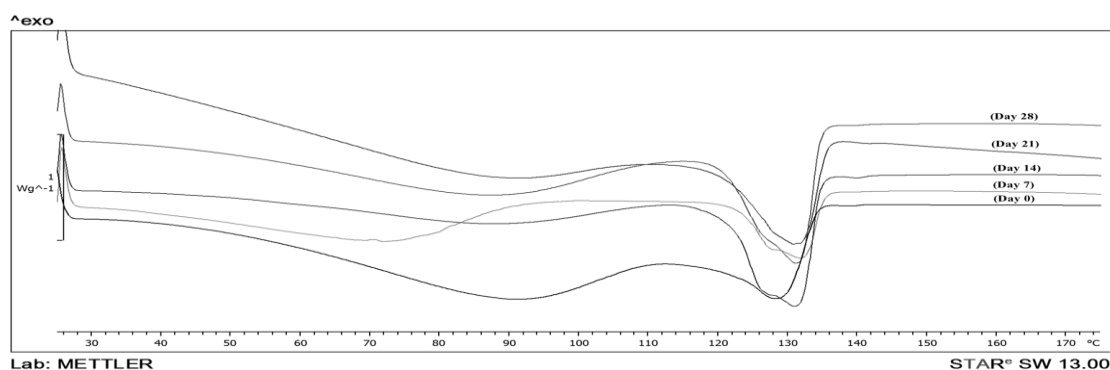
Figure 45. DSC thermograms of lyophilized LAIV vaccine products of G0S0 over 28 days at 30°C/75%RH

3.5. Crystallinity

Figure 46 gives evidence of changes in crystallinity of lyophilized products of G6S0. The reduction in intensity of all crystalline peaks is observed. In contrast, an appearance of peak at approximately 32° 2θ is observed when conducting experiment from day 7th onward. According to XRD pattern of mannitol before lyophilization, this peak is a peak of crystalline mannitol (Figure 27). However, the form that this peak corresponds to is unknown yet. Its intensity is increased gradually with time of stability study (Figure 46).
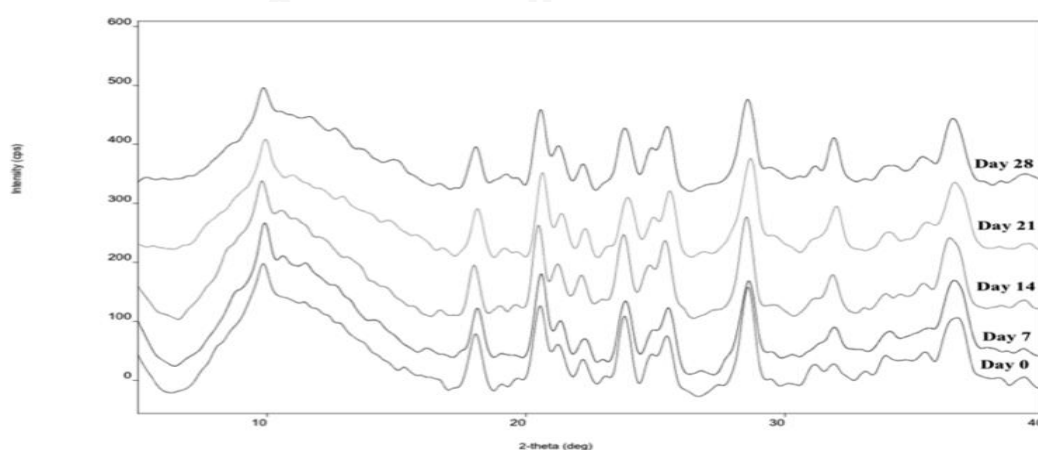
Figure 46. XRPD patterns of lyophilized LAIV vaccine products of G6S0 over 28 days at 30°C/75%RH

XRPD reveals that the intensity of δ-mannitol peaks at approximately 10 and 21° 2θ become more crystalline when lyophilized products of G6S1 stored at 30°C/75%RH for 28 days (Figure 47). Also, the peaks at approximately 18, 23, 25 and 28° 2θ show an increase in intensity. Another peak at approximately 32° 2θ correlated to mannitol appears and increases in intensity gradually throughout stability timeline (Figure 47). In addition, amorphous baseline from 5-17° 2θ is significantly reduced from day 21st onward.
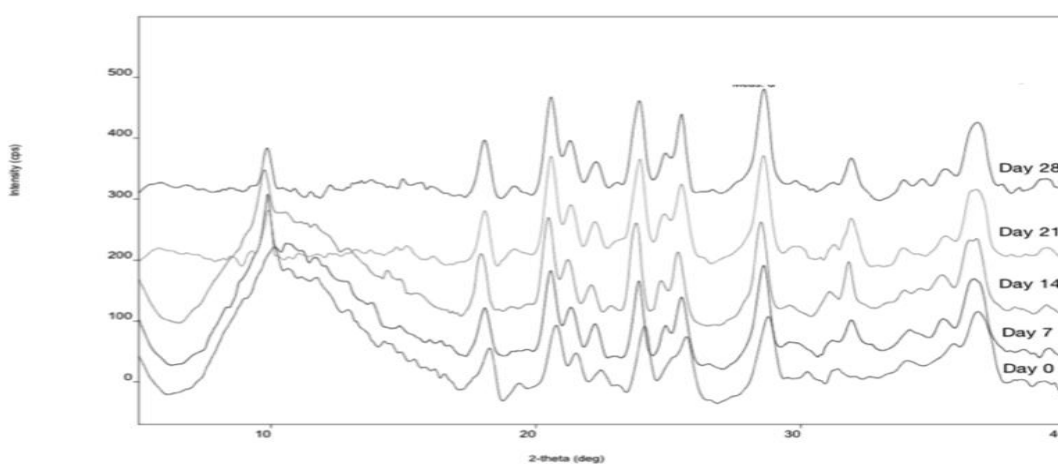
Figure 47. XRPD patterns of lyophilized LAIV vaccine products of G6S1 over 28 days at 30°C/75%RH

Figures 48 and 49 display the crystallinity of lyophilized LAIV vaccine products of G0S1 and G0S0 stored at 30°C/75%RH for 28 days. Peak intensity of G0S1 crystalline forms increased during the timeline of stability indicating higher crystalline products. However, the percentage of crystallinity is reduced in G0S0 lyophilized products over storage time.
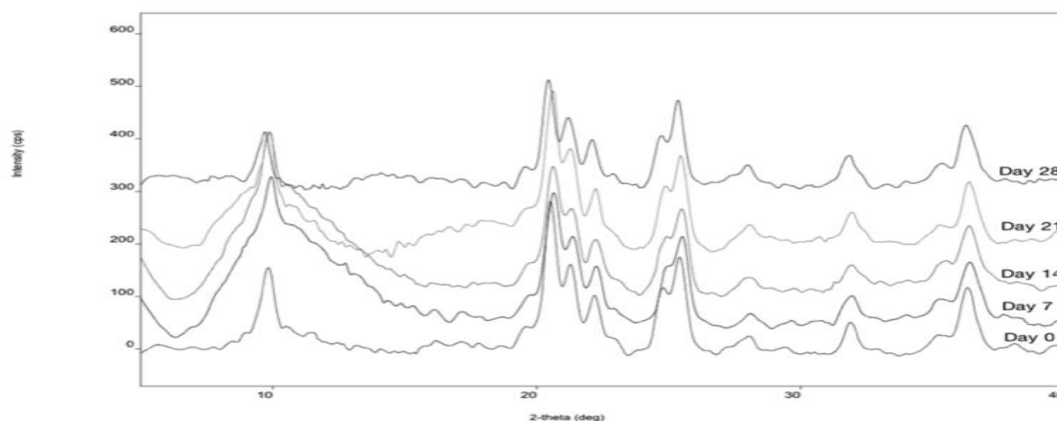
Figure 48. XRPD patterns of lyophilized LAIV vaccine product of G0S1 over 28 days at 30°C/75%RH
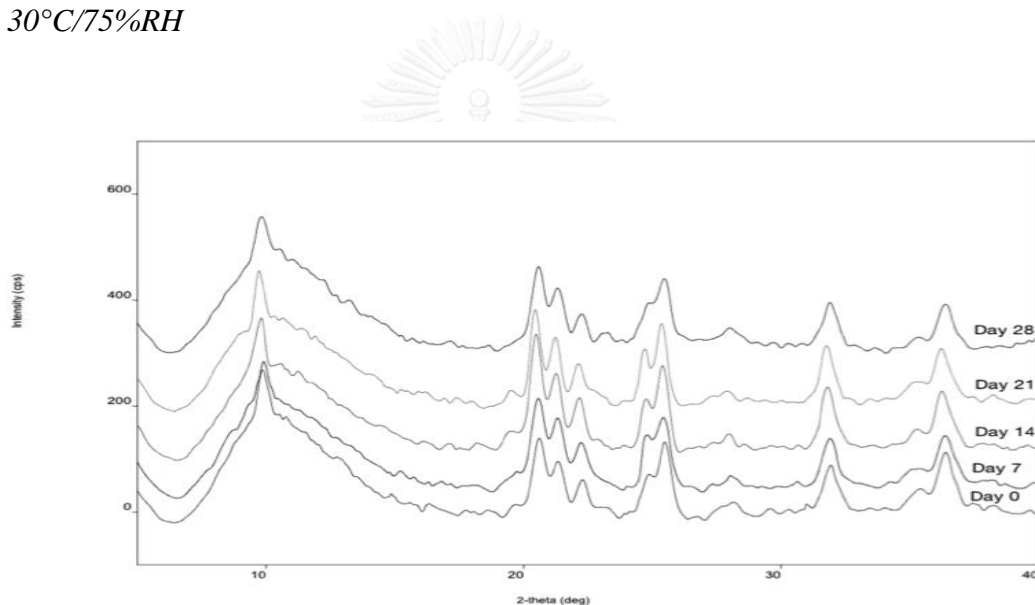
*Figure 49. XRPD patterns of lyophilized LAIV vaccine products of G0S0 over 28 days at 30°C/75%RH*

In summary, according to XRPD results, the solid-state structure of all four formulations is not stable over storage time of 28 days at 30°C/75%RH. It is found that without sucrose the crystallinity decreased overtime. But increase in crystallinity is found in formulations with sucrose. The high temperature, humidity and duration of storage condition are the reasons for this instability. Moreover, it should be noticed that the degree of crystallinity changed, whether increased or decreased, could result

in the variation in residual moisture content of lyophilized products during stability study.

3.6. Residual moisture content

- **TGA method**

Upon storage for 28 days at 30°C/75%RH, the moisture levels of all products did not show a trend along the timeline of 28 days storage at 30°C/75%RH (Table 21).

Table 21. Residual moisture of lyophilized LAIV vaccine products after 28 days of storage at 30°C/75%RH obtained from TGA

	Residual moisture content (%)			
Formulation	**G6S0**	**G6S1**	**G0S1**	**G0S0**
Day 0	5.95 ± 0.11	3.97 ± 0.53	3.37 ± 0.03	6.23 ± 1.24
Day 7	2.72 ± 0.66	5.50 ± 0.26	6.27 ± 0.57	3.52 ± 0.88
Day 14	5.32 ± 0.84	7.20 ± 0.75	5.98 ± 0.67	5.18 ± 0.31
Day 21	3.53 ± 0.33	5.09 ± 0.81	3.24 ± 0.66	10.46 ± 1.21
Day 28	2.52 ± 0.46	4.82 ± 0.28	2.28 ± 0.66	5.08 ± 0.06

* Data are expressed as mean value ± SD (n=3)

- **Karl Fisher method**

As shown in Table 22, lyophilized products of four formulations contain water contents of greater than 3%. On day 28, water contents of G6S0, G6S1, G0S1, G0S0 formulations are found to be 3.80 ± 0.24, 5.04 ± 0.46, 4.17 ± 0.22 and 7.86 ± 0.45%, respectively. The resulting trends obtained from Karl Fisher method is in line with TGA results. However, the moisture contents measured by Karl Fisher are slightly higher than those of TGA. The vial-to-vial heterogeneity, which probably occurs due to the time used for manual capping, could be one explanation for the differences in results. Another reason considered for these results is that the changes in degree of

crystallinity during stability storage may influence on the moisture uptake of lyophilized products once they are opened.

Table 22. Residual moisture content of lyophilized LAIV vaccine products after 28 days of storage at 30°C/75%RH determined by Karl Fisher titrimetry

	Residual moisture content (%)			
Formulation	G6S0	G6S1	G0S1	G0S0
Day 0	3.21 ± 0.44	4.10 ± 0.16	2.68 ± 0.29	7.96 ± 1.91
Day 7	3.53 ± 0.09	3.61 ± 0.13	3.65 ± 0.16	7.48 ± 1.53
Day 14	7.46 ± 2.99	5.87 ± 0.09	5.57 ± 0.33	6.94 ± 0.80
Day 21	5.37 ± 0.76	6.68 ± 0.86	5.29 ± 0.53	15.98 ± 7.02
Day 28	3.80 ± 0.24	5.04 ± 0.46	4.17 ± 0.22	7.86 ± 0.45

* Data are expressed as mean value $\pm$ SD (n=3)

3.7. Infectivity titer measurement

The titer of lyophilized products which are determined in tissue cells decrease over time when stored at 30°C/75%RH (Table 23). All four formulations lost their infectivity during stability study at 30°C/75%RH. All products show unsatisfactory titer when compare to standard limits (more than 6.5) starting from the first week of stability evaluation. On day 28, the $\log_{10}$ of $TCID_{50}$ of G6S0, G6S1, G0S1 and G0S0 are 4.47 ± 0.00, 4.57 ± 0.21, 5.33 ± 0.28 and 3.84 ± 0.41, respectively. This means that all four formulations lost their potency after 1 week of storage at 30°C/75%RH.

The findings demonstrated that lyophilized products of LAIV vaccine are not stable over storage at 30°C/75%RH of 28 days. The high temperature and humidity of storage condition are considered as the main causes of the instability due to the rapid loss in infectivity titer since the first week of stability study.

Table 23. Log$_{10}$ TCID$_{50}$/0.2 ml of lyophilized LAIV vaccine products at 30°C/75%RH

log$_{10}$ TCID$_{50}$/0.2 ml of lyophilized LAIV vaccine formulations				
Formulation	G6S0	G6S1	G0S1	G0S0
Day 0	7.51 ± 0.65	7.80 ± 0.00	7.33 ± 0.20	7.55 ± 0.00
Day 7	6.26 ± 0.41	6.33 ± 0.20	6.18 ± 0.06	6.36 ± 0.08
Day 14	5.86 ± 0.08	5.66 ± 0.04	5.91 ± 0.32	5.58 ± 0.16
Day 21	5.05 ± 0.35	5.35 ± 0.30	5.42 ± 0.00	4.59 ± 0.06
Day 28	4.47 ± 0.00	4.57 ± 0.21	5.33 ± 0.28	3.84 ± 0.41

* Data are expressed as mean value ± SD (n=2)

IV. Stability evaluation of lyophilized LAIV vaccine formulations (After 6 months at -20°C and 2-8°C)

4.1. Physical appearance

Based on visual observation, all the lyophilized powders are stable in terms of cake morphology under storage condition at -20°C and 2-8°C (Figure 50). All the formulations show rigid cakes over 6 months.
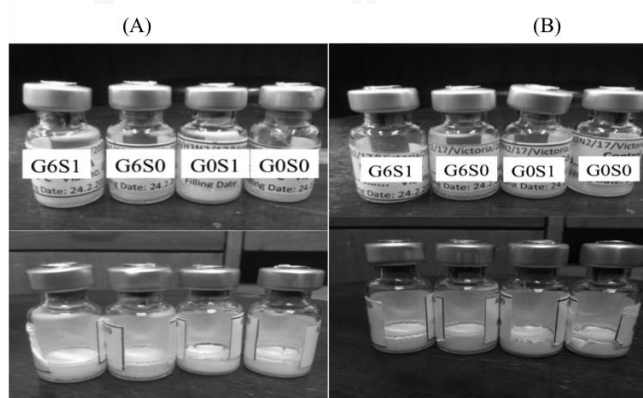

Figure 50. Physical appearance of lyophilized LAIV vaccine products when stored at (A): -20°C, (B): 2-8°C for 6 months

4.2. Reconstitution time

Reconstitution time of lyophilized products is illustrated in Figure 51. At -20°C is considered as the best storage condition for many biological products. Thus, products stored at -20°C are used as reference to compare to the desired storage temperature of 2-8°C. The two sets of products at two conditions are shown not to be significantly different from the initial values with p>0.05. The time for complete dissolution of G0S1 and G0S0 products are longer than those of G6S0 and G6S1 due to the existence of ionic bulking agent (glycine) in the formulations. It is found that the reconstitution times increased slightly over the period of stability study.

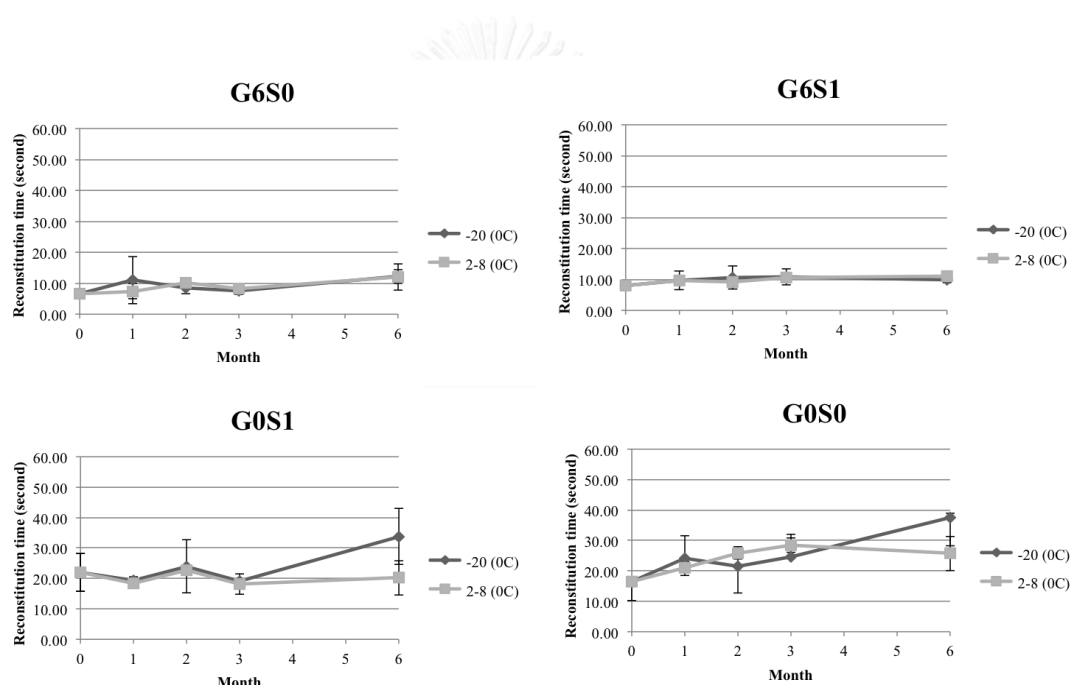*Figure 51. Reconstitution time of lyophilized LAIV vaccine products during 6 months storage at -20°C and 2-8°C*

* Data are expressed as mean value ± SD (n=3)

4.3. pH and osmolality

▪ **pH**

Figure 52 presents pH values of lyophilized LAIV vaccine products stored at -20°C and 2-8°C during stability study for 6 months. It can be observed that pH trend

of all reconstituted solutions of four lyophilized LAIV vaccine formulations exposed to 2-8°C are consistent with those stored at -20°C (p>0.05). After 6 months of storage, pH values do not change in all four formulations. The variations of pH values are within the interval of 6.7-6.9, which conforms to the initial specification.
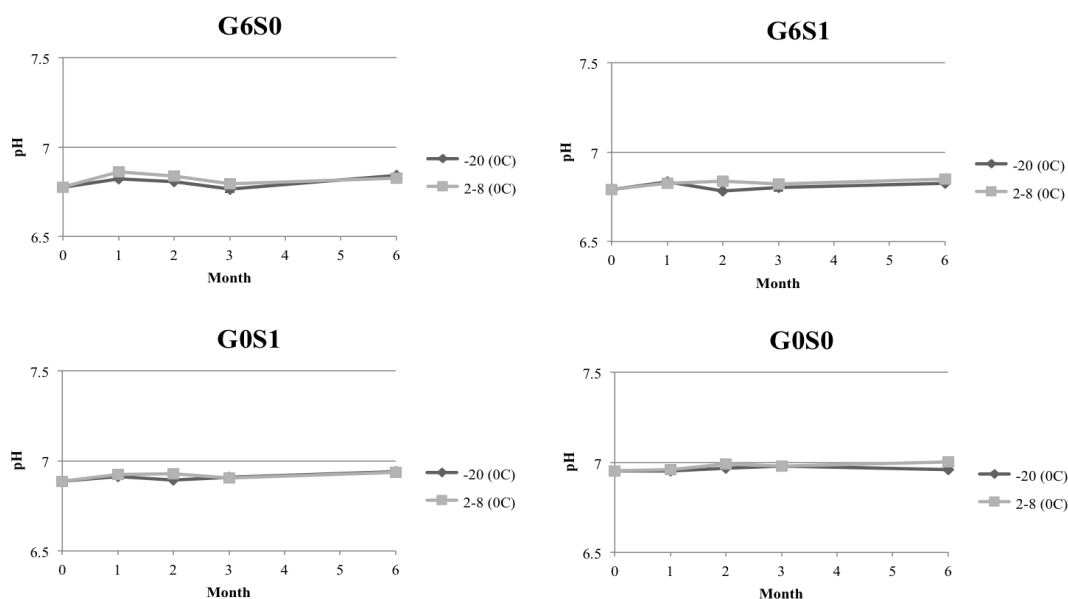

Figure 52. pH values of lyophilized LAIV vaccine products stored at -20°C and 2-8°C over period of 6 months
* Data are expressed as mean value ± SD (n=3)

- **Osmolality**

Under storage condition of 2-8°C, the osmolality values of reconstituted solutions of lyophilized powder products are consistent with the data obtained from the products stored at -20°C during 6 months of stability studies (p>0.05) (Figure 53). Moreover, the change in osmolality at each storage condition is insignificant. It could be stated that the osmolality of lyophilized LAIV vaccine products is stable over 6 months storage at -20°C and 2-8°C. For both conditions, only G0S0 formulation expresses osmolality of isotonic solutions. At month 6, the osmolality value of G0S0 is 335 ± 1mOsm/kg when stored at -20°C and 295 ± 1mOsm/kg when stored at 2-8°C. Other formulations present hypertonic solutions the same as the initial osmolality

measured after freeze-drying on day 0. However, all formulations possess osmolality within the acceptable range with only exception of G6S1 products that are exposed to 2-8°C had osmolality of 711 ± 1mOsm/kg at month 6.
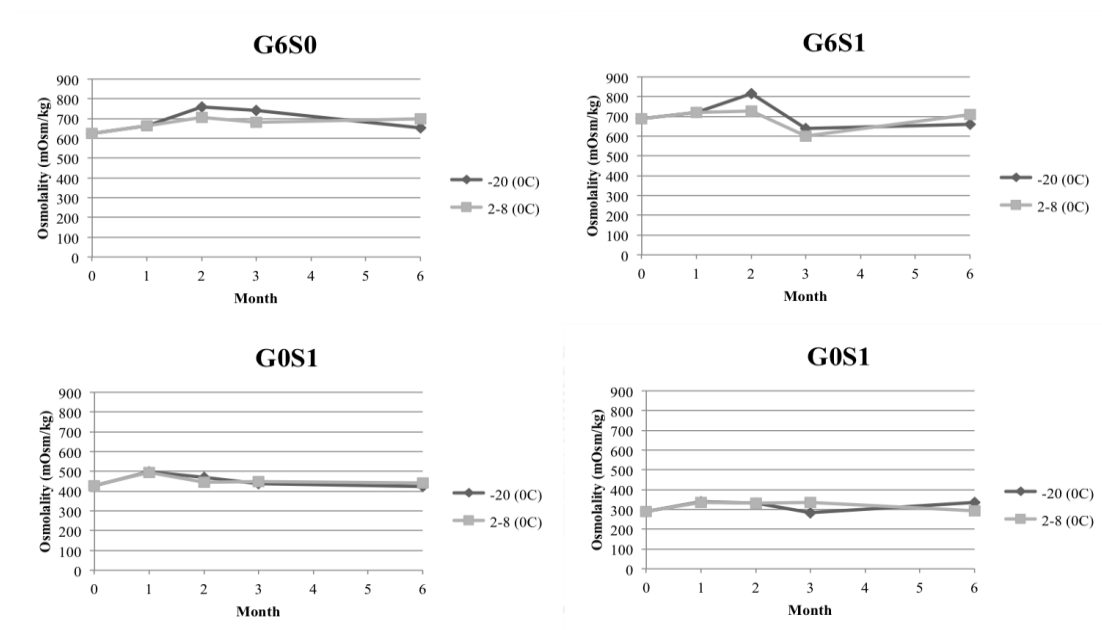

Figure 53. Osmolality values of lyophilized LAIV vaccine products stored at -20°C and 2-8°C over period of 6 months

 * Data are expressed as mean value ± SD (n=3)

4.4. Thermal behavior

DSC thermograms of G6S0 lyophilized LAIV vaccine products stored at -20°C and 2-8°C for 6 months are shown in Figures 54 and 55, respectively. At each storage time, endothermic peaks corresponding to dehydration are observed within the interval of 50-100°C with exceptions at months 2 and 6. For products obtained during 0, 1 and 3 months are found to show similar thermograms. Signifying that the structural change due to storage temperature of -20°C and 2-8°C are negligible. The similar results are also obtained from G6S1, G0S1 and G0S0 lyophilized products (Appendix A). This erroneous DSC results for months 2 and 6 are systematic in every product tested (Figures 54, 55 and Appendix A). Thus, DSC results for 2 and 6

months cannot be used in the thermal stability interpretation due to DSC technical error during that time of experiments.

Figure 54. DSC thermograms of lyophilized LAIV vaccine products of G6S0 stored at -20°C for 6 months
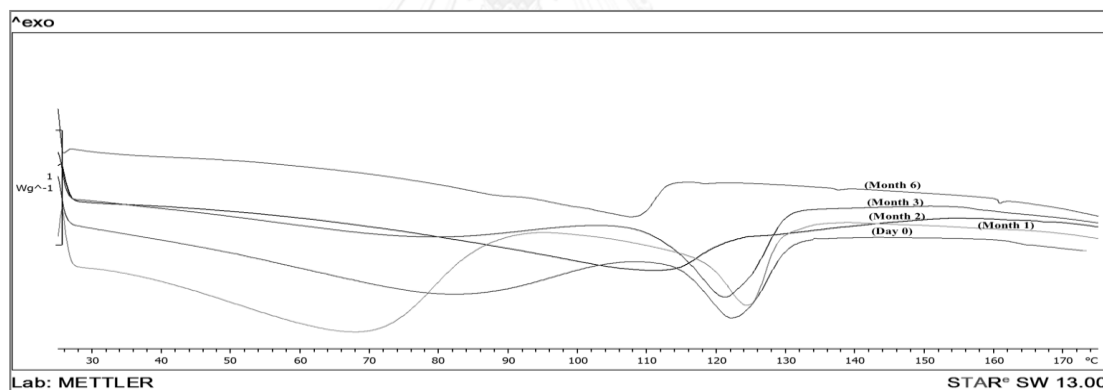
Figure 55. DSC thermograms of lyophilized LAIV vaccine products of G6S0 stored at 2-8°C for 6 months

4.5. Crystallinity

Figures 56 and 57 show the crystallinity of lyophilized LAIV vaccine product of G6S0 for 6 months stored at -20°C and 2-8°C. Minor reductions in crystalline intensity are observed throughout the storage time. The sharpness of crystalline δ-mannitol peak at $10°$ 2θ is reduced, indicating that the crystalline form somehow converted to an intermediate phase. As can be seen, at 2-8°C storage

condition, it exerts higher thermal energy in to the system when compare to -20°C. This higher energy induces more movements of the crystalline δ-mannitol to more random arrangements. Therefore, the reduction in degree of crystallinity of mannitol are observed (Figure 56). When -20°C is used, very small rearrangements are found (Figure 57). These similar results are also true for lyophilized LAIV vaccine products of G6S1, G0S1 and G0S0 (Appendix B).
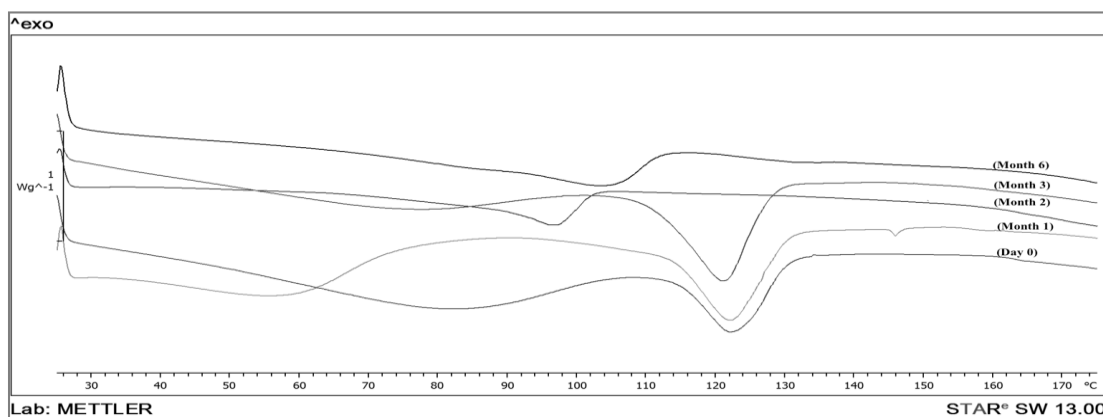

Figure 56. XRPD patterns of lyophilized LAIV vaccine products of G6S0 stored at -20°C for 6 months
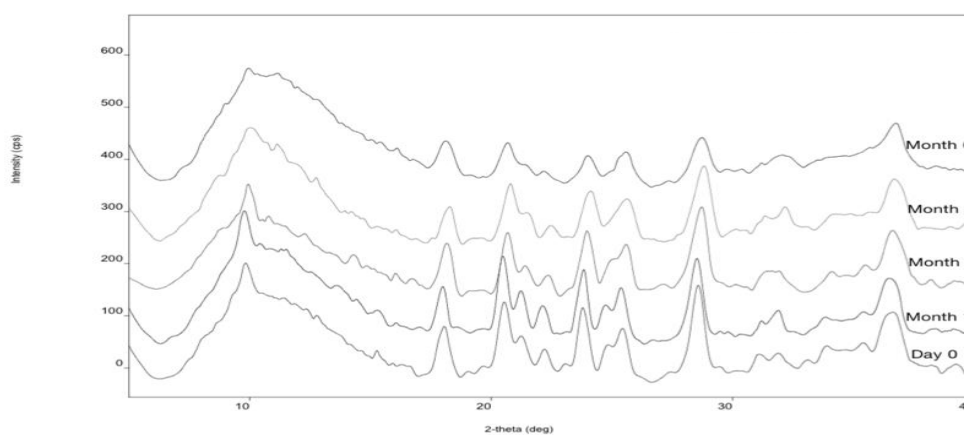
Figure 57. XRPD patterns of lyophilized LAIV vaccine products of G6S0 stored at 2-8°C for 6 months

4.6. Residual moisture content
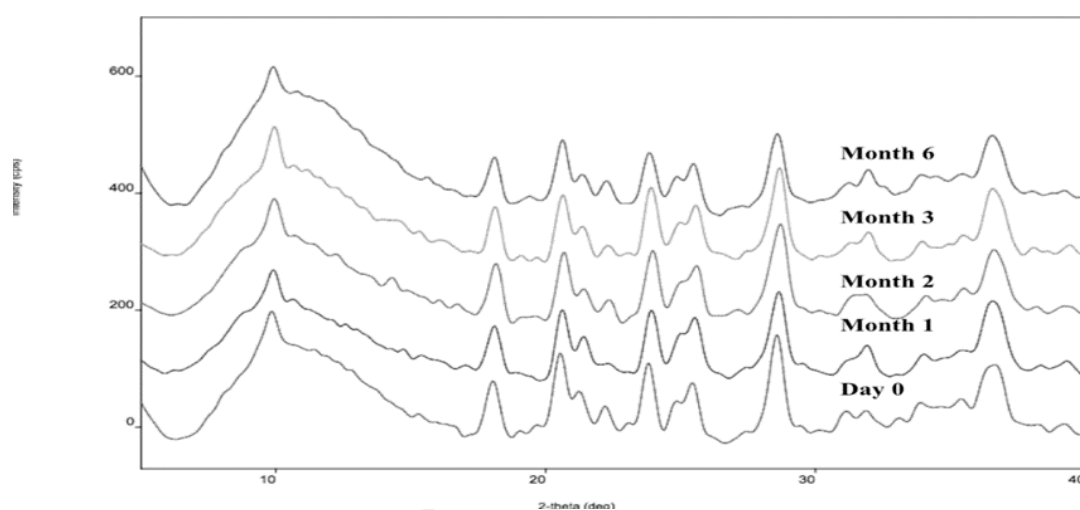
▪ **TGA Results**

Figure 58 illustrates the residual moisture content in lyophilized powder products over the period of 6 months storage at -20°C and 2-8°C. As mentioned previously that the percentages of water in all samples immediately after freeze-drying are higher than the acceptance range from the beginning. Therefore, the lyophilized products during stability tests apparently show water contents of greater than 3% throughout 6 months storage. According to statistical analysis of all formulations, p value is greater than 0.05, indicating that the differences in water contents between lyophilized vaccine products stored at 2-8°C and -20°C are not significant.

Figure 58. Residual moisture content of lyophilized LAIV vaccine products over 6 months storage at -20°C and 2-8°C (results obtained from TGA)

* The experiment at months 3 could not be conducted due to the technical problems of TGA.

* Data are expressed as mean value ± SD (n=3)

▪ **Karl Fisher method**

Karl Fisher method gives evidences that there are significant differences in moisture contents between samples stored at -20°C and 2-8°C (p<0.05) (Figure 59). All formulations show that the levels of residual moisture contents at 2-8°C are higher than at -20°C over the timeline of stability study. It could be noted that the moisture stability of lyophilized LAIV vaccine products is more preserved at lower temperature, -20°C. The water absorption rate of lyophilized powder is higher for products exposed to 2-8°C. XRPD results confirm the data of Karl Fisher by evidence of the reduction in degree of crystallinity at 2-8°C as mentioned earlier. Besides, Karl Fisher method is more sensitive to moisture in the bulk than TGA technique. Therefore, TGA presents lower values when compare to those obtained from Karl Fisher titrimetry.

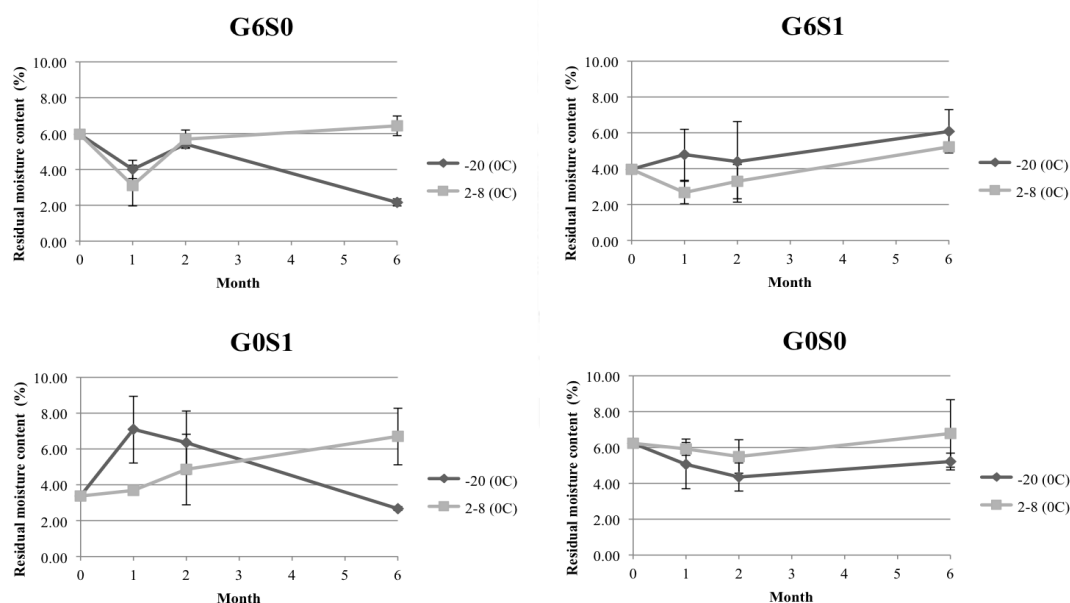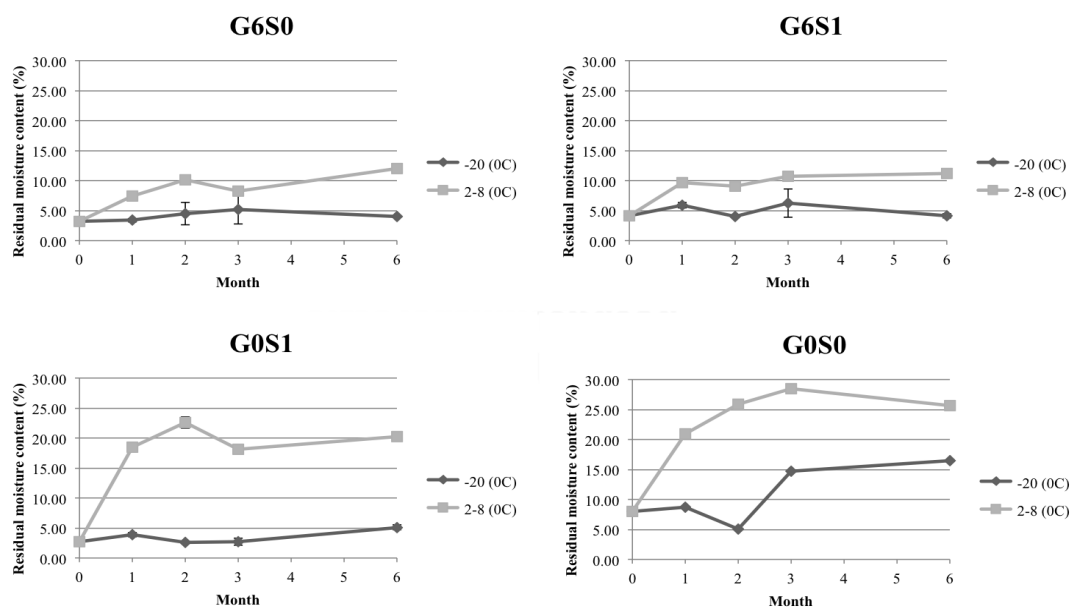*Figure 59. Residual moisture content of lyophilized LAIV vaccine products over 6 months storage at -20°C and 2-8°C (results obtained from Karl Fisher titrimetry)*
* Data are expressed as mean value ± SD (n=3)

4.7. Infectivity titer measurement

The infectivity titers obtained when products are stored at -20°C maintain their

abilities to infect tissue cells after 6 months of storage (Figure 60). The $\log_{10}$ $TCID_{50}/0.2$ ml values are 7.30 ± 0.08 for G6S0, 7.67 ± 0.35 for G6S1, 7.26 ± 0.06 for G0S1 and 7.43 ± 0.18 for G0S0.

According to statistical analyses, the differences in infectivity titers between storage conditions are only significant with G0S1 and G0S0 as evidence in p-value of less than 0.05. However, G0S0 may show significant difference in infectivity titer at 2-8°C compared to -20°C, but all values are still within acceptable specification range of not less than 6.5. Only G6S0 and G6S1 stored at 2-8°C show similar trend in infectivity titer to those stored at -20°C. At 2-8°C, the $\log_{10}$ $TCID_{50}/0.2$ ml of G6S0 and G6S1 at 6 months are 6.85 ± 0.24 and 6.9 ± 0.27, respectively. In conclusion, for G6S0, G6S1 and G0S0 are considered acceptable for infectivity titer values of LAIV vaccine formulations.
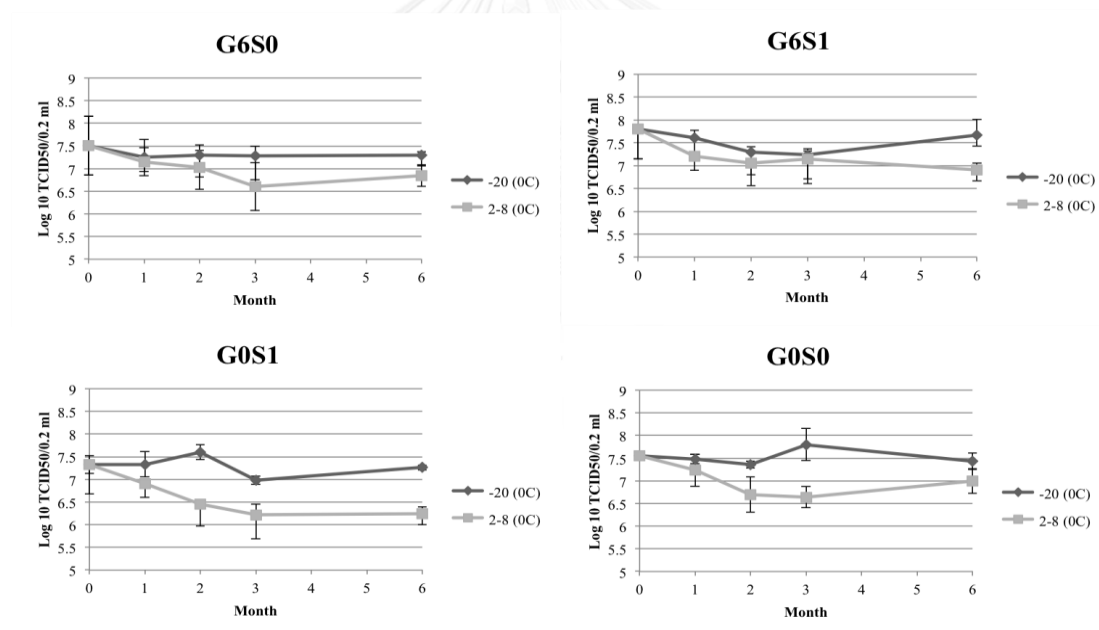

Figure 60. Infectivity titer of lyophilized LAIV vaccine products stored at -20°C and 2-8°C after storage of 6 months

* Data are expressed as mean value ± SD (n=2)

Table 24. Summary of the stability study of lyophilized LAIV vaccine products stored at -20°C and 2-8°C for 6 months

Parameter	Comparison between 2 conditions of storage (-20°C vs. 2-8°C)	Note
Physical appearance	Consistent	
Reconstitution time	Consistent	
Residual moisture content	Inconsistent (Karl Fisher)	Higher than acceptable specification of 3%
pH and osmolality	Consistent	
Thermal behavior	Consistent	Except technical errors at months 2 and 6
Crystallinity	Consistent	Degree of crystallinity: 2-8°C > -20°C
Infectivity titer	Inconsistent	-20°C: All formulations in acceptable range 2-8°C: Only G6S0, G6S1 and G0S0 in acceptable range

The summary shown in Table 24 proves that there exists a correlation between degree of crystallinity seen by XRPD and infectivity titer. The higher reduction in degree of crystallinity comparing to initial level induces the faster rate of degradation of lyophilized LAIV vaccine products. At 2-8°C, the lyophilized LAIV vaccine products of G6S0, G6S1 and G0S0 still retain their infectivity titers in acceptable specification. Wherein, only G6S0 and G6S1 show similar trends in infectivity titer of lyophilized products stored at -20°C and 2-8°C ($p > 0.05$). Combined with the

results obtained from previous sections, it could be concluded that glycine somehow plays an important role in lyophilized mannitol-based LAIV vaccine formulation by improving their physical and biological stability.

CHAPTER V - CONCLUSION

Measurements obtained from fluorescence spectroscopy, far-UV CD and near-UV CD indicated that various freezing rates (rapid rate 2.58°C/min, moderate rate 1.08°C/min and slow rate 0.50°C/min) affected the secondary and tertiary conformations of HA in aqueous solution. However, results from all four formulations of HA (G6S0, G6S1, G0S1 and G0S0) suggested that moderate freezing rate (1.08°C/min) induced minimum conformational change of HA in both secondary and tertiary structures. Therefore, the freezing rate of 1.08°C/min was selected to be used for the freeze-drying cycle development of all four LAIV formulations.

The lyophilization process, applied to this study using freezing rate of 1.08°C/min, provided acceptable final freeze-dried LAIV vaccine products in terms of physical appearance, reconstitution time, pH, osmolality, thermal behavior, crystallinity and infectivity titer. Only the residual moisture was higher than specified. The high water content may be due to the inadequate secondary drying time, hence longer secondary drying period should be used.

After 6 months storage at -20°C, lyophilized LAIV vaccine products of all four formulations exhibited outstanding infectivity titer level.

However, when lyophilized LAIV vaccine products were stored at 30°C/75%RH, physical stability and infectivity titer level of all four formulations were found to be unacceptable within only 1 week of storage. The desired storage temperature of these lyophilized LAIV vaccine products should be at 2-8°C. Stability determination at 2-8°C showed that only G6S0, G6S1 and G0S0 products were stable throughout the 6 months study period with excellent infectivity titers within acceptable criteria. In addition, only the formulations with glycine (G6S0 and G6S1) which were stored at 2-8°C showed the infectivity titer trend consistent to those stored at -20°C.

In conclusion, according to freezing, freeze-drying and stability studies, glycine expressed as the best protective agent for the conformational stability of HA as well as the infectivity of lyophilized mannitol-based LAIV vaccine products. Therefore, glycine of 6%w/v should be chosen for the future study on the scale-up of lyophilized LAIV vaccine manufacturing.

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APPENDICES

Appendix A – Thermal behavior of lyophilized LAIV vaccine products stored at -20°C and 2-8°C for 6 months
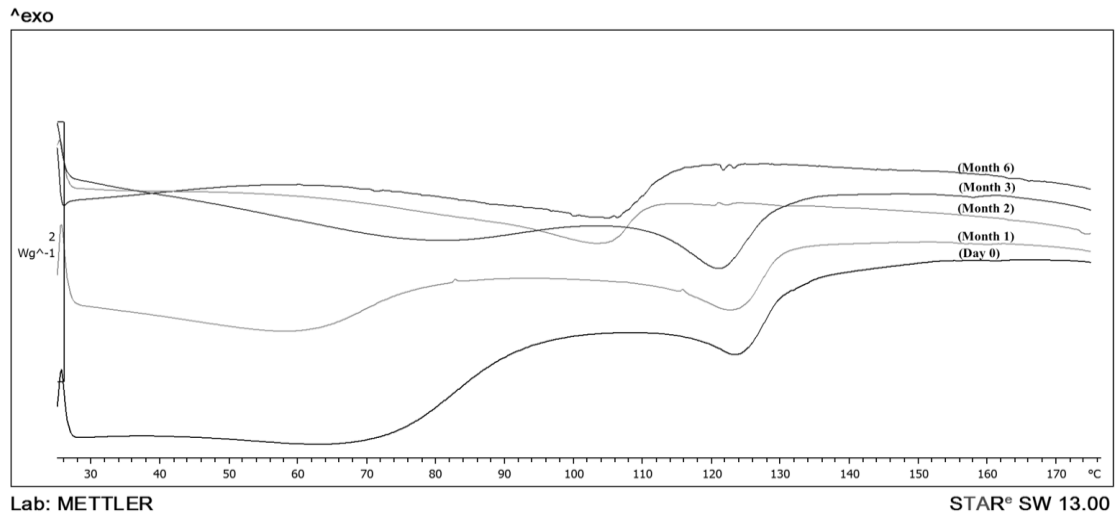
Figure 1. DSC thermograms of lyophilized LAIV vaccine products of G6S1 stored at -20°C for 6 months
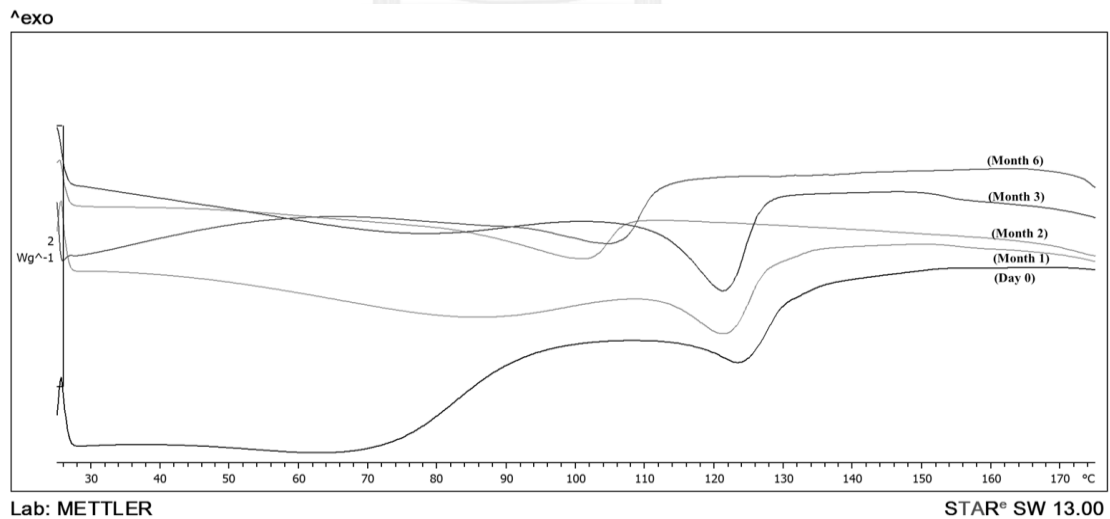
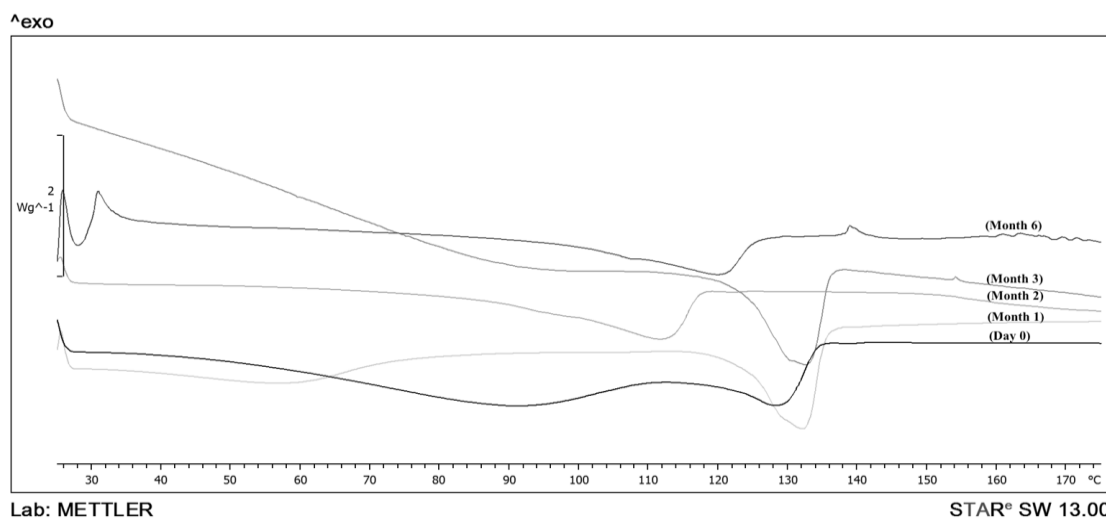*Figure 2. DSC thermograms of lyophilized LAIV vaccine products of G6S1 stored at 2-8°C for 6 months*
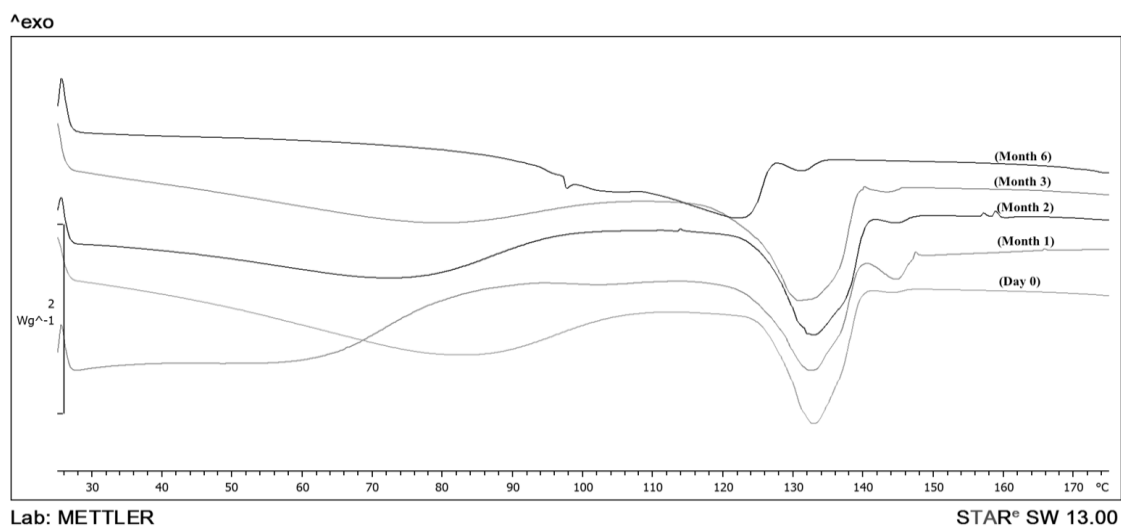
Figure 3. DSC thermograms of lyophilized LAIV vaccine products of G0S1 stored at -20°C for 6 months
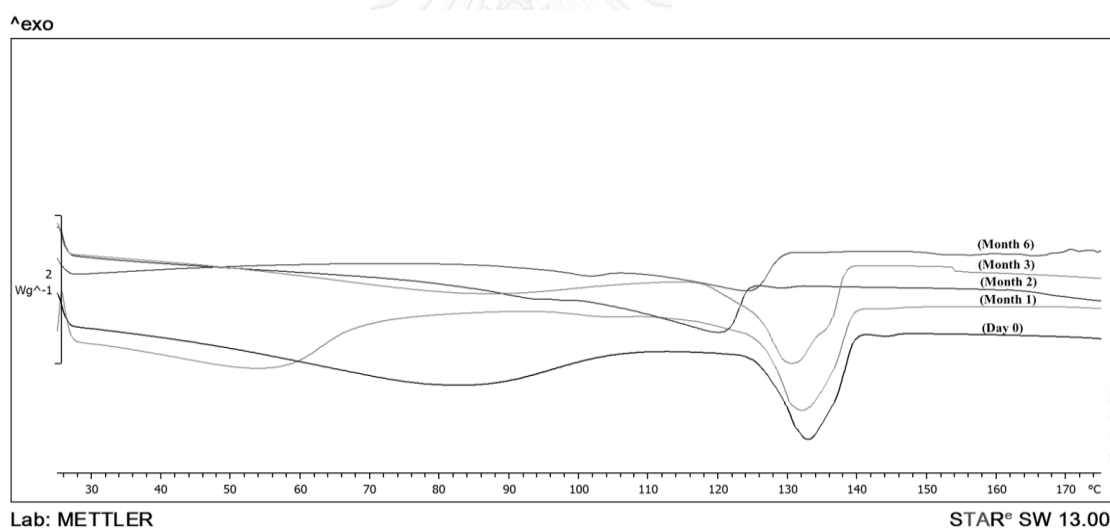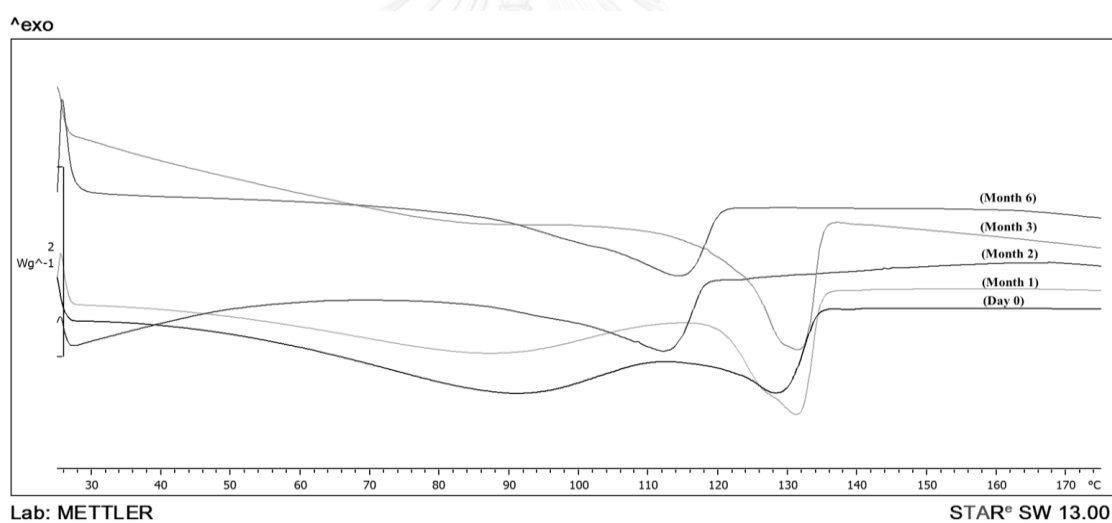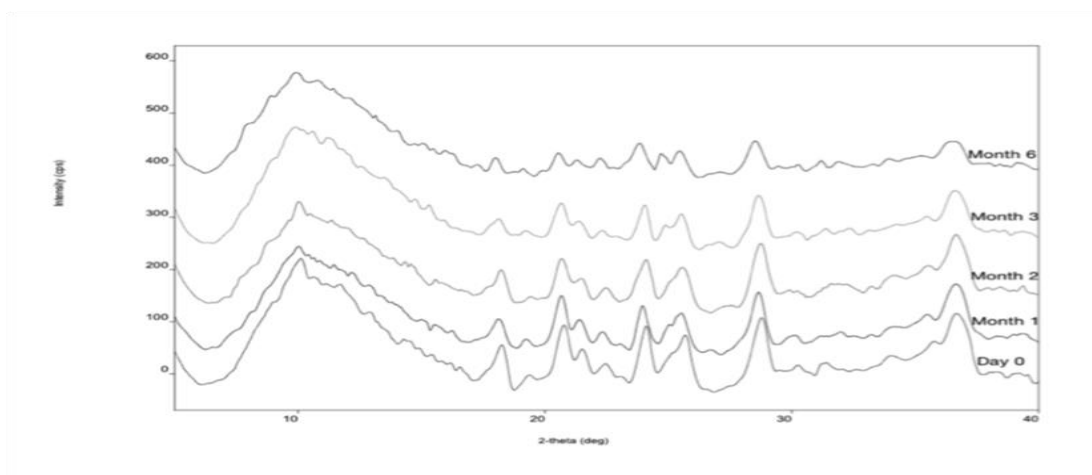

Figure 4. DSC thermograms of lyophilized LAIV vaccine products of G0S1 stored at 2-8°C for 6 months

Figure 5. DSC thermograms of lyophilized LAIV vaccine products of G0S0 stored at -20°C for 6 months

Figure 6. DSC thermograms of lyophilized LAIV vaccine products of G0S0 stored at 2-8°C for 6 months

Appendix B – Crystallinity of lyophilized LAIV vaccine products when stored at -20°C and at 2-8°C for 6 months

Figure 1. XRPD patterns of lyophilized LAIV vaccine products of G6S1 stored at -20°C for 6 months
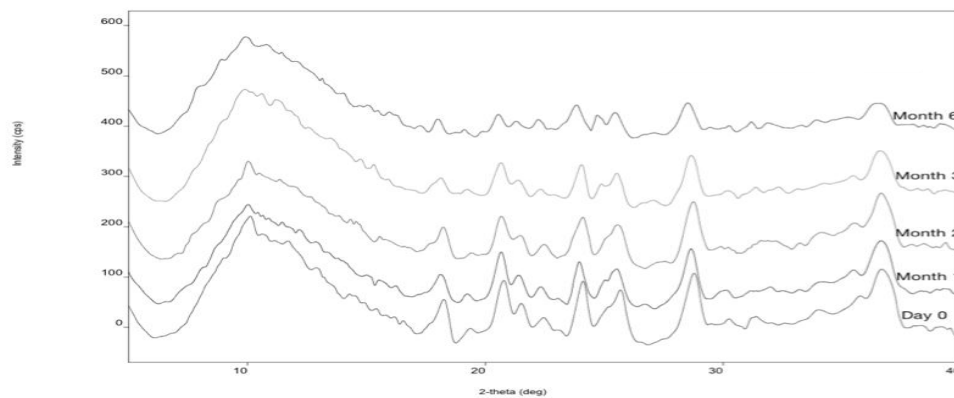

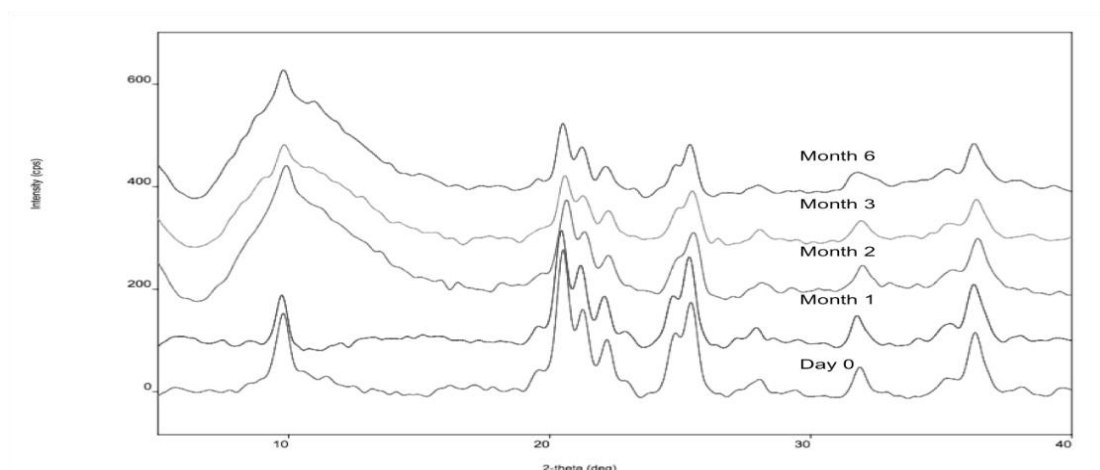*Figure 2. XRPD patterns of lyophilized LAIV vaccine products of G6S1 stored at 2-8°C for 6 months*

Figure 3. XRPD patterns of lyophilized LAIV vaccine products of G0S1 stored at -20°C for 6 months
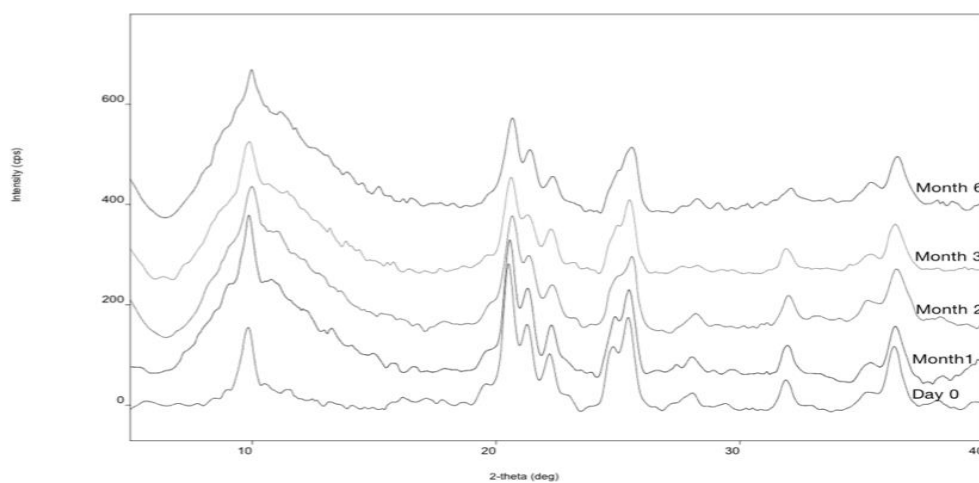

Figure 4. XRPD patterns of lyophilized LAIV vaccine products of G0S1 formulation stored at 2-8°C for 6 months
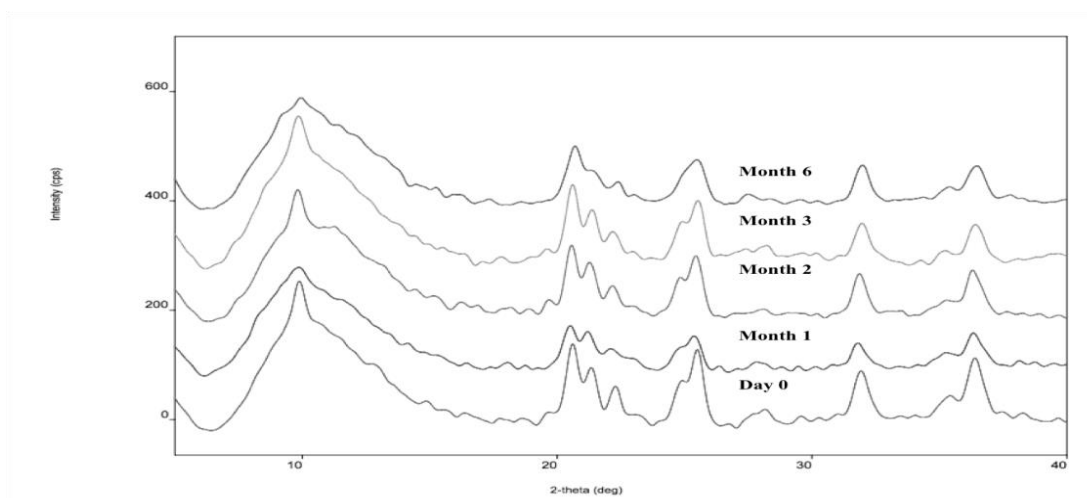
Figure 5. XRPD patterns of lyophilized LAIV vaccine products of G0S0 stored at -20°C for 6 months
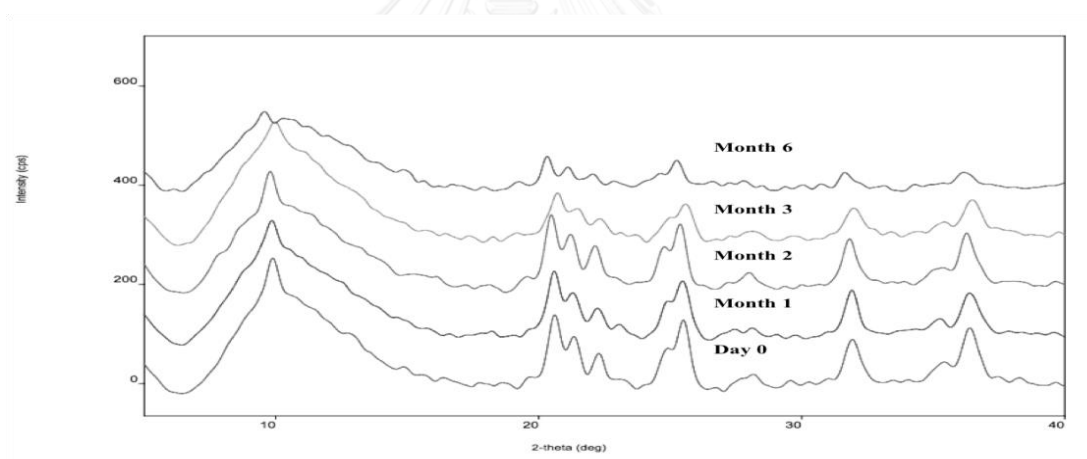

Figure 6. XRPD patterns of lyophilized LAIV vaccine products of G0S0 stored at 2-8°C for 6 months

VITA

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