การพัฒนาสูตรตำรับและกระบวนการทำแห้งเยือกแข็งของเซรุ่มแก้พิษงูเขียวหางไหม้

นายธรรมนูญ ด้วงโสน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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DEVELOPMENT OF FORMULATION AND LYOPHILIZATION PROCESS OF GREEN PIT VIPER ANTIVENIN

Mr.Thammanoon Duangsano

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Industrial Pharmacy

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Thesis Title	DEVELOPMENT OF FORMULATION AND LYOPHILIZATION PROCESS OF GREEN PIT VIPER ANTIVENIN
By	Mr. Thammanoon Duangsano
Field of Study	Industrial Pharmacy
Thesis Advisor	Narueporn Sutanthavibul, Ph.D.
Thesis Co-advisor	Sumana Khomvilai, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Associate Professor Uthai Suvanakoot, Ph.D.)

…….……. Thesis Advisor

(Narueporn Sutanthavibul, Ph.D.)

…….. Thesis Co-advisor

(Professor Sumana Khomvilai, Ph.D.)

…….. Examiner

(Professor Garnpimol Ritthidej, Ph.D.)

…….. Examiner

(Phanphen Wattanaarsakit, Ph.D.)

…….. External Examiner

(Associate Professor Satit Puttipipatkhachorn, Ph.D.)

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วัตถุประสงค์ของงานวิจัยนี้ เพื่อลดระยะเวลาในกระบวนการทำแห้งแบบเยือกแข็งของผลิตภัณฑ์เซรุ่มแก้พิษงูเขียวหางไหม้และเพื่อพัฒนาสูตรตำรับที่เหมาะสม ทำการประเมินผลขั้นตอนการเยือกแข็งต่อโครงสร้างทุติยภูมิของ F(ab')2 ของเซรุ่มแก้พิษงูเขียวหางไหม้ พบว่าขั้นตอนการเยือกแข็งที่เหมาะสมที่สุด คือ อัตราการเยือกแข็งที่ 1.86°C/นาที จนอุณหภูมิลดลงถึง -40°C และค้างไว้ที่อุณหภูมิดังกล่าวต่อไปอีก 30 นาที (Rapid3) นำผลิตภัณฑ์ทำแห้งแบบเยือกแข็งโดย Rapid3 มาวิเคราะห์ลักษณะทางกายภาพ โครงสร้างทุติยภูมิของ F(ab')2 ปริมาณความชื้น คุณสมบัติโครงสร้างผลึก และความคงตัวเป็นเวลา 3 เดือน ต่อจากนั้นจึงศึกษาและปรับขั้นตอนการทำให้แห้งในขั้นปฐมภูมิและทุติยภูมิ แล้วประเมินผลิตภัณฑ์ที่ได้ตามรายละเอียดในหัวข้อข้างต้นต่อไป กระบวนการทำแห้งเยือกแข็งที่เหมาะสมที่สุด ประกอบด้วยขั้นตอนการเยือกแข็ง Rapid3 การทำแห้งปฐมภูมิที่ -25°C เป็นเวลา 6 ชั่วโมง ต่อจากนั้นที่ 0°C เป็นเวลา 12 ชั่วโมง (ความดัน 300 mTorr) เพิ่มอุณหภูมิจาก 0^{o}C เป็น 30^{o}C ภายใน 1 ชั่วโมงแล้วทำให้แห้งขั้นทุติยภูมิที่ ที่ 30°C เป็นเวลา 2 ชั่วโมง ภายใต้ความดัน 100 mTorr กระบวนการทำแห้งแบบเยือกแข็งของผลิตภัณฑ์เซรุ่มแก้พิษงูเขียวหางไหม้ที่พัฒนาได้ใช้ระยะเวลารวมประมาณ 24 ชั่วโมง สูตรตำรับเซรุ่มแก้พิษงูเขียวหางไหม้ที่มีสารเพิ่มความคงตัวสำหรับการทำแห้งด้วยกระบวนการทำแห้งเยือกแข็งที่เหมาะสมที่สุด ประกอบ ด้วย F(ab')2 0.7 mg/ml และ glycine 2% w/v โดยนำสารละลายจำนวน 4 มิลลิลิตรบรรจุในขวดแก้วขนาด 6 มิลลิลิตร ทดสอบความแรงของเซรุ่มโดยทำการทดสอบการต้านพิษในหนูทดลอง พบว่าผลิตภัณฑ์สูตรตำรับข้างต้นให้ค่าความแรงของผลิตภัณฑ์ 0.966 mg/ml ซึ่งผ่านข้อกำหนดของเซรุ่มแก้พิษงูเขียวหางไหม้ของสถานเสาวภา สภากาชาดไทย (≥ 0.7 mg/ml) มีลักษณะทางกายภาพที่ดี ไม่มีการเปลี่ยนโครงสร้างทุติยภูมิ และมีความคงตัวที่น่าพอใจภายในระยะเวลาการทดสอบ 3 เดือน

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : LYOPHILIZATION / CIRCULAR DICHROISM / GREEN PIT VIPER ANTIVENIN/ CRYSTALLINE

THAMMANOON DUANGSANO : DEVELOPMENT OF FORMULATION AND LYOPHILIZATION PROCESS OF GREEN PIT VIPER ANTIVENIN. ADVISOR: NARUEPORN SUTANTHAVIBUL, Ph.D., CO-ADVISOR : PROF. SUMANA KHOMVILAI, Ph.D., 151 pp.

The objectives of this study are to reduce the total time used in the lyophilization process and to develop appropriate formulation of Green Pit Viper antivenin product. The effect of freezing steps on the secondary structure of F(ab')2 Green Pit Viper antivenin are evaluated. Optimized freezing step is found to have an average cooling rate of 1.86°C/min until -40°C is reached and annealed for 30 minutes (Rapid3). Lyophilized products obtain by Rapid3 freezing step are analyzed for their physical appearances, F(ab')2 secondary structures, moisture contents, crystallinity and stability after 3 months. Primary and secondary drying are studied and adjusted. Lyophilized products obtained are again analyzed similarly to the previous section. The optimal lyophilization process is Rapid3 freezing step, primary drying is done at -25°C for 6 hours and 0°C for 12 hours (pressure 300 mTorr). Heating time from 0°C to 30°C is 1 hour and final secondary drying at 30°C continues for another 2 hours under pressure of 100 mTorr. The total lyophilization time is approximately 24 hours. Green Pit Viper antivenin is formulated with proper stabilizing agent and freeze dried with optimal lyophilization process. The best formulation is 0.7 mg/ml F(ab')2 Green Pit Viper antivenin and 2% w/v glycine which 4 ml of the formula is filled into 6 ml vial. Potency test is done using "Mouse Neutralizing Test" which resulted in a potency of 0.966 mg/ml. The developed formula has the potency within the required specification of Queen Saovabha Memorial Institute for Green Pit Viper antivenin products ($\geq$ 0.7 mg/ml), good physical appearance, retain its initial secondary structure and shows satisfying 3 months stability results.

Department : Pharmaceutics and Industrial Pharmacy Student's Signature .....

Field of Study : Industrial Pharmacy Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	=	Percentage
β	=	Beta
α	=	Alpha
δ	=	Delta
θ	=	Theta
°/min	=	degree of angle per minute
°C	=	Degree of Celsius (centrigrade)
A	=	Angstrom
CD	=	Circular Dichroism
DSC	=	Differential scanning calorimetry
et.al.	=	et alli, and others
KD	=	Kilodalton(s)
mdeg	=	Milli degree
mg	=	Milligram(s)
ml	=	Milliliter(s)
mTorr	=	Millitorr
min	=	Minute(s)
MW	=	Molecular weight
nm	=	Nanometer
QSMI	=	Queen Saovabha Memorial Institute
RM	=	Relative humidity
sec	=	Second(s)
TGA	=	Thermogravimetry
UV	=	Ultraviolet
XRPD	=	X-ray powder diffractometry
F(ab')2	=	Fragment of antibody binding site which were produced by pepsin digestion of IgG
IgG	=	Immunoglobulin subclass G

SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
pH	=	The negative logarithm of the hydrogent concentration
Native F(ab')2	=	Solution of F(ab')2 of green pit viper antivenin 0.7 mg/ml in purified water
Ser	=	Solution of F(ab')2 green pit viper antivenin 0.7 mg/ml
SF	=	Mixture of F(ab')2 green pit viper antivenin 0.7 mg/ml with fructose 2%
SG	=	Mixture of F(ab')2 green pit viper antivenin 0.7 mg/ml with glycine 2%
SGU	=	Mixture of F(ab')2 green pit viper antivenin 0.7 mg/ml with glucose 2%
SM	=	Mixture of F(ab')2 green pit viper antivenin 0.7 mg/ml with mannitol 2%
SMd	=	Mixture of F(ab')2 green pit viper antivenin 0.7 mg/ml with maltodextrin
SS	=	Mixture of F(ab')2 green pit viper antivenin 0.7 mg/ml with sorbitol 2%
Sl	=	Slow freezing process
R1	=	Rapid1 freezing process
R3	=	Rapid3 freezing process
PD1	=	Primary Dry1 lyophilization process
PD2	=	PrimaryDry2 lyophilization process
PD3	=	PrimaryDry3 lyophilization process
PD4	=	PrimaryDry4 lyophilization process
PD5	=	PrimaryDry5 lyophilization process
PD6	=	PrimaryDry6 lyophilization process
PD8	=	PrimaryDry8 lyophilization process
PD9	=	PrimaryDry9 lyophilization process
PD10	=	PrimaryDry10 lyophilization process
SD1	=	SecondaryDry1 lyophilization process
4SM	=	mixture of F(ab')2 (0.7 mg/ml) with 4% mannitol formulation
2SG	=	mixture of F(ab')2 (0.7 mg/ml) with 2% glycine formulation
SGM	=	mixture of F(ab')2 (0.7 mg/ml) with 1 % glycine and 1.5% mannitol formulation
SGc	=	mixture of F(ab')2 (1.4 mg/ml) with 4% glycine formulation
SMc	=	mixture of F(ab')2 (1.4 mg/ml) with 8 % mannitol formulation

CHAPTER I

INTRODUCTION

Epidemiology of snake bite cases in Thailand is 13.18, 11.85, 13.25, 13.17, 11.98 and 13.06 persons per 100,000 populations in the year 2004, 2005, 2006, 2007, 2009 and 2010, respectively. For cases of fatality rate of snake bite are 0.01, 0.07, 0.06, 0.04, 0 and 0.02 percentages in the year 2004, 2005, 2006, 2007, 2009 and 2010. However, the number of cases may be unrecorded or death may accur before hospitalization. Most cases of snake bites are due to Malayan Pit Viper, Green Pit Viper and Cobra snakes. The snake venom in Thailand was classified into 3 groups as neurotoxin snake venom (Cobra, King cobra, Banded Krait, Malayan Kriat), hematotoxin snake venom (Green Pit Viper, Malayan Pit Viper, Russell's viper) and myotoxin snake venom (Sea Snakes). The clinical symptoms of patient may be local swelling, local necrosis, haemorrhage, thombosis, paralysis, shock or death. The treatment of snake bite is to receive a specific snake antivenin. The snake antivenin will neutralized and inactivate snake venom and decrease the danger of each venom. Snake antivenin is immunological products which are produced in the form of whole IgG, F(ab')2 or F(ab')2 fragments. The products are considered as protein materials. Immunoglobulin or antibody limitation is their instability (Chippaux and Goyffon, 1998 and Thankson, Wermel and Griffiths, 2003) both physical instability: denaturation (high temp, shear force or process), aggregation (high concentration, low temperature) and chemical instability : chemical reaction of disulfide exchange reaction, deamidation reaction or oxidation reaction. (Wang et al., 2007)

Most of snake antivenin products are currently being produced by polyclonal production method. By this method, the snake venom or antigen of optimal content and condition for immunization effect were injected to large animal such as horses (ovine). When animals received snake venom or antigen, they will produce immunological materials. After that serum or plasma of animal is drawn out and purified to standards and purity limit. The immunological materials are then formulated and produced as snake antivenin product.

The snake antivenin in solid dosage form are more stable, exhibits long shelf life, ease of transportation and storage than liquid dosage form. The choice of drying liquid antivenin, which

is protein material, to solid antivenin is freeze drying or lyophilization process which normally does not impact on stability of protein material.

Freeze dry or lyophilization process is drying process which freezes the aqueous solution to ice then sublimated ice to vapor within vacuum condition. This process applied low temperature to drying, thus suitable to protein and biological materials. Freeze dry or lyophilization composes of 3 stages: Freezing stage, which frozen the product form liquid to solid state. Primary drying, which sublimate ice to vapour by vacuum condition and low temperature. To determine the primary drying temperature of amorphous materials is from glass transition temperature. Crystalline materials, determination of primary drying temperrature is from eutectic temperature. Pressure inside chamber of freeze dryer must be control led below their eutectic points. Finally, secondary drying is to remove residual water by increasing temperature while pressure is under vacuum. Lyophilized product produced porous cake which is easily reconstituted. Futhermore, it is more stabled than liquid dosage form with longer shelf life.

Variables of on lyophilization process

1. Freezing stage is an important stage because it will affect primary drying, secondary drying, and characteristics of the lyophilized products.

1.1 Freezing rate affects form and structure of ice crystals. If the product is rapidly frozen then small ice crystals. These small ice crystals destroy protein structures less than large ice crystals which are producedby slow freezing rate. Fathermore, form and structure of ice affect the subsequent drying steps, circulation of water vapor from sublimination and quality of product. (Schwegman, Hardwick and Akers, 2005)

1.2 Temperature and duration of freezing. Freezing temperature was determined by glass transition temperature or eutectic temperature of the active ingredient in the product. Duration of freezing must be long enough to completely change liquid to solid form.

1.3 Volume of filling affects on thickness of the product. Thicker product requires more time for freezing and drying than thinner products.

1.4 Annealing process (Abdelwahed et al., 2006; Liu, 2006 and Tang and Pikal, 2004) applied at higher temperature than glass transition temperature for amorphous materials or eutectic temperature for crystalline materials. Annealing causes the ice crystal to form with the same size and reduced heterogenicity of product. Furthermore, annealing increased the size of the

ice crystal which causes optimal pore size in the product when drying. Primary drying will be shorter because of this bigger pores and reduced drying resistance and vapor can pass easily.

2. Primary drying

2.1 Temperature. If drying process occur at higher temperature, then the drying rate will be faster. Drying temperature must be lower than collapsed temperature which related to the glass transition temperature for amorphous materials or eutectic temperature for crystalline materials.

2.2 Pressure: Drying pressure must be lower than the eutectic point (crystalline) or glass transition (amorphous).

3. Secondary drying

3.1 Drying temperature affects most on this step which removed adsorbed water on crystal or solute phase. Proper temperature setting must be determined. Which fast heating rate or high temperature than required cause collapse of the product.

Formulation of lyophilized products composed of active ingredient and other additives such as : bulking agents (mannitol or glycine); structure modifiers (mannitol, glycine or sucrose); stabilizers or cryoprotectants which protect the product from freezing damage and lyoprotectants which protect the product from drying damage (glucose, dextrin, trehalose, mannitol or lactose); collapse inhibitors (glucose, dextrin or maltose); tonicity adjusters (sodium chloride, mannitol, or glycine).

Freeze drying of bulking agents or stabilizers, such as mannitol, found that amount or concentration and of freeze drying conditions impact on mannitol crystalline form (Kett, 2003). By freeze drying of glycine found that pH, ionic strengths effect on glycine crystalline form (Akers et al. 1995). Freeze drying of combined additives, such as mannitol with sucrose, found that sucrose decreased the crystallization rate of mannitol and decreased the glass transition temperature (Tg). Similarly effect is seen with sodium chloride. (Hawe and Friess, 2006 and Her, Deras amd Nail, 1995).

Studies were done on various single additives or combined additives for protein freeze drying. Single additives such as glycine, sorbitol, trehalose (Laio et al., 2005), maltodextrin (Passot et al., 2005). Combined additives, such as mannitol with sucrose, sucrose or trehalose with dextran (large molecule of carbohydrate) (Allison et al., 2000), sucrose or trehalose with hydroxyl ethyl starch (Garzon-rodeiguez, 2004). Sugar moiety preserves the structure of proteins

and increase stability of proteins. Large carbohydrate molecules increase glass transition temperature of the formulations (Allison et al., 2000) causing better lyophilized product stability at high temperature storage (Passot et al., 2005 and Prestrelski, Pikal and Arakawa, 1995). Water molecules are substituted with sugar molecules by hydrogen bonding with protein. Protein structures will be stabilize and protect the unfolding of protein that may cause decomposition by the drying process.

Freeze drying of antibody utilizes varieties of additives. Bulking agent or stabilizers that are crystalline materials produced good cakes and does not collapse for example, glycine and mannitol (Adams and Ramsay, 1996 and Mayer et al., 2009). Sugars, for example sucrose, increased the stability of protein. It found that the higher concentrations of proteins increased the glass transition temperature (Tg) of product. (Cleland et al., 2001 and Duddu and Dal Monte, 1997).

The study of lyophilization process found that freezing step is very important process which would be effect on physicochemical property of product, lyophilization process and finished product. Freezing step is effect on size of ice crystal and several factors are impact on freezing step: freezing rate, size of container, type of container and thickness of filling volume (Hottot, Vessot and Andrieu, 2007). The important process about freezing step is annealing. The study of mannitol and trehalose lyophilization found that annealing process was prolonged primary drying time. Because annealing process is increased size of mannitol and sodium chloride crystal which the big size of mannitol crystal blocked the circulation of water vapor from sublimination. Although the process without annealing may be partial collapse of cake but its is increased the circulation of water vapor and given shorter drying time. The study of sucrose or PVP (Abdelwahed et al., 2006) lyophilization found that annealing is increased water ice crystal and increased water vapor circulation rate which given the faster primary drying.

The study of antibody lyophilization found that the high concentration of protein induced the many of difference temperature between collapse temperature with glass transition temperature which given drying temperature for primary drying higer than glass transition temperature and absence collapse product (Colandene et al., 2007). Because the high concentration of protein increase viscosity and rigidity of structure which is difficult to collapse.

For lyophilization process development researchers must develop the formulation along with the process. Formulation greatly affects the optimal process obtained and the reproducibility.

However, there are very few studies that investigate the lyophilization process suitable for Green Pit Viper antivenin product. Also, the current lyophilization processfor Green Pit Viper antivenin product from QSMI uses total lyophilization time of over 50 hours per cycle. The objectives of this study are to:

1. Reduce the total lyophilization time used by QSMI in the production of Green Pit Viper antivenin product.
2. Develop appropriate formulation of Green Pit Viper antivenin lyophilized product.
3. Stability study of Green Pit Viper antivenin lyophilized products.

CHAPTER II

LITERATURE REVIEW

1. Venomous snake in Thailand

Thailand is a tropical country which has many kinds of snakes.(More than100 kinds are found in Thailand) Most snakes in Thailand are non-venomous snakes. More than 40 kinds of venomous snakes are found which can be classified by toxin properties into 3 groups. Snakes in neurotoxin group are such as Cobra, King cobra, Banded kraite and Malayan kraite snake.Snakes in haematotoxin group are such as Malayan pit viper, Green pit viper, Russell's viper snake.Snakes in myotoxin group such as sea snakes. (ไพบูลย์ จินตกุล, 2543)

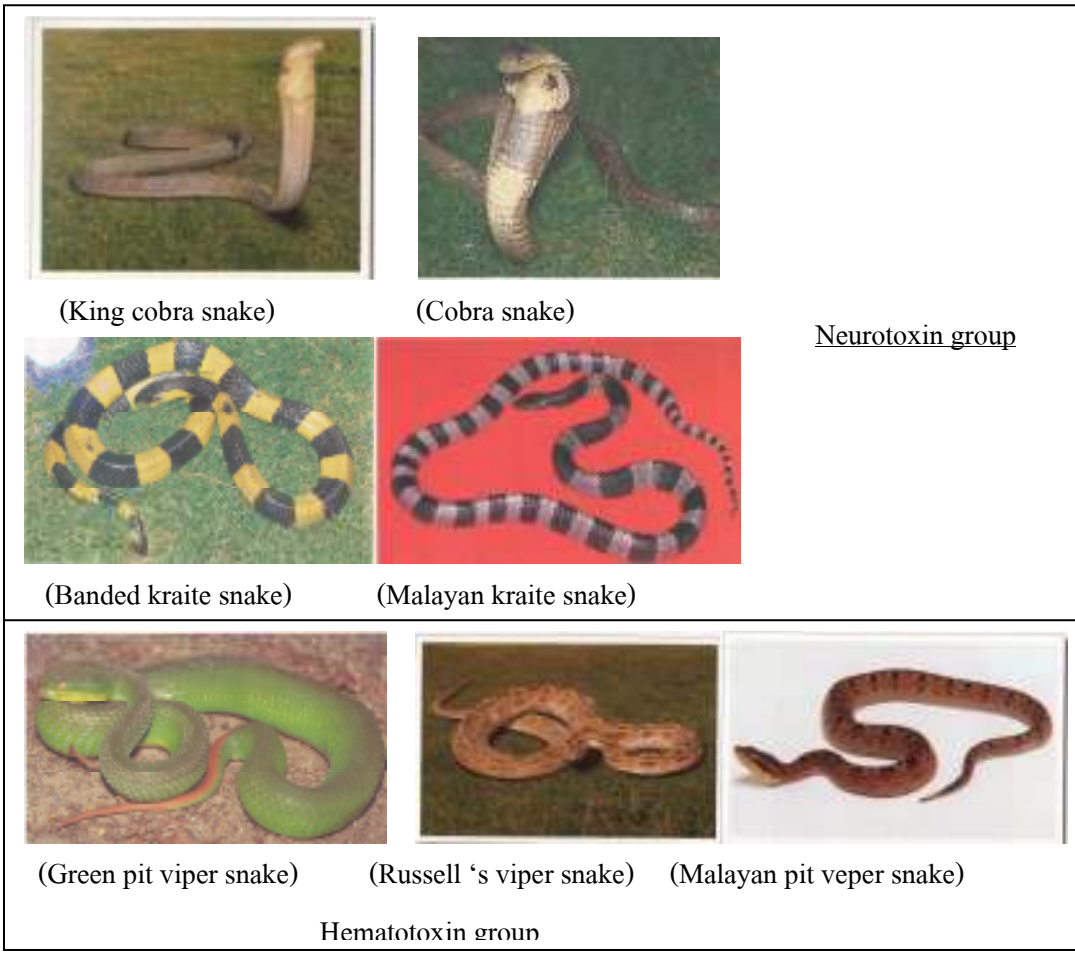


Figure 1 Venomous snakes in Thailand

(ไพบูลย์ จินตกุล, 2543; กระทรวงสาธารณสุข, กรมวิทยาศาสตร์การแพทย์, 2551)

When snake bited, snake venom would distribute and activate specific target organs of the victims. The symptoms of snake bites depend on toxin properties; neurotoxin or hematotoxin or myotoxin.

The symptoms of neurotoxic: the venom will completely bind with acetyl chlorine at actylchlorine receptor between muscular-nerve junctions then promote to paralysis. Patients have the following symptoms: droopina, sleeplike paralysis, spasm, excess salivation and vomitting, tissue necrosis. Patients die from respiratory system failure because of the muscle paralysis.

The symptoms of haeamatoxin: this venom has properties of hemarrhagins, procoagulant enzymes, proteolytic and haeamatolytic enzyme. The symptom of patients are such as bleeding, great pain, swelling, bruising, drop of blood pressure, bleeding in brain, gastro intestine system, renal failure and death.

The symptoms of myotoxin: The main symptoms are muscle pain in the leg, hip and shoulder, low volume of urine and may have renal failure.

Snake venom

The venomous snake has venom gland which is behind both eyes of snake and near the cheeks of snake. The venom gland produces snake venom and secretes the venom by muscle contraction.The venom is transported pass venom duct to venom flags. The venom flags are sharp for ease to insert on skin of the prey and inject the venom. Snake venom is weapon for self protection and hunting.(ไพบูลย์ จินตกุล, 2543) It composes 2 groups of chemical. About 90% is protein component which had toxin. Another group is enzymes. (Chippaux and Goyffon, 1998; ไพบูลย์ จินตกุล, 2543)

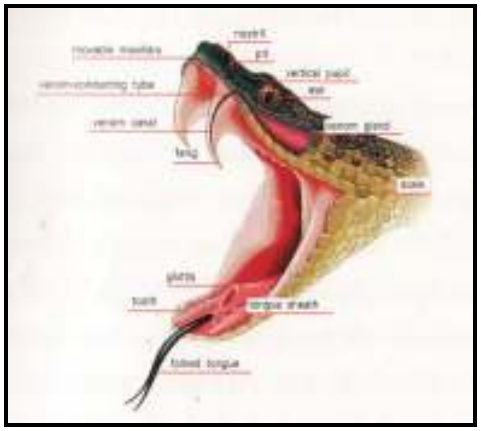


Figure 2 Venom gland of snake
(กระทรวงสาธารณสุข, สำนักยาและวัตถุเสพติด, 2551)

Snake bite in Thailand

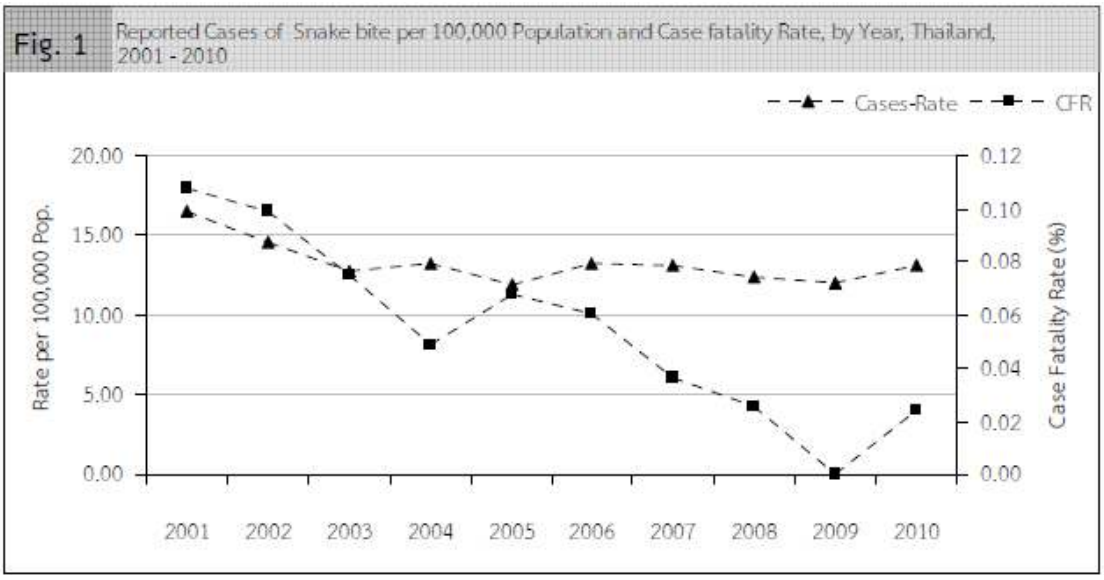


Figure 3 graph of reported case and case fatality rate of snake bite per 100,000 population in Thailand: 2001-2010 (Bureau of epidemiology, 2010)

The rate of snake bite in Thailand from Bureau of epidemiology in 2009 was 11.98 per 100,000 populations. The retrospective data about 10 years ago found that trend of snake bites and death rate was low. Most of snake bites were Malayan Pit Viper, Green Pit Viper and Cobra. Although snake bite rate and fatality rate were decreasing trend but it is significant since some snakes have dangerous venom which patients may cause death or severe symptoms: handicapped, disabled or paralysis. Treatment of snake bite can be done by using snake antivenin. (กระทรวงสาธารณสุข, สำนักพัฒนาวิชาการแพทย์, 2547) The snake antivenin is specific for the kind of snakes. The snake antivenin will contact and combine with snake venom, therefore venom is inactivated. The neutralized venom can not combine with receptor and do not biological effect on patient. The treatment with snake antivenin is necessity for decreasing severity of symptom and fatality of pateint.

Snake antivenin

Snake antivenin is immunoglobulin that could neutralized specific snake venom. It is prepared from plasma or serum of animal, for example horse, sheep, goat which were injected and immunized by snake venom. The snake venom is prepared to approp concentration and condition

which can only induce immunoglobutlin generation but do not cause any injury to the animal. (กระทรวงสาธารณสุข, สำนักยาและวัตถเสพติด, 2551)

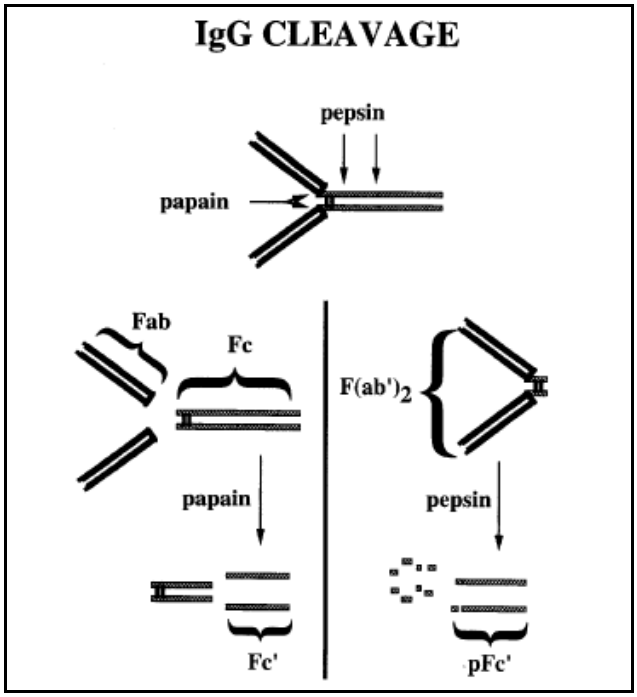


Figure 4 Immunoglobulin G, F(ab')2 and Fab Fragments which were enzyme digested. (Chippaux and Goyffon, 1998)

The snake antivenin was produced in form of whole IgG, Fab or F(ab')2 fragments. Those active structures have an antibody binding side which can contact and combine with snake venom. Each form of antivenin has different pharmacokinetic properties.

Table 1 Comparison between IgG, F(ab) and F(ab')2 fragments (Chippaux and Goyffon, 1998)

Characteristics	IgG	F(ab')2	F(ab')2
Obtainment	Precipitation	precipitation+pepsin	Precipitation+papain
Distribution (h)	>3	3	1
Elimination (h)	>100	60	10
Tissue affinity	1	2	5
Complement reaction	yes	no*	no
Immunologic affinity	1 to 2	1 to 2	1
Excretion	immune tissue	immune tissue	kidney

*Complement activated through the alternate pathway

The antivenin production

Most of snake antivenin was produced by polyclonal production method. The polyclonal production is antibody production by animal then purification and formulation. The first stage of polyclonal method is the animal immunisation. The animal immunisation is induced and promoted to immunoglobulin production in animals. The animals used in immunization of immunoglobulin are horse, sheep, goat. The snake venom was prepared to optimal dose for immunize animals for immunoglobulin production which may reduce toxicity effect but have immunizing effect and sustain immunized effect. The snake venom was injected to animal then repeated injection for immunization.After that the animal will produce immunoglobulins which had a specific action on snake venom. The second stage is concentration or purification of neutralizing fraction. In this process the plasma or serum of animal will be collected. The plasma or serum of animal which contained immunoglobulin or neutralizing fragments was purified and concentrated. The important process is the enzyme digestion/cleavage for F(ab')2 or F(ab) fragment production. The process involved purification of immunoglobulin as follows: precipitation with ammonium sulfate, caprylic acid, thermocoagulation including concentration or purification process example; for centrifugation, ultrafiltration or chromatography. The manufacturer will assign the type of fragments of antivenin and develop optimal process for purification. At the end of the process, fragments of antivenin are obtained which may be in form of concentrated bulk that is ready for preparation of final products. The final process is formulation and finised products production. The neutralizing fragments produced, (whole IgG, F(ab) or F(ab')2), was formulated and diluted to desired concentration. Neutralizing fragment was diluted and mixed with other excipients. The final products are dividen into 2 groups; liquid preparation in solution dosage form and solid preparation in lyophilized products. Generally, liquid dosage form has shorter shelf life compare to solid dosage form. Storage condition of liquid dosage form is in cold condition but solid dosage form is in ambient condition. Solid dosage form has adventage in storage, tranportation and shelf life, however, it has high cost of production and high production investigation.

Antibody

Antibody is substance is generated by human body to act against antigen or foreign particles that entered to the body. Immunoglobulin or antibody is glycoprotein material composed of about 82- 96 % of polypeptide and about 4-18 % of carbohydrate. Ig is classified in to 5 classes: IgG, IgA, IgM, IgE and IgD. Each class of immunoglobulin has a different number of basic unit molecule. IgG, IgD and IgE are monomers which compose one basic unit. While IgM is a pentamer (consist with 5 basic unit molecules of immunoglobulin) and IgA may be monomer or polymer.

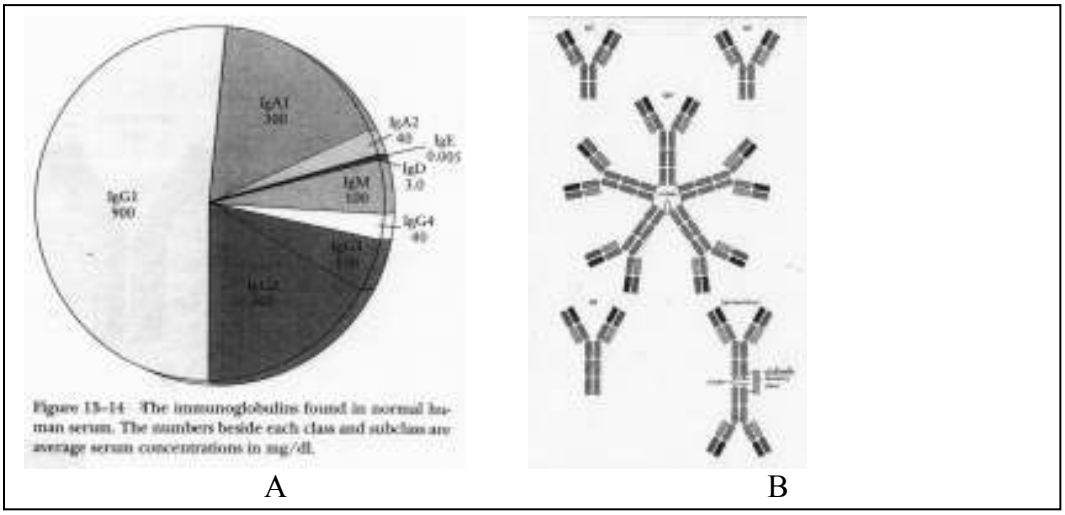


Figure 5 Amount of immunoglobulins in each class (A) and molecule of each Immunoglobulins class(B).(Tizard, 1999)

Table 2 The major immunoglobulin classes of humans. (Tizard, 1999)

	IgG	IgM	IgA	IgE	IgD
Molecular weight (kDa)	160	900	360	200	160
% Carbohydrate	3	12	7	12	12
Electrophoretic mobility	γ	β	β--γ	β -- γ	γ
Heavy chain	γ	μ	α	ε	δ
Heavy chain domains	4	5	4	5	4
Subclasses	γ1, γ2, γ3γ4	None	α1, α2	None	None
Half-life (days)	21	5	6	2	3

The immunoglobulin in each class has various biological properties and localization. IgG is the most important immunoglobulin and highest content in the body.

Table 3 Biological properties and localization of the 5 classes of Immunoglobuin. (Rodney, 2003)

Class	Heavy Chain	Light chain	MW (kDa)	Properties	Loacalization
IgG	γ	κ or λ	150	Toxin neutralizing, agglutinating, opsonizing, bacteriolytic(with aid of complement system): antigen-IgG antibody complexes may cause tissue injury)	Serum, amniotic fluid
IgM	μ	κ or λ	~950	Similar to IgG can also serve as antigen receptor on B-lymphocytes	Serum
IgA	α	κ or λ	160 340 (dimer)	Toxin-neutralizing agglutinating, opsonizing, secretory immunoglobulin	Serum, secretion, colostrum, siliva, tears, GI tract
IGD	δ	κ or λ	170	Antigen recepter on B-lymphocytes; Marker for master B cell	Serum, B cell surface
IgE	ε	κ or λ	190	Mediates change in vascular permeability due to allergy, hypersensitivity, or anaphylactic reaction	Serum

Structure of Immunoglobulin

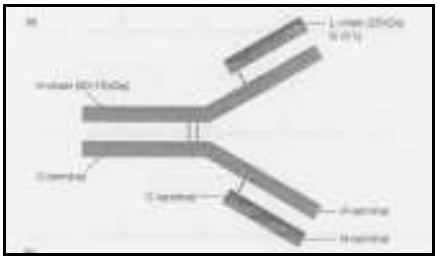


Figure 6 Basic subunit of Immunoglobulin molecule. (Wood, 2002)

Each type of Immunoglobulin molecule composes of main basic unit which consist of long chain polypeptide 2 chains (Heavy chain) and short chain polypeptide 2 chains (Light chain). The heavy chain (H- chain) and light chain (L-chain) linkage together with disulfide bonds.

The heavy chain consists of about 450 amino acids and has molecular weight about 50-70 KD. This chain is dividen 2 part

- Variable region (V). It is amino acid end terminal and has various amino acid

- Constant region (C) It is a carboxylic group end terminal which has constant amino acid. However, constant region is dividen into 3-4 domains; CH1, CH2, CH3 and CH4 domain. These domain are used to assign class of Immunoglobulin (IgG, IgA,IgM, IgE and IgD)

The light chain consists of about 214 amino acids and has molecular weight about 25 KD. Each light chain is divided into 2 parts: Variable region (VL) and Constant region (CL). Light chain has 2 types : kappa (κ) and lamda (λ), which the ratio of κ and λ are different according to type of animals.

The secondaty structure of Immunoglobulin.

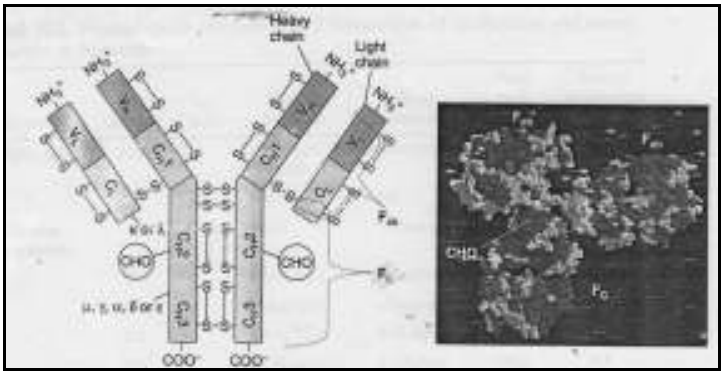
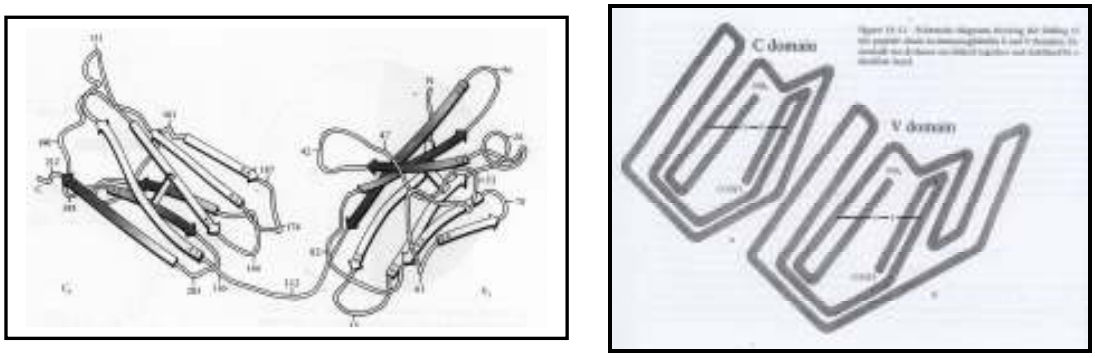


Figure 7 Structure of immunoglobulin and tri-dimension of molecule.

(Rodney, 2003)



A B

Figure 8 Secondary structure of β- sheet direction (A) and top-view of Immunoglobuline molecule (B). (Tizard, 1999)

The chains of immunoglobulin fold into loop which is call "domain". The domain is compact globulin structure. Each domain consists of amino acid about 100-110 amino acids. The fold of immunoglobulin chain shows secondary structure of protein. Inner domain consist β-barrel and β- plate sheet link together with disulfide bonds, but direction of β- plate sheet is opposite and has hydrophobic amino acid in center of domain.

The variable region both Light (VL) and Heavy chains (VH) have various difference of amino acid arragement. The difference of amino acid is assign to the specific of antibody and used for combination with antigen.

The hinge region

In heavy chain, CH1 and CH2 parts have a longer connection domain than other parts and is called "hinge region". These areas consist of about 60 amino acids. Because a lot of proline amino acid protect the fold of chain to globular structure and support to elastic of this part, including curliness of molecule look like Y-shape.

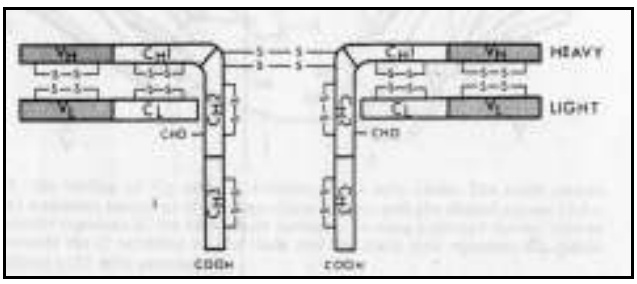


Figure 9 Hinge region of immunoglobulin.(Wood, 2002)

Lyophlization process

Lyophilization or Freeze dry is a drying process which product is dried at low temperature and under vacuum condition. This process was developed about world war 2nd and continously develop to now. This process composed of 3 steps follows: freezing, primary drying and secondary drying.

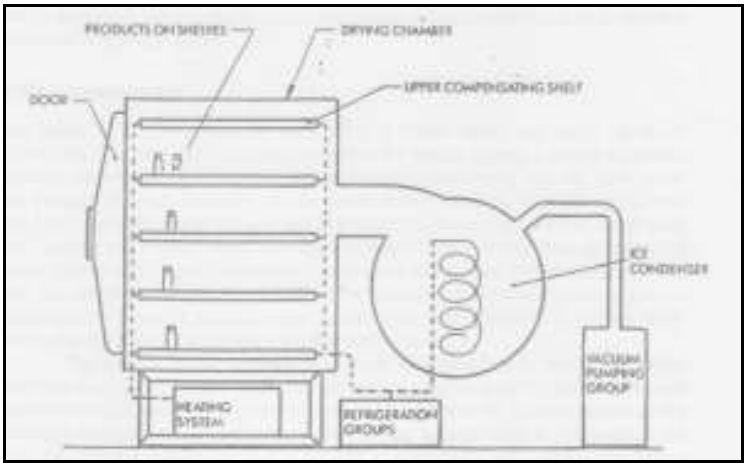


Figure 10 Lyophilizer or freeze dryer with basic unit machine

Lyophilizer or freeze dryer composes of chamber which has a controlled shelf for freezing and drying of products. Refrigeration system genearate cooled system and reduced temperature of the shelf. Heating system operate the heating up for primary and secondary drying steps. Vacuum pump system reduces pressure and maintains vacuum condition. These units are necessary for lyophilization principle and lyophilization process.

Lyophilization principle

Lyophilization process development has the objective to produce good lyophlilized products. Also it must consider formula of formulation together. Lyophilization process has 3 steps: freezing, primary drying and secondary dying. In process development there must be controling of parameters such as of temperature, pressure, duration time to conform to the formula. The Freezing step has an objective to freeze liquid form to solid state. Therefore freezing must be setting up to freeze the liquid form to become solid state completely. Temperature, freezing rate, duration time and step of freezing step must be considered since. Freezing step is

very important for lyophilization process. It is the first step and the result of which has impact on primary and secondary dried as well as quality of lyophilized products.

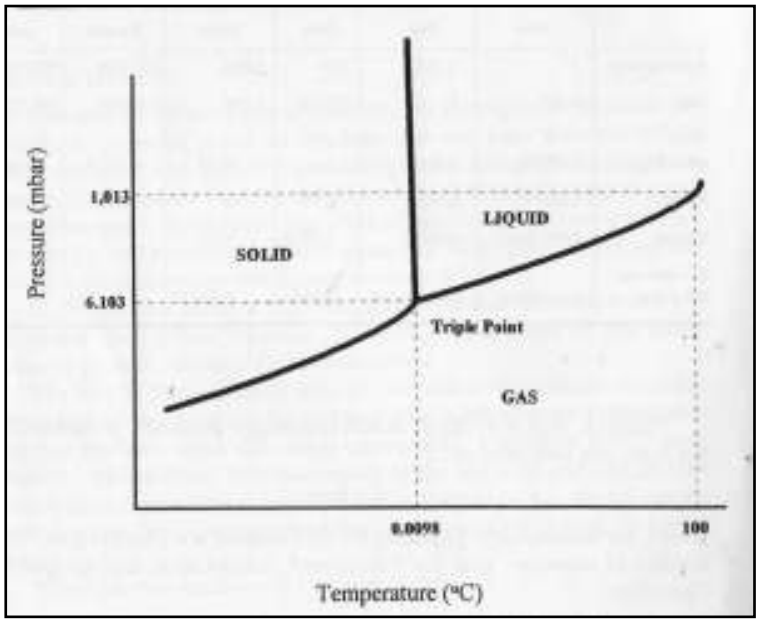


Figure 11 The triple point of water (Cameron, 1996)

From figure 11 show the triple point of water that is generally solvent of solution in pharmaceuticals. Water is liquid phase in normal pressure and temperature. When water is heated or boiled, it will change from liquid to gaseous phase. When water is frozen, until temperature of water is about 0°C, it changes to solid phase (ice). Those events are in normal pressure. The figure shows the black line which displayed boundary of phase changing. The important principle is the area which is between solid phase and gas phase where temperature is under 0.0098 °C and pressure under backline below 6.103 mbar. This zone the solid phase transforms to gaseous phase with condition at low temperature and low pressure (vacuum condition) or it is called "sublimation". Sublimantion process is the main process of lyophilization or freeze drying. The triple point is mean point that water can change to solid, liquid or gaseous phase.

Lyophilization or freeze drying is drying process which use sublimated ice crystal to vapor gas and do not changed to water or liquid phase. Sublimation of ice to water vapor produced to remain of structure of solute and created pores or hold of ice crystals. Primary drying will sublimate ice crystal to water vapor which used a longest time of all step. Secondary drying will drying by heating up which cake of products will heat and water which is unfrozen water will

remove out of products. Finally, the products produced from freeze dryer or lyophilizer is solid dosage form, is call “freeze dried products” or “lyophilized products”

The adventage of lyophilization process (Cameron, 1996)

1. Maintain the activity of active ingredient

The freezing / low temperature is used in order to protect and to preserve the active ingredient which is heat labile material. This is because, when freezing, the molecule of all formula is restricted, immobilized and prevented chemical action between materals. At low temperature there is low speed of degradation reaction. In drying process, under the vacuum conditioned, the air including oxygen gas is removed out of the system; therefore, the oxidation reaction is decreased.

2. Remain the form of active ingredient

Lyophilization process composes of freezing step. When the freezing step is completed, the liquid form is transformed to solid phase. In solid phase, all molecules are restricted and fixed, therefore, freezing step prevent the migration of molecules which may transform or change the form of active ingredient, expecially protein group.

3. Ease to reconstitute / soluble

The lyophilization products have many pores in the texture. The porous in texture of products are created from ice crystal sublimation. When the products have pores, they are easily soluble or reconstituted. This is because pores increase surface area which will contact with water.

4. Long shelf life

Lyophilized products are solid dosage forms which have low moisture and low chemical reaction. Therefore, the shelf life of lyophilized products is longer than liquid products.

5. Ease for storage and transportation

Solid dosage forms of lyophlilized products are more stable than liquid dosage form which can be storage at high temperature and maintain at high temperature conditioned.

Whilestorage and transportation of liquid products must be in cold conditioned where lyophilized products are not necessary.

6. Accuracy dose

Since lyophilized products are filled into vial before lyophilizattion. The liquid filling can controlled easily; in term of volume and dosing of filling. Therefore, lyophilized products have accurate dose.

The disadvantage of lyophilization process

1. High capital cost of equipments
2. High energy costs
3. Lengthy process

The main process of lyophilization

1. Freezing step. Freezing is the first step of lyophilization process. The freezing step is a critical and important step as it will have impact on primary drying, secondary drying and final products are affected also. The products are freezed from this step and change to solid state.In this step solution or liquid form is frozen and change to solid phase. The control of this step must be take on freezing rate, freezing temperature, duration of freezing and pattern of step of freezing. When temperature of the shelf is decreased, the product (liquid state) becomes cooler. Until temperature of product reaches "nucleation temperature" that is when water is crystallized to first ice crystal. The ice crystal is created and becomes bigger. When water changes to ice, the concentration of solute also changes. The concentration of solute will increase. This solutes include active ingredient and excipients. The temperature will decrease until it reachs phase transition for amorphous material (glass transition temperature); from rubby state to glassy state. However, this condition is not normal; it is freeze concentrate and high concentration. This temperature is called "glass transition temperature of freeze concentrate or Tg' ". For crystalline materials, when temperature of system is decreased to eutectic temperature, solute will become crystallized to crystal form. The freezing step has impact on the excipients both crystalline materials and amorphous materials. When water changes to ice, the concentration of solute is

increased, and also pH changes, protein or active ingredients concentration change, crystallization or phase transition.

- Increasing of concentration of proteins makes molecules of solute closer which may increase chemical raction.

- Changes of pH, espectially some kinds of buffer, may loss buffer activity after passing freezing step. This may result in quality of the active ingredients. Freezing step also has impact on crystallization of ingredients.

- Most of water in solution is changed to ice crystal. However there will be some water which does not change to solid phase. It wills absorp on the surface of the glassy materials or crystalline materials. This water which does not frozen, is called “unfrozen water”

Freezing or cooling rate is very important and can effect on frozen product. It was found that the high freezing rate produces to smaller ice crystal and may slow down primary drying step. The degree of supercooling means the difference of temperature between equilibrium freezing point and the nucleation temperature. The high degree of supercooling shows high freezing rate in freezing step and created smaller ice crystals.

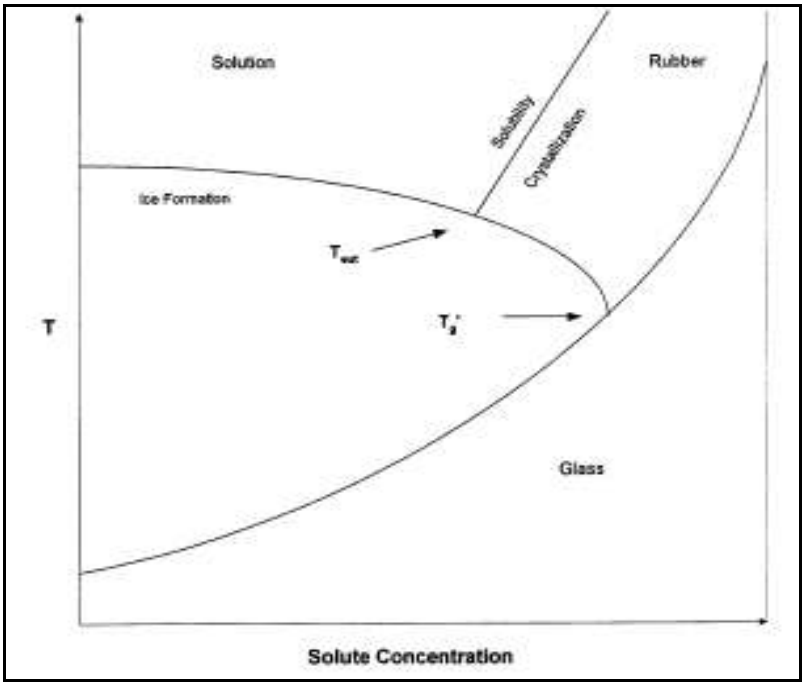


Figure 12 The phase diagram of binary solute: ice formation, crystallization, eutectic point and glass transition during freezing (Wang, 2000)

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2. Primary drying. In freezing step, the solution is frozen and turns to solid phase which are ice crystal and glassy phase or crystal material. Primary drying step takes longest duration time. In this step temperature of the shelf is increased and pressure of chamber is reduced to vacuum condition. The important point is sublimatation which changes ice crystal from solid phase to water vapor without passing liquid phase. The temperature of drying must below than collapse temperature of products. The collapse temperature is the temperature that the solid phase will melt or not stable and loss its structure, the final products that do not pass this criteria will be undesirable products. The collapse temperature of products is related to eutectic temperature or glass transition temperature of products. For crystalline materials, the collapse temperature is nearby eutectic temperature of products. Other amorphous materials, the collapse temperature is nearby glass transition temperature of products. However, several studies show the collapse temperatuer is higher than eutectic temperature or glass transition temperature of about 2°C. During primary drying it is needed to maintain and keep product temperature below collapse temperature. The water vapor in gaseous phase will increase and pressures of system will also increase. Therefore, vacuum condition is generated by vapor circulation to remove water vapor out of system. Several studies suggess about vacuum condition that pressure is typically set to keep between ¼ - ½ fold of saturated vapor pressure at product drying temperature. (Colandene et al 2007) When shelf temperature is increased, the primary drying starts and the ice crystal will be sublimated to vapor gaseous. The vapor gas transfer to upper side of bottom or drying layer in upper zone. When primary drying is proceeding, the drying layer on upper of the cake is increaseing level and generated ice-vapor boundary zone. During primary drying ice crystal generates pores which hold of ice crytal that sublimeted out products. The big ice crystal sizes produce big pores of which vapor gas pass easily.But smaller ice crystal generates smaller pore which is high drying resistance. Final of primary drying stage may remain the moisture content about 5-7 %. The end of primary drying may decise when temperature of product is equal to shelf temperature.

When primary drying proceed, it will separate generate product 2 parts; upper zone (dry product) and the lower zone (frozen product). Any positions of vials have varous temperature.

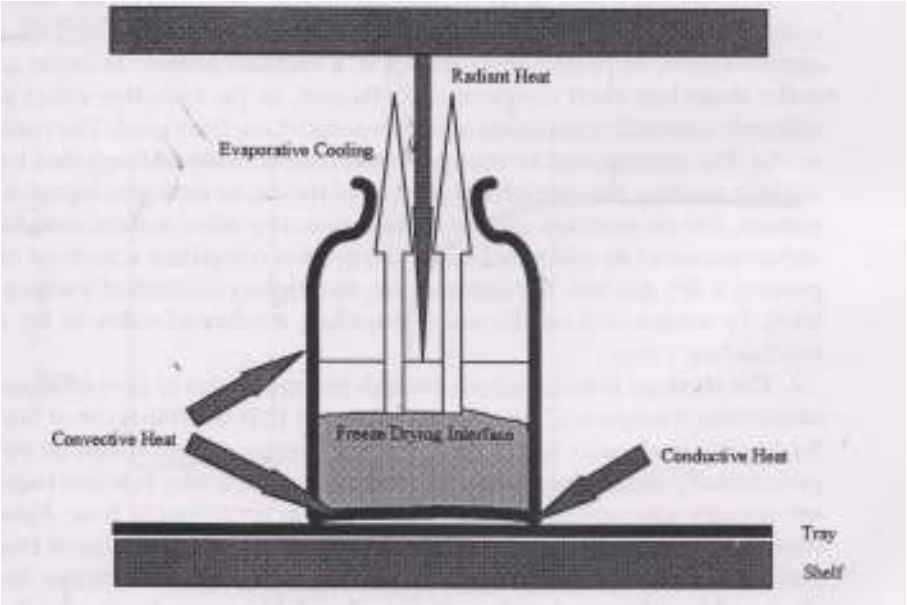


Figure 13 Heating processs and heating force during drying (Cameron, 1996)

The heating force from primary drying are convective heat at bottom of vial, conductive heat at glass or container sides and radiant heat from upper of vial. The main heat is convective heat.

3. Secondary drying. Secondary drying is the process of removing unfrozen water from the products. This step is the final step. The main principle is to increase temperature in order to remove unfrozen or bound water out of products. The glass transition temperatute of products is important for this step because final temperature should be setting up below glass transition temperature to prevent product melt of phase transition. Using high temperature of secondary drying will shorter duration time than using low temperature. The vacuum pressure for secondary drying may set up to 30 – 300 mTorr. (Abdelwahed, 2006)

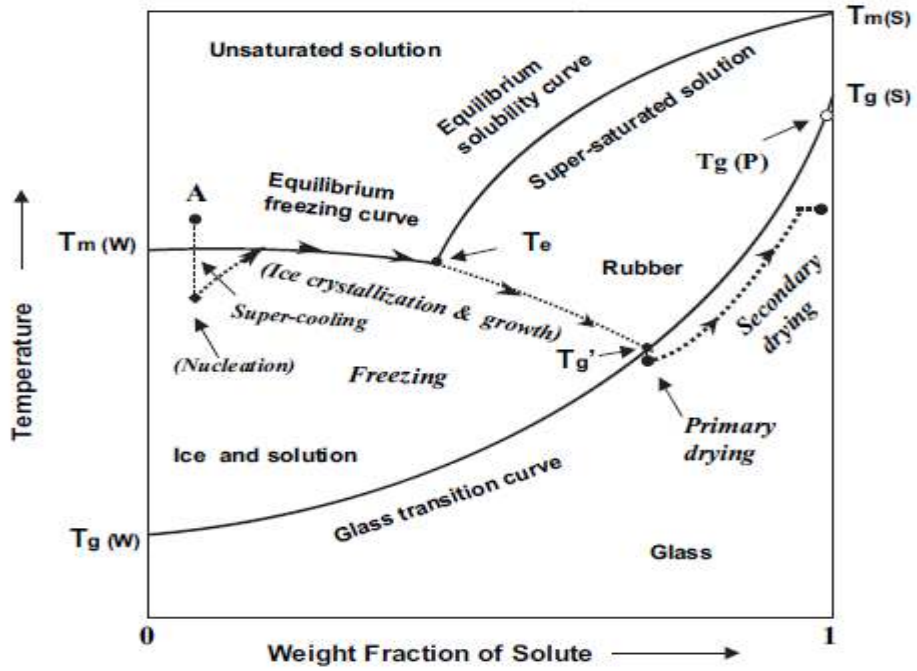


Figure 14 Lyophilization process, phase, condition and parameter involvement

(Liu, 2006)

The lyophilization process begin with freezing step which generated ice crystal nucleation and super-cooling, including ice crystallization and crystal growth. Solute may occur phase transition (rubber to glass phase) or may be crystallized to crystal form. Primary drying uses the lower temperature than Tg' or Te to remain rigid structure and prevent collapse of cake. And then secondary drying increase temperature which lower Tg of products.

<u>The products of lyophlization</u>

The lyophilization process is use in the pharmaceutical, biological, fine chemical and biotechnology fields; i.e.

-Protein
-Peptide
-Complex synthetic organic molecules
-Antibiotics
-Antitoxins
-Bacteria
-Blood coagulants
- Pathological samples
- Plasma
- Reagents
- Standards
- Tissue
- Vaccine
- Viruses

-Enzymes
-Fine chemicals
-Growth factors
-Hormones
-Media
- Vitamins
- Yeast
- Food and fruits

The summarized control of freeze drying process and formula (Franks, 1998) are as follows:

1. Products : Composition/ formulation/ concentration /solid content/ fill volume
2. Containers : type (vial, ampoule, syringe), geometry, stoppers
3. Equipments : freeze drier model, loading (shelf, tray), probes
4. Process : shelf temperature, chamber pressure, time (cooling, annealing, Primary /secondary drying cycles)

Factors which have impact on lyophilized products

1. Freezing step. Process development of this step should adjust this parameters : Freezing rate, freezing temperature, pattern of freezing, anneal and duration time
2. Primary drying should adjust parameters: duration time, drying temperature, pressure or vacuum pressure which determined glass transition temperature or eutectic point and means to collapse temperature.
3. Secondary drying determines glass transition temperature of products.

Formulation of lyophilzed products

Lyophilized products composed of active ingredients and other excipients that is necessary for stability of physical, chemical and biological properties. In formulation, it should be determined from the first active ingredient especially content of active ingredient. If the active ingredient has low content, the lyophilized products will not form to cake. For low active ingredient content, the bulking agent should be use for increasing content. Other additives should be determined as well as property of active ingredients. (Sadikoglu et al 2006; Abdelwahed, 2006)

Bulking agents

Bulking agents help increase the content or bulk of product and is used when concentration of active ingredient is very low. Bulking agents provide the body/ structural stregth to the cake, enhanced physical stability and attractive appearance. The bulking agents are crystalline materials or amorphous materials. Example: mannitol, glycine, sucrose, hydroxyethyl starch, trehalose, lactose.

Stabilizers

Stabilizers such as cryoprotectant which can protect proteins from freezing stresses and lyoprotectant which can stabilize protein in the freeze dried state. The cryoprotectants are sugar group: trehalose, sucrose, glucose, mannitol. Those cryoprotectants form highly viscous amorphous sugar glasses during freezing step to prevent aggregation of protein, undesirable reaction and protect stresses of ice crystals. Lyoprotectant involves water replacement by forming of hydrogen bonds between a lyoprotectant and polar group of active ingredient. When drying starts, the water is removed while active ingredient is stable and restricts structure from bonding between lyoprotectants.

Example of stabilizers: sucrose, lactose, glucose, trahalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, dextran.

Tonicity agents

Tonicity agents make to isotonic solution and control osmotic pressure of formula. Tonicity agents are salts (sodium chloride, etc), mannitol, glycine, sucrose and glycerol

Buffers

Buffers adjust and control pH of formula during freeze dry process and upon reconstitution.Examples of buffer: Tris HCl, citrate, histidine and phosphate. Some buffers like sodium phosphate can crystallize out of the formula during freezing so it rather shift the pH. Other, acetate and imidazole buffer should be avoid for freeze drying because the pH of formula is shift during freeze drying and after reconstitution.

Collapse inhibitors

Collapse inhibitors help increasing the collapse temperature of formula which can dry at higher temperature. Collapse inhibitors are glucose, dextran, maltose, maltoriose, PEG, etc.

Structure modifiers

Structure modifiers enhance product elegance and also are used to prevent blowout of cake structure. These additive are mannitol, glycine, sucrose,...etc.

Preservatives

Preservative is used for multi-dose formulation and provides to protection against microbial growth in formula. Example: benzyl alcohol, phenol and m-cresol.

Freeze drying or lyophilization process was studied about condition of process important factors, crystallization of products, annealing, formulations and excipients which may divide 2 main groups follow as process and formulation. For lyophilization process, the freezing step was often studied. Freezing step is the first step and critical step. Liu et al (2006) and Hottot et al (2007) summerized that freezing step is the key step and was impact on the other steps and also on final products. The important factor of freezing step was studied as follows: freezing rate, annealing, glass transitions temperature, crystallization. Freezing changed liquid formulas to solid state. The decrease of temperature with various cooling rate given the differ of ice crystals size distribution, crystal of solute, morphology of freezing solid and stability of active ingredients. Freezing impacted on active ingredient from 3 factor follow as increasing of concentration of active ingredient/ protein, increasing of protein-protein intermolecular reaction and changes of pH. The wording which involed with freezing step: Nucleation temperature that the first ice crystal is cystallized, Annealing which held freezing step at temperature higher than Tg' of formula but below freezing point of formula which promoted crytals growth, crystallinzation increasing. Annealing process decreased in vial-to-vial heterogeinity of product and ice crystal size distribution (Schwegman, J.J., Hardwick, L.M., and Asker, M.J., 2005)

Tang and Pikal (2004) found that the slow freezing rate will result in more protein damaged than fast freezing rate because the large ice crystals may easily damaged the protein and the longer contact time with slow freezing rate.

Primary drying step must use drying temperature below collapse temperature(Tc) which invloved with glass transition temperature(Tg) for amorphous material or eutectic temperature(Teu) for crystalline material. Tang and Pikal (2004) and Sadikoglu, H., Ozdemir, M., and Seker, M. (2006) report that temperature safety margin (The temperatute difference between product temperature and Tc) of drying is 2 $^{\circ}$C for longer freeze drying (about 2 days)

Passot et al.(2005) studied physical characterization of formulation for two stable freeze dried protein(Clostidium difficle toxins) and found that the optimal cystalline formulation composed of 4%mannitol and 1%sucrose and amorphous formula composed of 4% maltodextrin and 0.02% Tween80 or 5% BSA could remained the activity of active ingredients longer storage time.

Garzon-Rodriguez et al.(2003) reported that using mixture of disaccharide and polymeric carbohydrate preserved stability of lyophilized product. The combine additives were synergistically effect which disachharide inhibited protein unfolding and hydroxyehtylstarch (HES) were increased the Tg of formula.

Kodoya et al.(2010) studied freeze drying of protein and found that some oligosaccharide-derived sugar alcohol (eg. Maltitol, lactitol) formed amorphous cake which protected secondary structure perturbation for BSA and remained stability of LDH activity.

Allison et al.(2000) found that the formulation s of Actin lyophilized products composed of combination disaccharride and dextrin were optimal formula which preserved activity of actin for stability studied time.

Matejtschuk et al.(2009) found that the optimal formulation for lyophilised tetanus toxoid composed of 5mg/ml of trehalose with 0.1 M of sodium chloride.

Ronzi et al.(2003) studied lyophilization of factor VIII and factor IX concentrated found that the optimal freeze drying cycle composed freezing step about 1 hr at cooling rate -3°C/ml; primary drying step at -35 $^{\circ}$C for 9 hrs and secondary drying about 10 hrs.

Corveleyn and Remon (1996) found that maltodextrin protected LDH activity during freeze drying process that depend on the dextrose equivalent (D.E.) and concentration of

maltodextrin. The lyophilzed products were amorphous cake and may combine with PEG8000 for improved preserve activity.

Liao et al (2002) studied lyophilization of lysozyme and catalase found that the small molecular excipients (eg. Glycerol, sorbitol, sucrose, trehalose) stabilized the activity and / or native structure of catalase and lysozyme lyophilized products.The combination of sucrose with dextran was synergistically preserved native structure.

Her, Deras, and Nail (1995); Hawe and Freiss (2006); and Franks (1998) reported sodium chloride or electrolyte in formula decrease glass transition temperature of formula and suggested that salts addition is to be avoided.

Circular dichroism

Circular dichroism is method for studying the conformation of peptide and protein. This method is very sensitive to protein conformation and is a convenient for monitoring structural changes in proteins. CD instrument (known as spectropolarimeter) measure the differential absorption of the left- and right- handed circularly polarised light by the chiral molecule,ΔA = A_L-A_R. For chiral molecule, A_L does not equal A_R sothe resulting ΔA will be non-zero and canhave either a positive or negative sign. The CD signals depend on the relative intensities of the left- and right-handed absorbances.

Circular dichroism has a great sensitivity to change in conformation. So it is an excellent technique for studying the effect of changes in environment conditions such as pH, temperature or ionic strength; for determination protine stability using chemical or thermal denaturation.

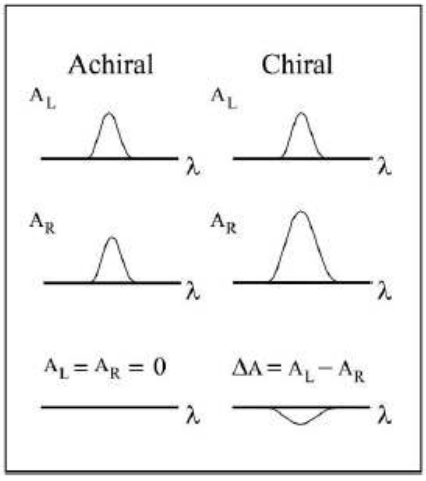


Figure 15 The differing effect from achiral and chiral molecule on the absorption of left- and right-handed cirulcularly polarised light. (Janes and Wallace, 2009)

Wavelenght of light used for CD measurement

1. The near-UV region : from ~360 nm to ~260 nm for the aromatic side chains of proteins
2. The far-UV region : from ~ 260 nm to ~190 nm for the protein polypeptide backbones
3. the vacuum UV region : from ~190 nm to ~120 nm

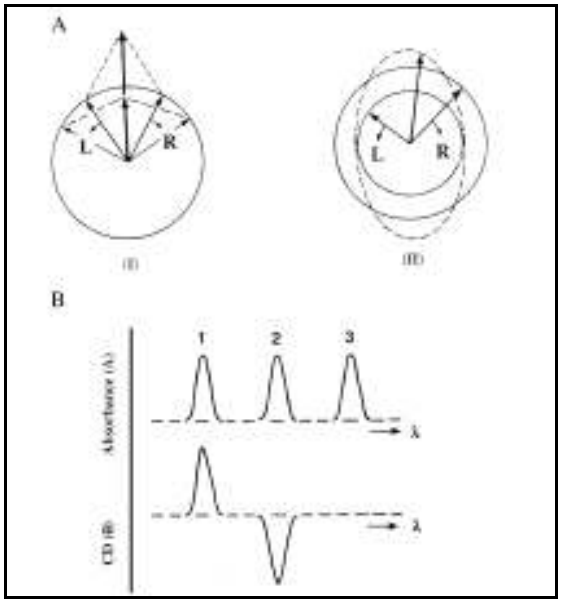


Figure 16 A: the left(L) and right(R) circularly polarised components of plane polarised radiation (I) the two components have the same amplitude and when combined generate plane polarised radiation. (II) the components have different amplitude ; B : the relation between absorption and CD spectras. Band 1 has a positive CD spectrum which L absorption more than R, band 2 has a negative CD spectrum which R absorption more than L, band 3 is achiral chromophore.

(Kelly and Price, 1997)

<u>The unit of measurements</u>

1. Ellipticity (ε) is basic unit from commercial CD instrument and show in millidegrees. The ellipticity units are not the most useful units for comparisons of different samples which their magnitudes differ depend on the amount of protein present in sample. (example : the concentration, the pathlength of cell)

2. Molar cirular dichroism (delta epsilon)

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = (A_L - A_R) /(dl)$$

Where ε_L and ε_R are defined as the left and right extinction coefficients, respectively. l is the pathlength for the sample in centimetres, and d the molar concentration of the sample. For macromolecules the extinction coefficients are usually, although not away, defined per amino acid residue. The delta epsilon referred to as molar dichroism.

3. Mean residue ellipticity

The other major parameters use to reportCD data are mean residue and molar ellipticities, whichca be link to delta epsilon . Mean residue ellipticity, $[\theta]_{MRE}$ or MRE is defined as :

$$[\theta]_{MRE} = 3298\Delta\varepsilon$$

MRE has the dimensions degrees cm^2 $dmol^{-1}$ $residue^{-1}$. Molar ellipticity, $[\theta]$, has dimensions cm^2 $dmol^{-1}$, and calculated as above except using extinction coefficients derived from the whole protein rather than per residue. The adventage of reporing with the MRE value instead of the molar ellipicity is that this then means the magnitude of the spectrum will be independent of the size (molecular weight) of the protein, so comparisons between large and small protein are easier.

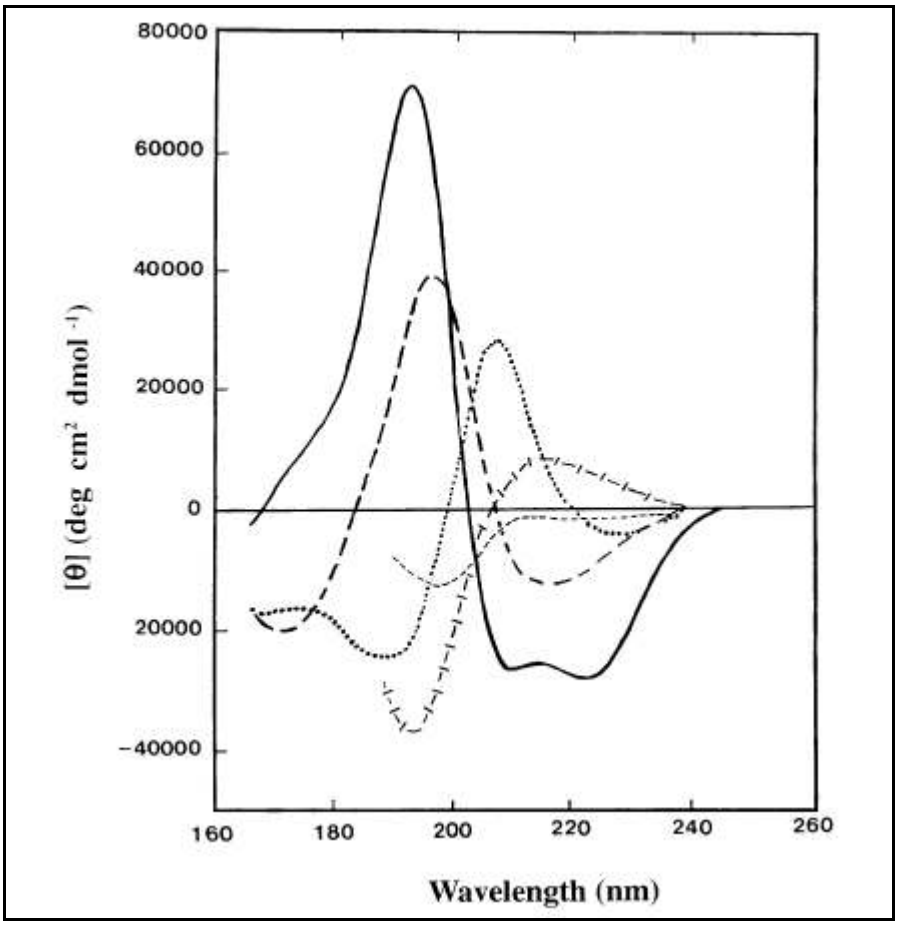


Figure 17 The type of secondary structure of protein from Far-UV spectra :
α-helix(solid line),anti-parallel β-sheet(long dasheh line), β-turn(dotted line) and poly(Pro)II helix(cross dashed line) and irregular structure(short dashed line). (Janes and Wallace, 2009)

Utilization and application of CD data for protein studies.

1. Secondary structure composition. (% helix, sheet, turns, etc.)

The secondary structure of protein is analyse by spectropolarimetry at far-UV region (190 -250 nm). Polypeptide chains form diverse types of secondary structures that are themselves chiral. The α-helix structures produce two negative CD peaks at ~222 and ~208 nm and a positive at ~190 nm. The β-sheet structures produce the transition at ~215 nm for the n to π^* transition and ~195 nm for the π to π^* transition.

2. Tetiary structure from the near-UV CD of protein (aromatic amino acid dichroism)

The near-UV CD of proteins arises from the environments of each aromatic amino acid chain. Each of the aromatic amino acid tend to have a characteristic wave length profile : typtophan, peak close to 290 nm with the fin structure between 290 and 305 nm; tyrosine , a peak between 275 and 282 nm; phenylalanine, sharp fine structure between 255 and 270 nm. The factors influence on internsities of aromatic amino acid CD band follow as: the rigidity of the protein which had more highly mobilities side chains will be lower intensities; interactions between aromatic amino acid; and the number of aromatic amino acid

3. Integrity of cofactor binding sites

Generaly organic cofactors such as haem, flavin, etc. show little if any CD signals when in free solution. But when bound to protein partner in sites which confer chiarality, the CD signals are thus excellent indicatord of the integrity of the cofactor-binding site.

4. Conclusion about the overall structure features of proteins.

-CD measure ments in the far UV can give quantitative estimates of secondary structure. The CD spectra in different spectral region are invaluable for assessing the structural relationship between native and recombinant protein, and between wild-type and mutant proteins. The loss of CD signal may effect on addition of denaturation agents or by increasing of temperature.

- Study about protein stability, denaturation, protein unfolding
- Changes of conformation on protein

The previous studies found that secondary structure of immunoglobulins G (IgG) was determined and analyzed in several reports. The secondary structural conformation of IgG was analyzed by CD or spectopolarimeter. Szenczi et al.(2006) found that the conformational stability of human polyclonal IgG in solution decrease with decreasing pH and the optimal pH range for storage was 5.0 -6.0. The CD spectra of IgG are in a negative band at 217 nm and representing a high content of β-sheet. This result is same result of Schüle et al. (2007) which found that the CD spectra of IgG1/mannitol solutions have aminimum at 217 nm and consist mainly β-sheet elements.

Dutta, U., Cohenford, M.A., and Dain, J.A. (2006); Vermeer, A.W.P., Bremer, M.G. and Norde, W. (1998) studied IgG conformational structure found that the CD spectra of IgG show a primarilly β-sheet secondary structure, minima at 217 nm and a zero intensities at about 206 nm. Another results, Vermeer and Norde (2000) found that the intact IgG are that of typical immunoglobulin, representing a highly content of β-sheet with a negative band at 217 nm. While the ellipticity of CD spectra in far-UV region is slightly affected by temperature change between 20 and 55 °C which the IgG secondary structure is stable within this temperature.

However Hawe et al. (2009) found the different of minimal band of IgG. The result of them found that IgG was characterized by negative maxima at 218 nm, a zero intensities at 210 nm and positive at 202 nm. These spectral characteristics are high β-sheet content.

The studying about antivenin or F(ab')2 fragments was studied by Rodrigues-silva et al (1997) and Kanavage ett al(2006). Kanavage studied antivenom of commercial prodcucts which found that the far UV CD spectra of antivenoms were mainly β-sheet structure, exhibited minima around 218 nm and maxima in the 190-204 nm range. While Rodrigues-silva (1997) found that the CD spectra of F(ab')2 fragments and whole IgG showed a principle band at approximately 216 nm and mainly β-structure.

CHAPTER III

MATERIALS AND METHODS

1. Materials

The following materials were used

1.1 Active ingredients and diluents

- Concentrated bulk F(ab')2 of Green Pit Viper antivenin lot no. 09002TA and 10002TA were supplied by Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand
- Glycine (Lot No. AF507086, Ajax Finechem, Australia)
- Mannitol (Lot No. AF509221, Ajax Finechem, Australia)
- Maltodextrin (Dextrose equivalent 13.0-17.0) (Batch No. 33596PJ, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- Sorbitol (Lot No. M430197, Srichand United Dispensary Co.,Ltd, Thailand)
- D-Glucose anhydrous (Lot No.F2H113, Asia Pacific Specialty Chemicals, Australia)
- Fructose (Lot No. 324173/1 21297, Fluka Chemie AG, Switzerland)

1.2 Materials for testing

- Hydranal Composite-5 (Lot no.SZE9138D, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- Methanol (HPLC grade) (Lot no.J9AG1H
- Selenium reagent mixture (Lot No. F1578130 915, Merck GaA, Dammstadt, Germany)
- Sulfuric acid 95-97% (Lot No. K40132831 919, Merck GaA, Dammstadt, Germany)
- Glycine (Lot No. K32086501 435, Merck GaA, Dammstadt, Germany)
- Boric acid (Lot No. K30864165 243, Merck GaA, Dammstadt, Germany)

- Sodium Hydroxide (Lot No.B0485398028, Merck GaA, Dammstadt, Germany)
- Green Pit Viper venom (Lot no. TV144-s)
- Gonotec GmbH; Calibration solution for Osmomat 010/030 300 mOsm/kg NaCl/H2O (Lot no. 2372C47, Berlin, Germany)

2. Equipments

- Lyophilizer (Model Lyolab ST4B, Lyolab, USA)
- Spectropolarimeter (Model J-715, 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)
- Balance (Model A200S, Sartorius analytic, Germany)
- Balance (Model XP205, Mettler Toledo, Switzerland)
- Osmometer (Model Osmomat O30-DM, Gonotec, West Germany)
- pH meter (Model 210A, Thermo Orion, USA)
- Karl Fisher titrator (Model DL38, Mettler Toledo, Schwerzenbach, Switzerland)
- Balance (Model BP 210D, Sartorius, Goettingen, Germany)
- Kjeldahl apparatus as follows :
    - Digestion unit (Model K-435, Buchi, Flawil, Switzerland)
    - Distillation unit (Model B-324, Buchi, Flawil, Switzerland)
    - Titrator (Model DL50 Rondolino, Mettler Toledo, Switzerland)
    - Extraction and Nutralization unit (Model Scrubber B-414, Buchi, Flawil, Switzerland)
    - Hood (Model NewLab Fume cupboard, Official Equipment, Manufacturing, Bangkok, Thailand)

-Balance (Model AG204, Mettler Toledo, Switzerland)

-Oven cabinet (Model BM600, Memmert, Germany)

-Oven cabinet (Model UM400, Memmert, Germany)

-Microbiological safety cabinet Class II (Model Safe/Maxi Safe2010, Holten)

-SDS-PAGE as follow

- Dual gel caster (Model SE250, Hoefer scientific instruments, califonia, USA)
- Electrophoresis power supply (Model LKB EPS500/400, Pharmacia® , Califonia, USA)
- Microsyringe (25 mcl, Hamilton, Bonaduz, Switzerland)
- Platform Shaker (Model KS125 B, IKA labortechnik, Staufen, Germany)

- Pipette 10-100 μl (Socorex, Switzerland)
- Pipette 100-1000 μl (Socorex, Switzerland)
- Pipette (Model SL-5000, Rainin Instrument, USA)

3. Methods

3.1 Preformulation

Preformulation studies were done on the Green Pit Viper antivenin commercial product (Queen Saovabha Memorial Institute, QSMI) and the lyophilized F(ab')2 Green Pit Viper antivenin concentrated bulks were produced by concentration adjustment with purified water and lyophilized.

3.1.1 Solid State Characterization

Green Pit Viper antivenin commercial product and the lyophilized F(ab')2 Green Pit Viper antivenin concentrated bulk were characterized by various solid state analytical techniques.

3.1.1.1 X-Ray Powder Diffractometry

Crystallinity of the samples were determined by X-ray powder diffractometer (Model Miniflex II, Rigaku, Japan). Measurements were carried out from 5 to 60°2θ, at an angle scan speed of 2.0 °/min by CuKα source with wavelength of 1.541841 A.

3.1.1.2 Thermal analysis

a. Differential Scanning Calorimetry (DSC)

Thermal analyses of samples were determined by using differential scanning calorimeter (Model DSC822e, Zurich, Switzerland). Samples of 2-5 mg were hermetically sealed in aluminum crucible. An empty aluminium crucible and lid were used as reference. The sample were heated from 25 to 280°C at heating rate of 20 °C/min. DSC thermograms were determined and compared with QSMI product.

b. Thermogravimetry (TGA)

Approximately 3 mg of lyophilized product was heated in an alumina crucible from 25 to 250 °C, at 10 °C/min under nitrogen purge using thermogravimetric analyzer (Model TGA/SDTA851e, Switzerland). TGA thermograms were analyzed to determine the moisture contents from percent weight loss in the sample.

3.1.2 Conformation Evaluation by Circular dichroism

Secondary conformational structure of F(ab')2 was determined by circular dichroism using spectropolarimeter (Model J-715, Jasco, Kyoto, Japan). The lyophilized samples were reconstitued with purified water and diluted to 1:100. Potency adjustment was done on the concentrated bulk to 0.7 mg/ml with purified water (the sample at this stage is called "native F(ab')2") and diluted to 1:100. Samples were analyzed from wavelength 190 to 250 nm with response time 2 sec, sensitivity 20 mdeg, bandwidth 2.0 nm and scan accumulation of 3 scans. The CD values are determined at 217 nm on CD spectral pattern. The circular dichroism data will be uesd as reference to evaluate the future studies.

3.2 Preliminary Lyophilization Process Development

3.2.1 Freezing point determination of F(ab')2 Green Pit Viper antivenin by DSC

Freezing point of sample solutions [F(ab')2 solution and mixture solutions of F(ab')2 and 2% various stabilizers were determined by differential scanning calorimetry (Model DSC822e, Zurich, Switzerland)]. Approximately 20 μl of sample solution was hermetically sealed in an aluminium pan. An empty sealed aluminium pan was used as reference. Samples were cooled from 25 to -40 °C at a cooling rate of 2 °C /min and hold at -40°C for 5 min. Then samples were heated from -40°C to 25°C at heating rate of 5°C /min. The DSC thermograms during analyzing were recorded and determined freezing point of each mixtures.

3.2.2 The effect of freezing patterns on secondary structure of F(ab')2 Green Pit Viper antivenin.

Samples of F(ab')2 Green Pit Viper antivenin solution (0.7 mg/ml) and solution mixtures of F(ab')2 Green Pit Viper antivenin and stabilizers 2% w/v (glycine, mannitol, maltodextrin, sorbitol, glucose and fructose) were prepared. Samples of 4 ml were placed in vial then were frozen using various freezing patterns (Figure 19) by Lyophilizer (Model Lyolab ST4B, Lyolab,

USA). Post frozen samples were defrost to room temperature and analyzed for their secondary structure using spectropolarimeter (Model J-715, Jasco, Kyoto, Japan) according to the method in 3.1.2

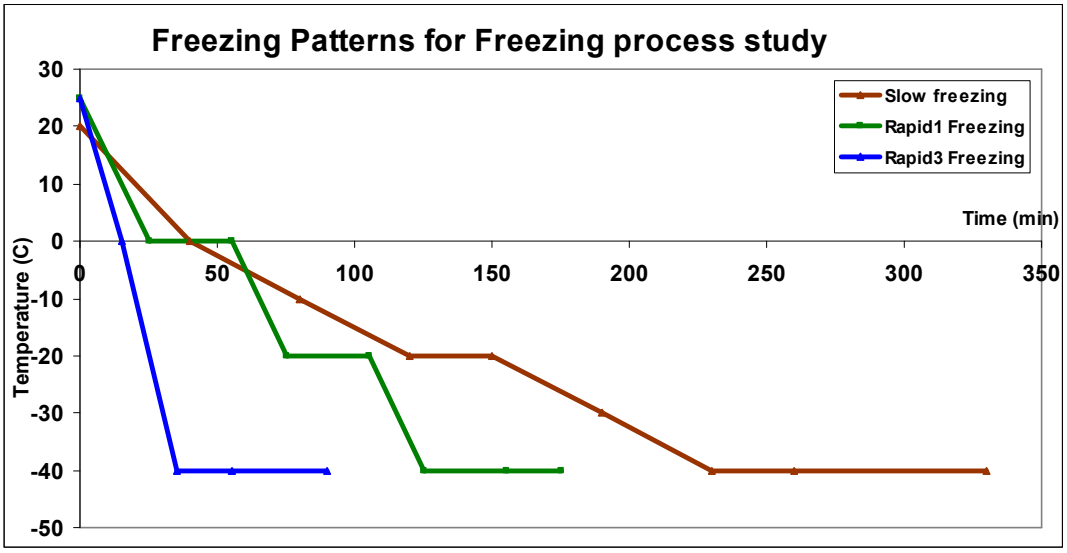
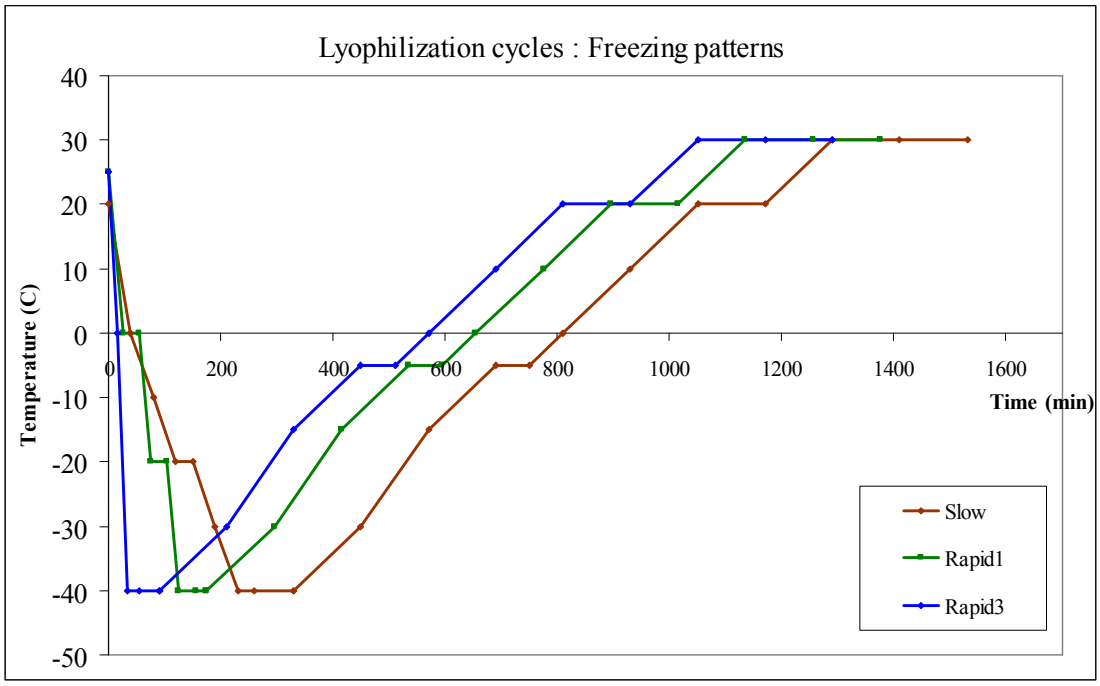


Figure 18 Freezing patterns (Slow, Rapid1 and Rapid3) for freezing process study on secondary structure of F(ab')2 Green Pit Viper antivenin

3.3 Lyophilization process development.

3.3.1 Freezing Process Evaluation

Samples of F(ab')2 Green Pit Viper antivenin (0.7 mg/ml) solution, solution mixtures of F(ab')2 and 2 % stabilizer (glycine, mannitol, maltodextrin, glucose, sorbitol, fructose) of 4 ml were placed into 6 ml glass vial and were lyophilized by varying the freezing patterns.The primary and secondary drying cycles were kept constant as shown in Figure 20 by lyophilizer (Model Lyolab ST4B, Lyolab, USA).



* Pressures of primary and secondary drying were controlled at 300 mtorr and 100 mtorr, respectively.

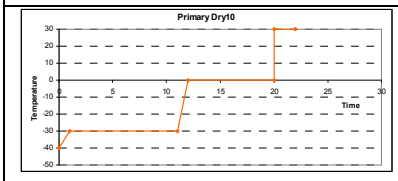
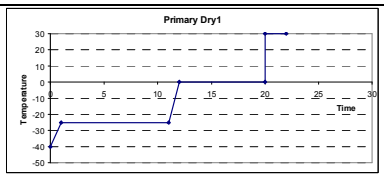
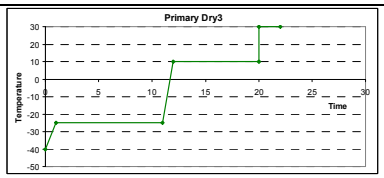
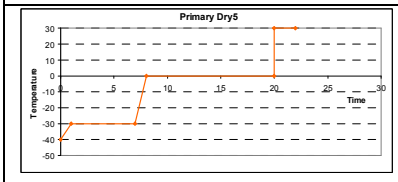
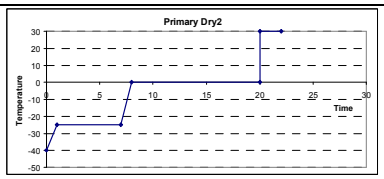
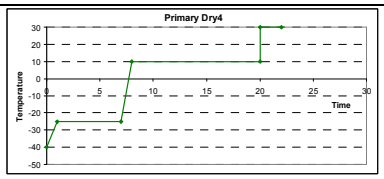
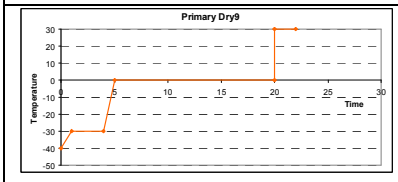
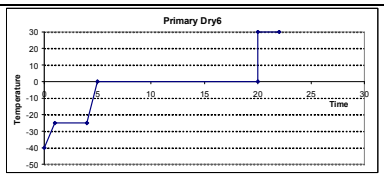
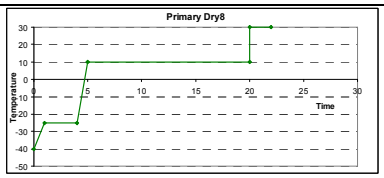
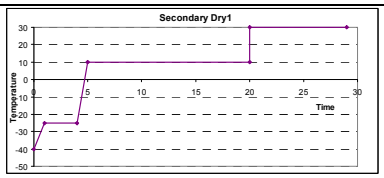
Figure 19 Lyophilization process cycle for freezing process evaluation

Lyophilized products which were produced by each freezing patterns were analyzed for their secondary structures, moisture contents, thermal properties, crystallinity compared with the initial condition (day0). In addition, lyophilized products were kept at 2-8°C (cold condition) for 3 months then were analyzed for their secondary structures, moisture contents, thermal properties, crystallinity for the evaluation on their stabilities.

3.3.2 Primary and Secondary Drying Process Evaluation

Freezing patterns of lyophilization process is set up as mentioned in 3.3.1. Primary and secondary drying were designed as seen in the follow my table 4. The total time primary and secondary drying time were controlled at 22 hours. Samples were lyophilized under designated lyophilization process programs by lyophilizer (Model Lyolab ST4B, Lyolab, USA).

Table 4 Lyophilization processes of primary and secondary drying optimization study.

Lyophilization process cycle : Primary and secondary dry		
Primary dry temp :-30 °C, 0 °C	Primary dry temp :-25 °C, 0 °C	Primary dry temp :-25 °C, 10 °C
 Primary Dry10	 Primary Dry1	 Primary Dry3
 Primary Dry5	 Primary Dry2	 Primary Dry4
 Primary Dry9	 Primary Dry6	 Primary Dry8
		 Secondary dry1

Lyophilized products which were produced by each lyophilization process were analyzed for their secondary structure, moisture contents, thermal properties and crystallinity. In addition, lyophilized products were stored at 2-8 °C for 3 months and were analyzed as stated above.

3.4 Formulation development of F(ab')2 Green Pit Viper antivenin lyophilized product.

Green Pit Viper antivenin formulations were formulated into solid dosage form by lyophilization. Formulas contained 0.7 mg/ml of F(ab')2 Green Pit Viper antivenin and stabilizers which produced good cake and retained the secondary structure of F(ab')2 as the native F(ab')2. The formulas were formulated to have osmotic pressure of 300 mOsm/kg. Stabilizers used in the formula was selected from the result in 3.2. There are 3 groups formulated

1. Formula of 0.7 mg/ml of F(ab')2 with stabilizer (filled volume of 4 ml)
2. Formula of 1.4 mg/ml of F(ab')2 with stabilizer (filled volume of 2 ml)

3. Formula of 0.7 mg/ml of F(ab')2 with combined stabilizers.(filled volume of 4 ml)

After formulas were prepared then lyophilized with optimized lyophilization process obtained in 3.3. At the end of the lyophilization process, the dried products were manually sealed with rubber stoppers.

3.5 Evaluation of lyophilized Green Pit Viper antivenin products

Lyophilized Green Pit Viper antivenin products were analyzed for their physical appearance, secondary structure, crystallinity, moisture contents, thermal properties, protein contents, F(ab')2 contents, purity and potency of antivenin

3.5.1 Physical Appearance

Lyophilized cakes were evaluated visually. The good lyophilized cakes show cake volume the same as filled volume, no collapse occur. The numbers of good products were recorded.

3.5.2 Conformation evaluation by Circular Dichroism

Circular dichroism was used to determine the secondary structure of F(ab')2. Samples were reconstituted to liquid solution and diluted with purified water (1:100). Samples were added to quartz cuvette with a 1 mm pathlength and measured from 190 to 250 nm at the scan speed of 50 nm/min, a response time 2 seconds, a band width of 2 nm, sensitivity of 20 mdeg, and an accumulation of 3 scans. The CD spectra were evaluated at the CD value of 217 nm.

3.5.3 Crystallinity

X-ray diffraction patterns of lyophilized products were obtained by X-ray diffractometer (Model Miniflex II,Rigaku, Japan), with Cu Kα radiation at 30 kV/ 15mA. The samples were measured from of 5° to 60 °2θ at an angle speed of 2 ° /min.

3.5.4 Moisture contents

a) Thermogravimetric Analysis (TGA)

Approximately 3 mg of lyophilized product was heated in an alumina pan from 25 to 250 °C, with 10 °C/min under nitrogen purge gas by thermogravimetric analyzer (Model TGA/SDTA851e, Switzerland). TGA thermograms were collected and analyzed for their % moisture contents.

b) Karl Fischer Titration

Residual moisture of the samples were determined by Karl Fischer titration (Model DL38, Mettler Toledo, Schwerzenbach, Switzerland). Lyophilized products of 10-20 mg were transferred into the titration vessel. Residual water contents were shown as ratio (%) to compare with sample weight.

3.5.5 Thermal analysis

Differential scanning calorimetry (Model DSC822e, Zurich, Switzerland) was used in the analysis. Approximately 5 mg of the lyophilized products were placed in the aluminium pan and hermitically sealed. An empty aluminium pan was used as reference. Samples were heated from 30 to 280 °C at 20 °C/min. DSC thermogram were collected and evaluated.

3.5.6 Protein content

Kjeldahl method was used to determine protein contents of the lyophilized products. Lyophilized products were reconstituted. Sample solutions were mixed with selenium reagent then sulfuric acid were added. Proteins in the mixture were degraded by heating. Mixtures were distilled with sodium hydroxide which converts the ammonium salt to ammonia and the end of condenser was dipped into boric acid solution. Ammonia reacted with acid. Then distilled mixture was titrated with sulfuric acid by a titrator. Percentage of protein contents were calculated from the volume of sulfuric acid used. Glycine was used as standard protein.

3.5.7 Purity of product

SDS-PAGE analysis was used to determine the purity of the products and F(ab')2 contents. Polyacrylamide gel (10%) of resolving and stacking gel was prepared from these components : water, 30% acrylamide solution (acrylamide : bisacrylamide = 29:1), 1.5M Tris-Hydrochloride buffer pH 8.8, 10% Sodium dodecyl sulphate solution, 1.0M Tris-hydrochloride buffer pH 6.8, 10% ammonium persulphate solution, Tetramethylethylenediamine (TEMED). Samples (reconstituted lyophilized products) and standards [standard albumin (MW 60 KD), standard immunoglobulin (MW 150 KD) and standard F(ab')2 (MW 100 KD)] were mixed with SDS-PAGE sample buffer. Mixtures were applied to the stacking gel by microsyringe. Gels were subjected to applied electric field until color band of sample moved to the bottom of the gel. Then gel stained with Coomassie blue staining solution. Sample bands of SDS-PAGE were compared with standard bands and checked for any other bands that appeared on the sample line.

3.5.8 F(ab')2 contents

F(ab')2 contents were evaluated by SDS-PAGE. SDS-PAGE was scanned and recorded on the computer. F(ab')2 content was determined by using Image Master program which used intensity of band color and band position for the calculation.

3.5.9 Antivenin potency

The potency of lyophilized product was determined by "mouse neutralizing test". Green Pit Viper venom solution was prepared by normal saline dilution and was prepared in serial dilution that covers the concentration of antivenin. Green Pit Viper antivenin lyophilized products were reconstituted into solution. Mixtures of venom and antivenin solutions were mixed and incubated at 37 °C for 30 minutes. Mixtures of venom and antivenin were injected into mice. Each dilution used 6 mice and 0.5 ml of dilution was injected intravenously into each mouse. The mice were observed for 48 hours after injection and number of survival mouse were recorded. The potency of antivenin was calculated by Probit Analysis method.

3.6 Stability study of lyophilized Green Pit Viper antivenin products.

Green Pit Viper antivenin lyophilized products were stored under 3 conditions, cold (2-8 °C), ambient (30°C) and accelerated (45°C and 75% RH) conditions for 3 months. After 3 months, lyophilized products from each condition were determined for their physical appearances, protein conformations, crystallinity, moisture contents, thermal properties, protein contents, purity and F(ab')2 contents. (refer to methods in 3.5.1-3.5.8)

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preformulation

Preformulation studies were done on Green Pit Viper antivenin commercial products and lyophilized product of F(ab')2 Green Pit Viper antivenin.

4.1.1. Solid State Characterization

4.1.1.1 X-ray Powder Diffraction

-Green Pit Viper antivenin commercial products (QSMI)

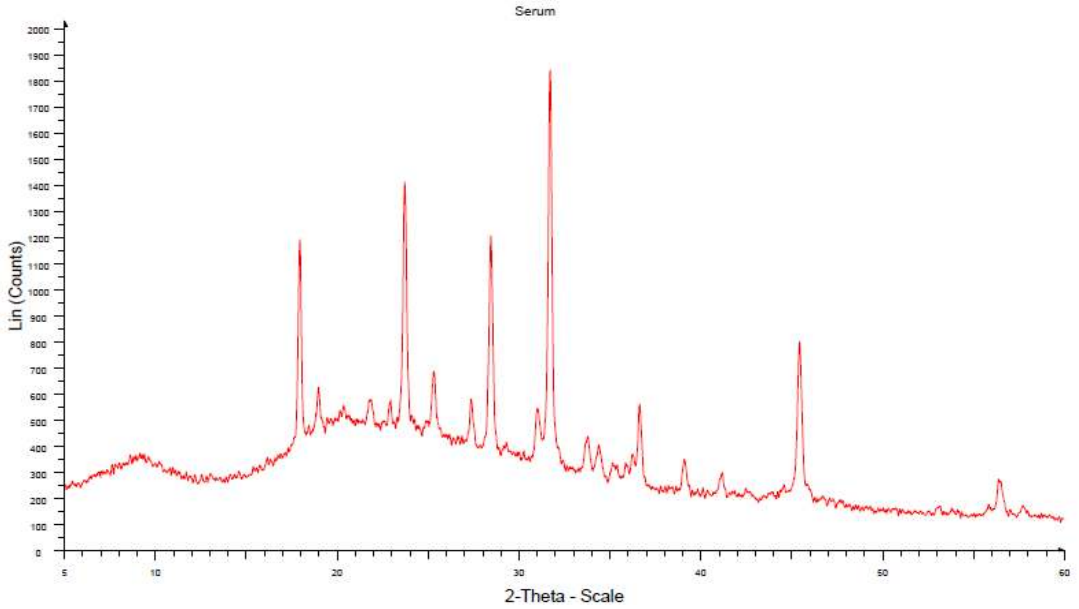


Figure 20 XRPD diffractogram of Green Pit Viper antivenin product (QSMI).

Figure 20 shows XPRD pattern of Green Pit Viper antivenin product from QSMI. XRPD pattern displays sharp peaks at various 2-theta degrees which are the signature of crystalline material. While the underlying pattern base line is character of amorphous solid. Green Pit Viper antivenin contained F(ab')2 as protein active ingredient of immunoglobulin type. The active ingredient or F(ab')2 is assumed to be the cause of amorphous pattern in Figure1. The crystalline peak may be due to additives or excipients in the formulation of QSMI product.

-Lyophilized F(ab')2 Green Pit Viper antivenin bulk concentrate.

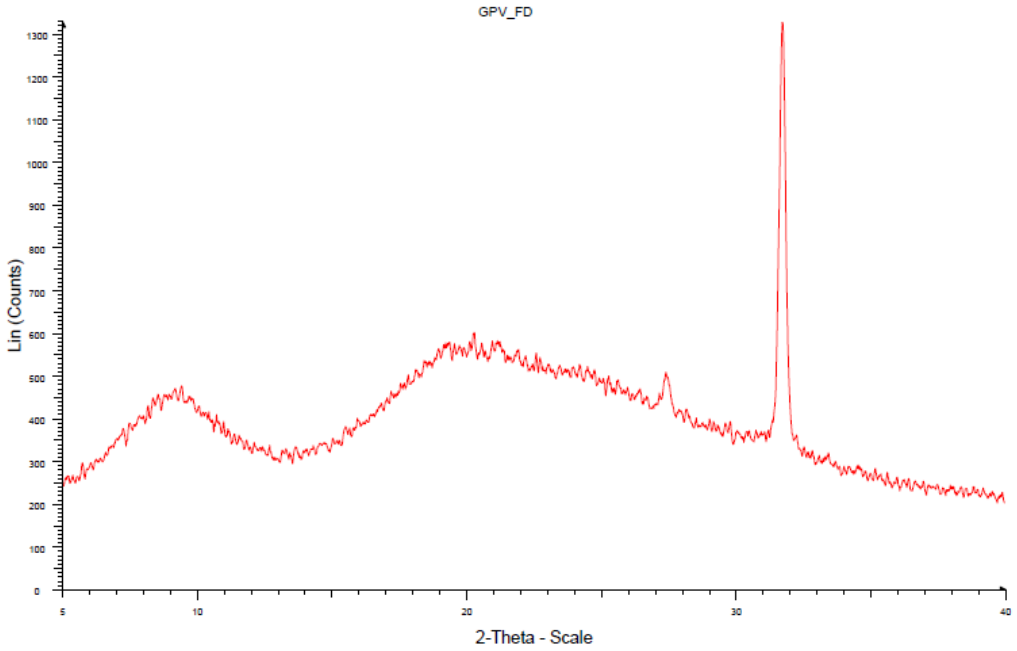


Figure 21 XRPD diffractogram of lyophilized F(ab')2 Green Pit Viper antivenin bulk concentrate.

XRPD diffractogram of lyophilized F(ab')2 Green Pit Viper antivenin bulk concentrate has single characteristic crystalline peak at approximately 32° 2Θ and F(ab')2 amorphous baseline. Crystalline peak may be contributed to tonicity adjusting agent such as sodium chloride, which shows XRPD diffraction peak at 31.8 ° 2Θ (Hawe and Frieb, 2006 and Dixon, 2008).

4.1.1.2 Thermal Analysis

a. Differential Scanning Calorimetry (DSC).

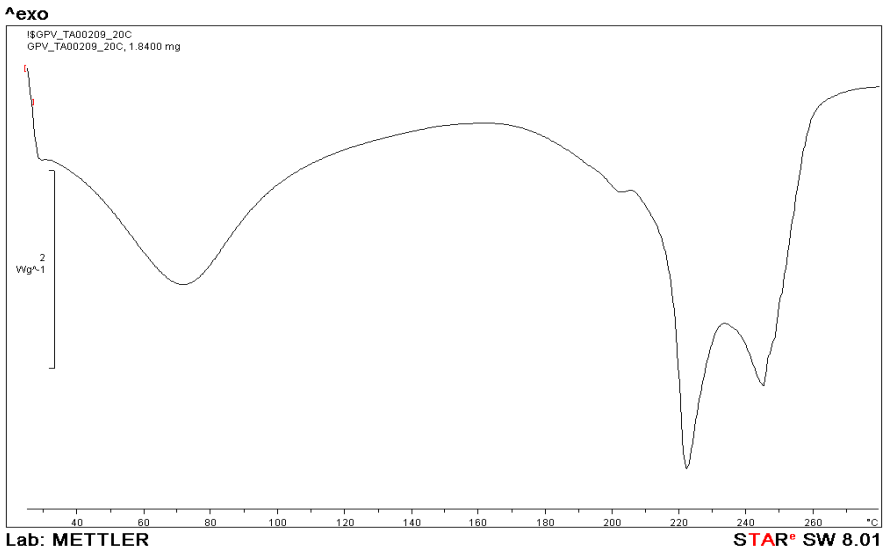


Figure 22 DSC thermogram of Green Pit Viper antivenin product (QSMI).

Figure 22 presents DSC thermogram of Green Pit Viper antivenin product of QSMI which was heated from 25°C to 280°C at 20 °/min. DSC thermogram shows broard endothermic event starting from 40°C to 140°C due to water evaporation. At approximately 220°C and 250°C show endothermic peaks that may be due to the melting of excipients and decomposition of Green Pit Viper antivenin at high temperature, respectively.

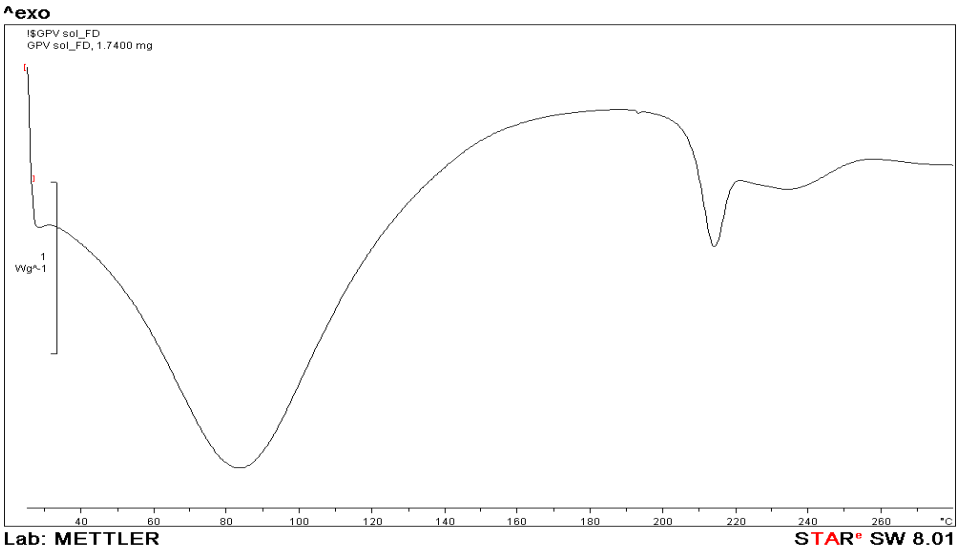


Figure 23 DSC thermogram of lyophilized F(ab')2 Green Pit Viper antivenin bulk concentrate.

DSC thermogram shows broad endothermic event with onset at 40°C and endset at 160°C for the desolvation of water. A minor glass transition temperature of amorphous Green Pit Viper antivenin concentrated bulk is observed at 190°C. When heat was increased until 210°C, endothermic melting peak of sodium chloride is seen and a decomposition endotherm of lyophilized Green Pit Viper antivenin concentrated bulk occurs between 230-240°C.

b. <u>Thermogravimetic analysis (TGA)</u>

Table 5 Moisture contents of Green Pit Viper antivenin QSMI product from TGA method (n=2)

Sample	Moisture (%w/w)
Sample 1	3.914
Sample 2	3.290
Average	**3.602**

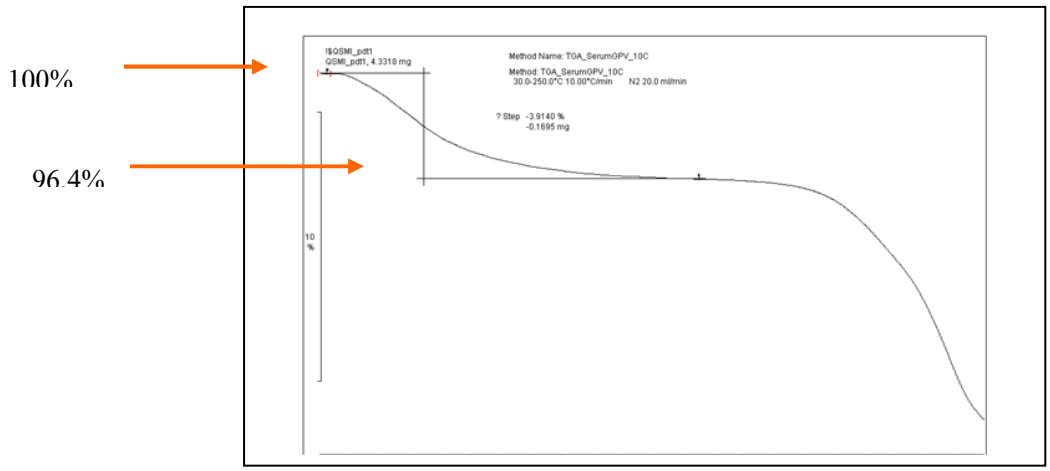


Figure 24 TGA thermogram of Green Pit Viper antivenin QSMI product.

Table 5 and Figure 24 show avearage moisture content of Green Pit Viper antivenin QSMI products from thermogravimetric analysis (TGA) of 3.602 %w/w. However, the moisture content of QSMI product Lot no. TA00209 obtained by Karl Fischer Titration is 0.5%. The difference in moisture values obtained may be due to the difference in analytical methods used. (Yoshii and Tonogai, 2004)

4.1.2 Conformation Evaluation by Circular Dichroism

4.1.2.1 Green Pit Viper antivenin QSMI product

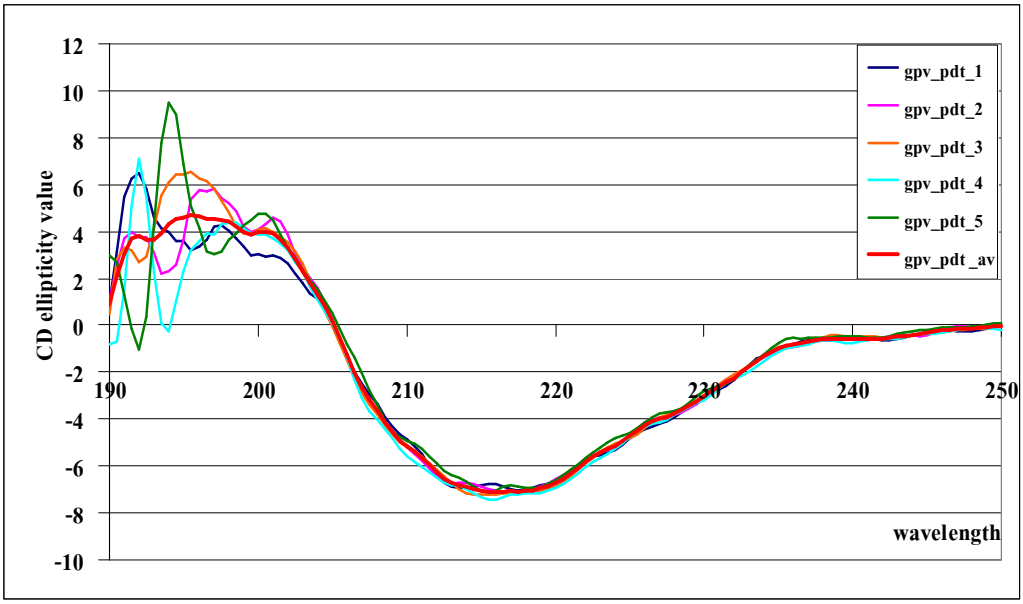


Figure 25 CD spectra of Green Pit Viper antivenin QSMI product after reconstitution and analyzed by spectropolarimeter.

Figure 25 shows CD spectra of Green Pit Viper antivenin QSMI product which displayed predominantly β-sheet secondary structure. CD spectra show minima at wavelength of 217 nm and a zero intensity at wavelength of 206 nm. The average ellipticity of Green Pit Viper antivenin at 217 nm is -7.067 ± 0.149 mdeg. These results agree well with previous research of IgG characterized by CD which mainly comprised of β-sheet secondary structure with minimum at 217 nm. (Vermeer and Norde, 2000; Kanavage et al., 2006 and Szenczi et al., 2006). These CD spectra will be used as reference to compare and determine the property of future products.

4.1.2.2 Lyophilized F(ab')2 Green Pit Viper antivenin bulk concentrate.

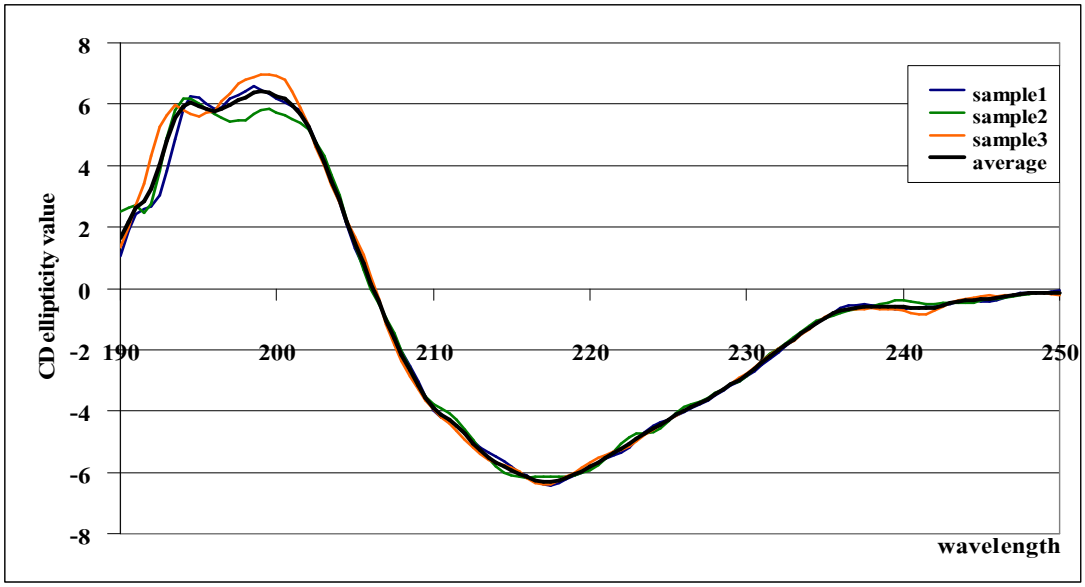


Figure 26 CD pattern of lyophilized F(ab')2 Green Pit Viper antivenin bulk concentrate after reconstitution.

Figure 26 shows CD spectra of F(ab')2 Green Pit Viper antivenin bulk concentrate which displays predominantly β-sheet secondary structure. CD spectra have minima at wavelength of 217 nm with average ellipticity of -6.303 mdeg. A zero ellipticity value is seen at 206 nm. This result agrees well with previous research (Vermeer and Norde, 2000; Kanavage et al., 2006; Szenczi et al., 2006). CD spectra of lyophilized F(ab')2 is the same as CD spectra of Green Pit Viper antivenin QSMI product which show mainly the character of β-sheet conformation. However, CD ellipticity values at 217 nm are slightly different which may be due to the formulation or differences in the production process of lyophilized F(ab')2 Green Pit Viper antivenin which may have impact on the secondary structure and its CD ellipticity value.

4.1.3 Bulk Concentrate Conformation Evaluation by Circular Dichroism.

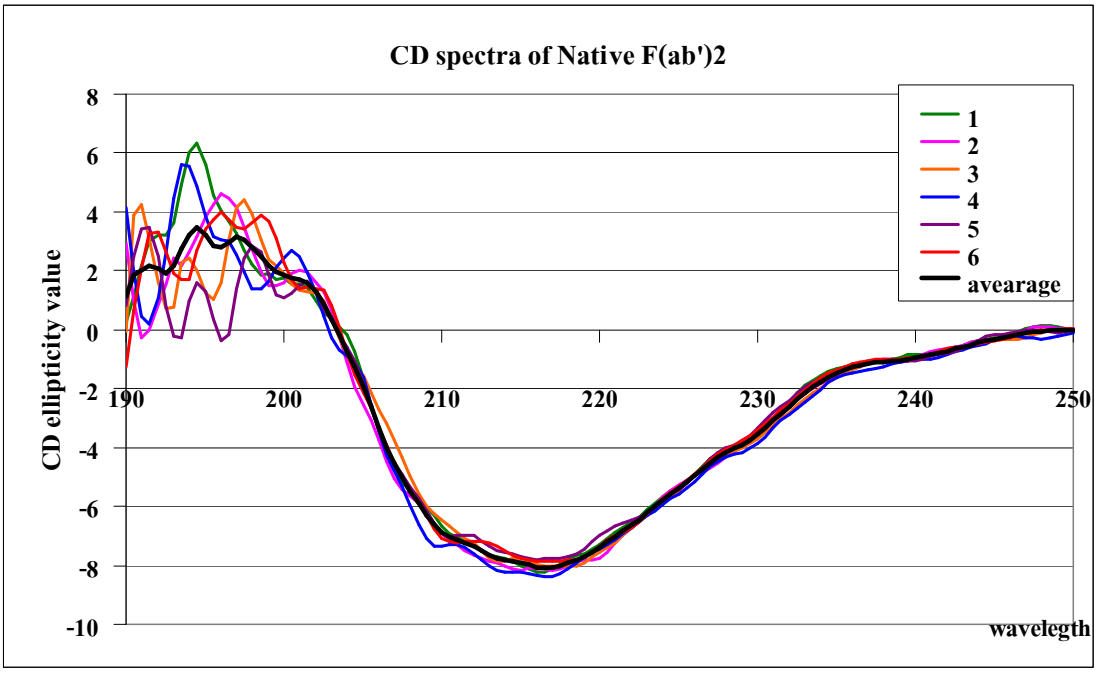


Figure 27 CD spectra of F(ab')2 Green Pit Viper antivenin solution at concentration of 0.7 mg/ml of water, called "native F(ab')2" (n=6)

Figure 27 shows CD spectra of native F(ab')2 which exhibit mainly β-sheet conformation and average CD ellipticity value at 217 nm is -8.060 ± 0.211 mdeg (n=6). This data is used as reference for comparison in future studies.

Comparison of Green Pit Viper antivenin QSMI product and lyophilized F(ab')2 Green Pit Viper antivenin from bulk concentrate.

DSC thermogram

Figure 30 displays comparative DSC thermograms of two lyophilized products, Green Pit Viper antivenin QSMI product and lyophilized F(ab')2 Green Pit Viper antivenin from bulk concentrate. Both products show similar sequence of events in the diffractograms. First broad dehydration endotherms are between 40-140 °C. Glass transition temperatures at approximately 190-200°C. Melting of major tonicity adjusting agent, sodium chloride, is at 210-220°C. Finally, decomposition of F(ab')2 in lyophilized Green Pit Viper antivenin in both products at approximately 240°C.

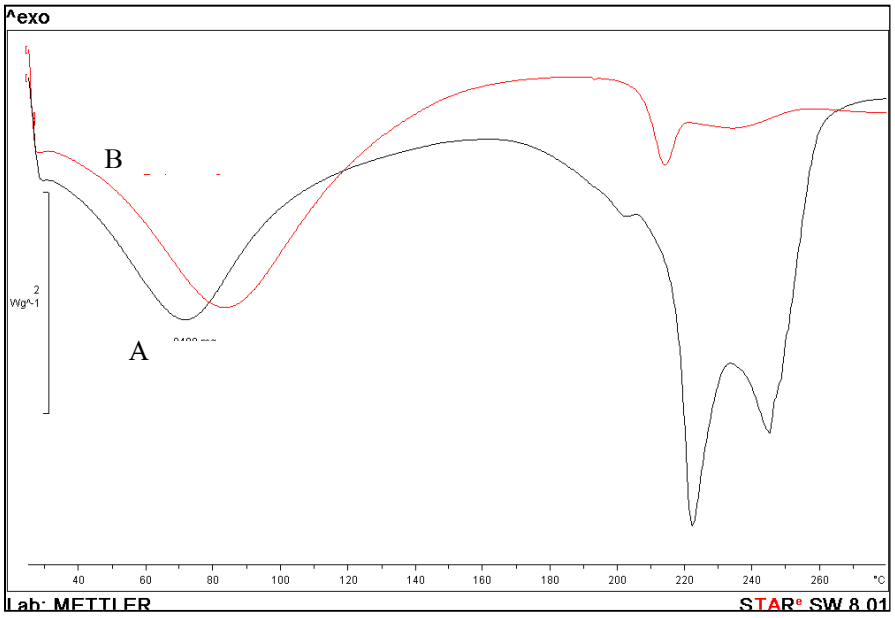


Figure 28 DSC thermogram comparison between Green Pit Viper antivenin QSMI product(A) and lyophilized of F(ab')2 Green Pit Viper concentrated bulk(B).

Preformulation results of Green Pit Viper products (QSMI) found that Green Pit Viper antivenin products (QSMI) show crytalline peaks of XRPD difraction pattern which may be additives in formula and amorphous base line of F(ab')2. when comparison between Green Pit Viper antivenin products(QSMI) and Active ingredient (F(ab')2 Green Pit Viper antivenin) by DSC thermograms found that display endothermic peaks of water evaporation (at approximately 40 -140 °C). The glass transition temperature of amorphous Green Pit Viper antivenin exhibit about 190°C. The endothermic peaks of additives melting and decomposition show at peak of 220 and 250°C according to formulation.

The secondary structure conformations of Green Pit Viper antivenin products (QSMI) display predominanly β-sheet conformation. Both F(ab')2 Green Pit Viper lyophilized products and Green Pit Viper antivenin bulk concentrate exhibit mainly β-sheet conformation together. However, the ellipticity CD values of each sample are slightly different and ellipticity CD values at 217 nm were used for evalution results. The CD spectra of native F(ab')2 was analyzed and used to reference for next study.

4.2 Preliminary Lyophiization Process Development

4.2.1 Freezing Point Determination of Green Pit Viper Antivenin by DSC

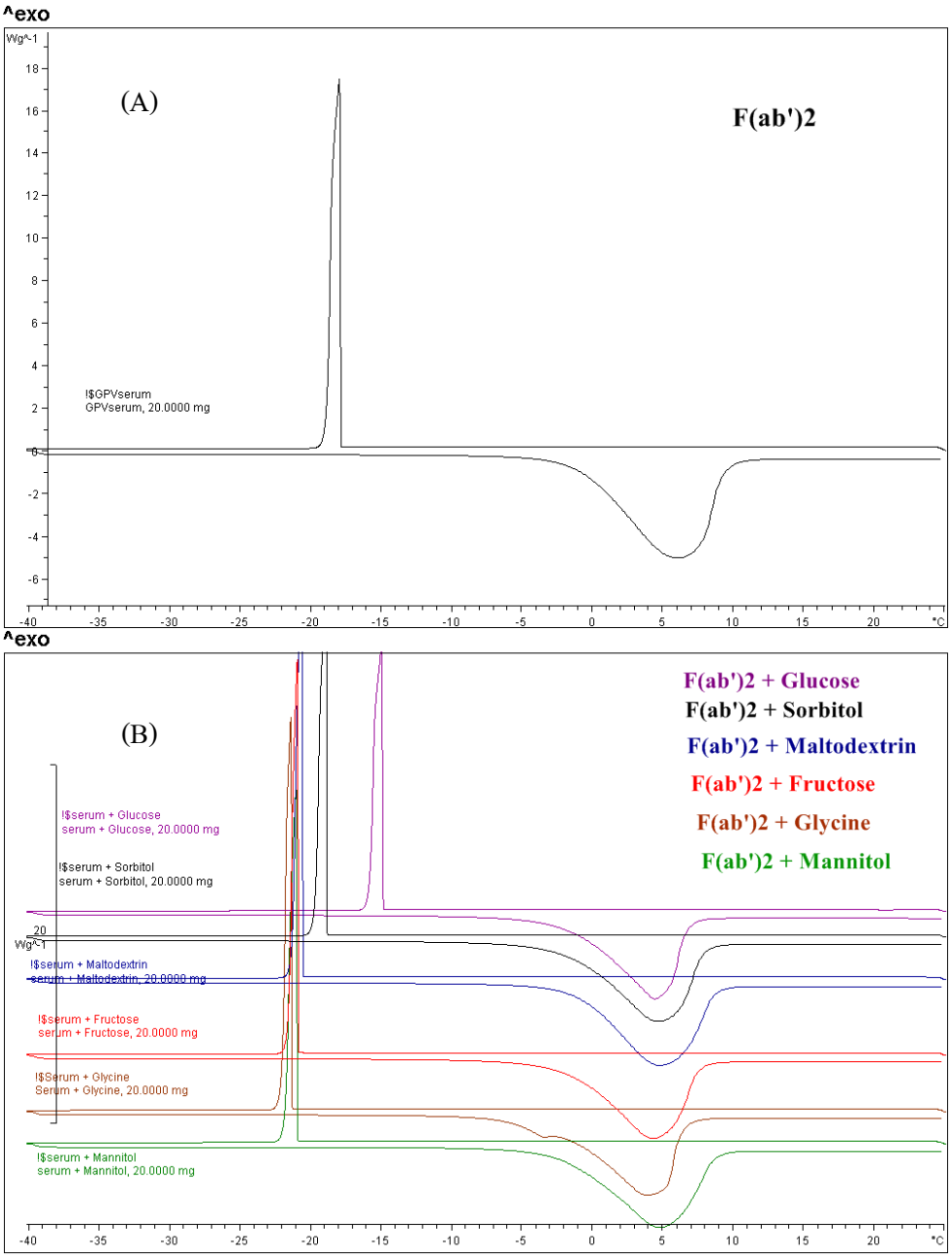


Figure 29 DSC thermogram of F(ab')2 Green Pit Viper antivenin (A) and mixtures of F(ab')2 Green Pit Viper antivenin with stabilizers (2% w/v) (B) after frozen to -40 °C and gradually heated up at the rate of 2 °C/min

Figure 29 shows DSC thermograms of F(ab')2 Green Pit Viper antivenin and mixtures of F(ab')2 Green Pit Viper antivenin with stabilizers that were frozen to -40 °C. DSC thermograms show exothermic freezing point when cool samples until freezing, product transforms liquid to solid and released their energy (exothermal peak). Our data found that freezing points of F(ab')2 are at about -18 °C, freezing point of mixture F(ab')2 + glucose is about -15 °C, freezing point of mixture F(ab')2 + sorbitol is about -19 °C, freezing point of mixture F(ab')2 + maltodextrin is about -20 °C, freezing point of mixture F(ab')2 + glycine is about -22 °C, freezing point of mixture F(ab')2 + fructose and freezing point of mixture F(ab')2 + mannitol are about -21 °C. So, range of sample freezing points [F(ab')2 and mixture of F(ab')2 with stabilizers] were about -15 to -22 °C. In our study, freezing step was set at -40 °C which all samples transformed from liquid to solid phase. Thus, freezing step chosen for lyophilization of Green Pit Viper antivenin should be below -22 °C.

4.2.2 The Effect of Freezing Patterns on Secondary Structure of $F(ab')_2$

In Figure 30 and Table 6 show freezing patterns of each cycle of freezing process. The 3 freezing patterns are Slow, Rapid1 and Rapid3 freezing process. Each process has difference cycle, rate and total time.

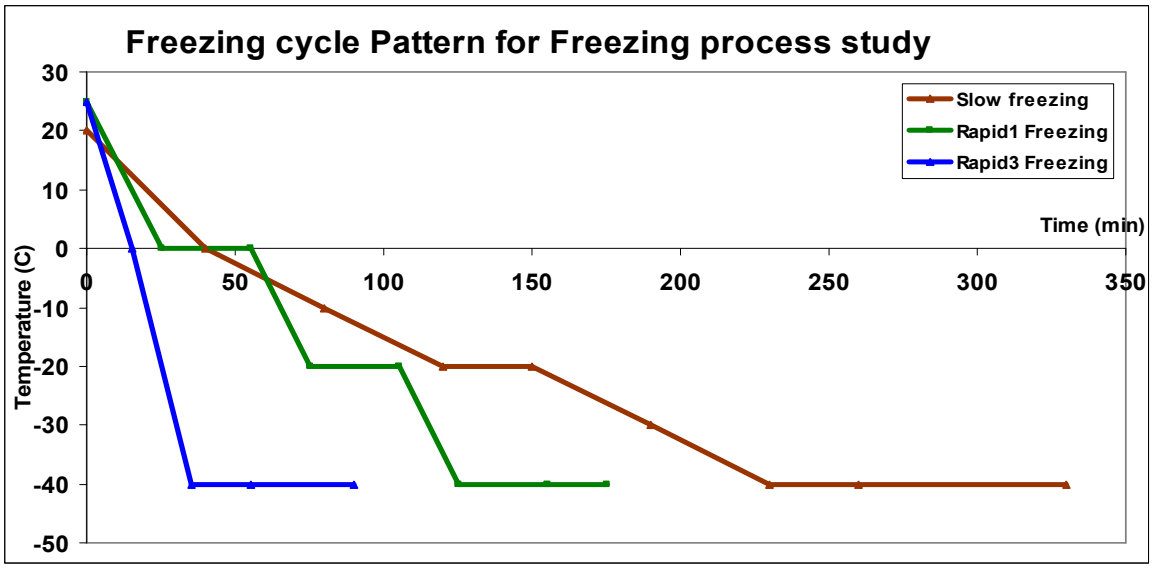


Figure 30 Freezing cycle pattern for freezing process study.

Table 6 Freezing cycle pattern

Cycle name / Step parameter	**Slow**		**Rapid1**		**Rapid3**	
	Temperature (°C)	Time (minute)	Temperature	Time (minute)	Temperature	Time (minute)
Freezing	20	0	25	0	25	0
	0	40	0	25	0	15
	-10	40	0	30	-40	20
	-20	40	-20	20	-40	20
	-20	30	-20	30	-	-
	-30	40	-40	20	-	-
	-40	40	-40	30	-	-
	-40	30	-	-	-	-
	-40	70	-40	20	-40	35
Average Cooling rate (°C /min)	0.26		0.52		1.86	
Total time (min)	330		175		90	

Effect of Freezing Patterns on Secondary Structure of F(ab')2

Samples are frozen by various freezing patterns. At the end of freezing process, the frozen samples are kept at room temperature until melted. Samples are analyzed with circular dichroism using spectropolarimeter and the secondary structures of F(ab')2 are evaluated.

Figures 31-33 show CD spectra of native F(ab')2 compared to mixtures of F(ab')2 Green Pit Viper antivenin with various stabilizers 2% w/v and frozen by various freezing patterns. It is found that the structure of CD spectra of every samples are similar to native F(ab')2 but different in magnitude. CD spectra had positive intensity (ellipticity) at wavelength from 190-206 nm, had zero intensity at wavelength of 206-207 nm and a negative intensity (ellipticity) at wavelength of 207 -250 nm where the mimimum intensity remained at 217 nm. Although they show slightly different ellipticity values from native F(ab')2, but every patterns of frozen mixtures of F(ab')2

with stabilizers represent mainly β-sheet secondary structures. This result is consistent with previous study that the CD spectra at the far UV region of the antivenom consisted mainly of β-sheet structure (Vermeer and Norde, 2000; Kanavage et al., 2006; Szenczi et al., 2006).

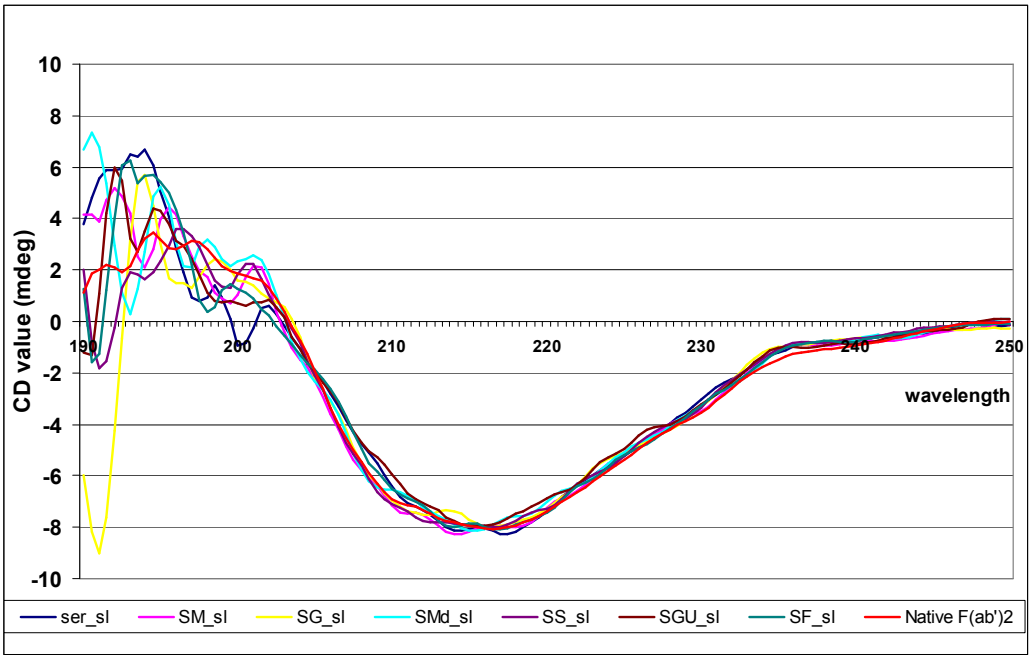


Figure 31 CD spectra of mixtures of F(ab')2 Green Pit Viper antivenin with various stabilizers (2% w/v) after frozen by "Slow" freezing pattern.

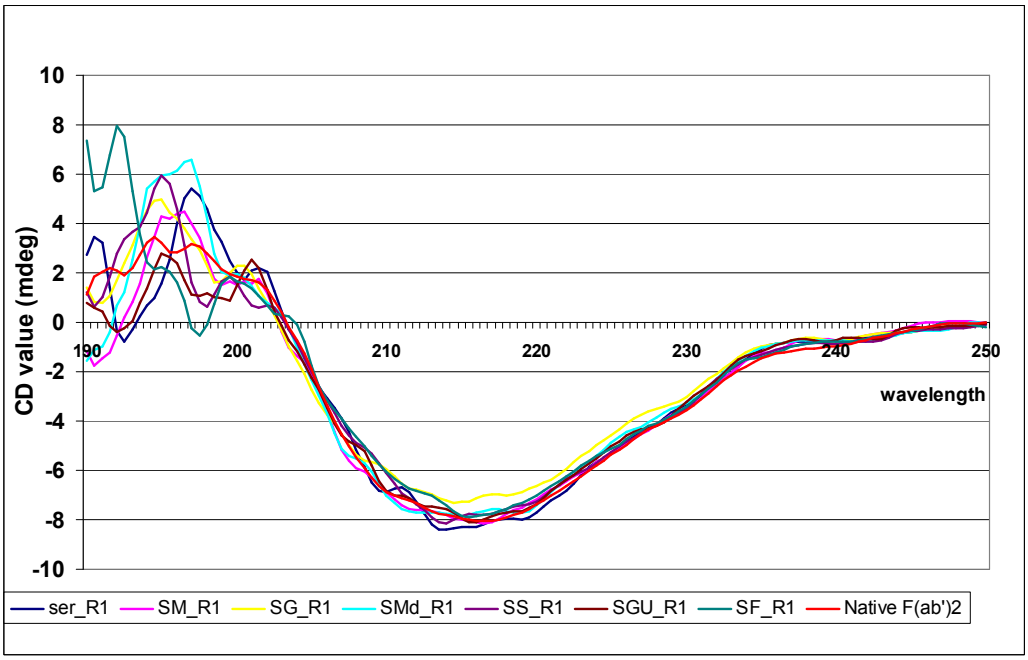


Figure 32 CD spectra of mixtures of F(ab')2 Green Pit Viper antivenin with various stabilizers (2% w/v) after frozen by "Rapid1" freezing pattern.

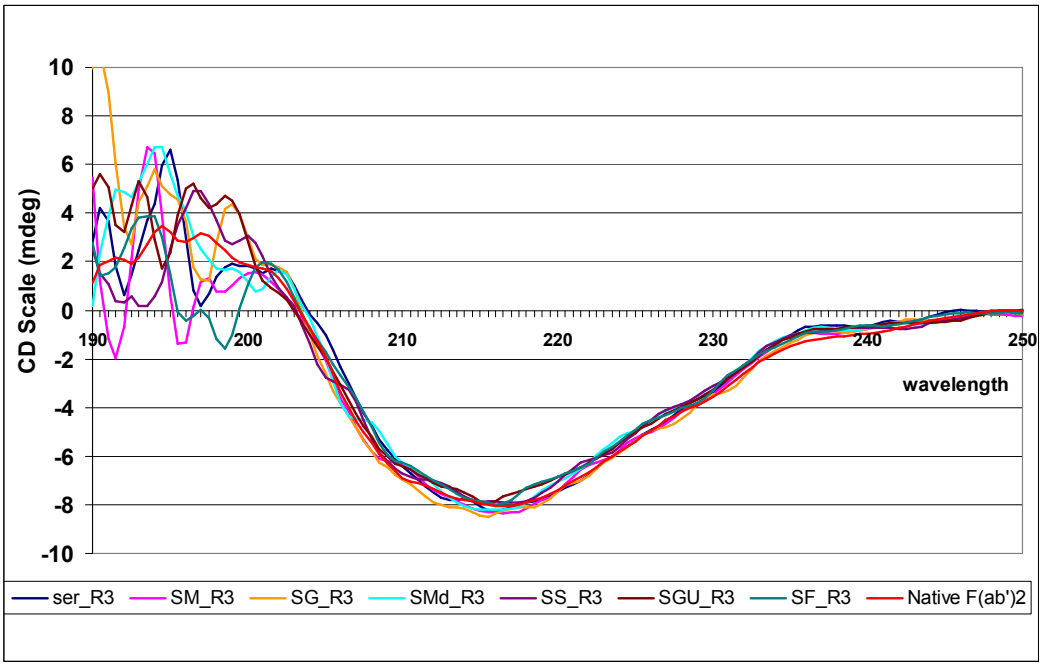


Figure 33 CD spectra of mixtures of F(ab')2 Green Pit Viper antivenin with various stabilizers (2% w/v) after frozen by "Rapid3" freezing pattern.

Table 7 CD ellipticity values (at 217 nm) of mixtures of F(ab')2 Green Pit Viper antivenin and various stabilizers after different freezing patterns.

Sample	Ellipticity values at 217 nm of frozen F(ab')2 mixtures (mdeg)		
	Slow freezing	Rapid1 freezing	Rapid3 freezing
F(ab')2 (Ser)	-8.267	-8.064	-8.088
F(ab')2+Mannitol (SM)	-7.941	-8.080	-8.323
F(ab')2+Glycine (SG)	-7.963	-6.990	-8.145
F(ab')2+Maltodextrin (SMd)	-7.754	-7.546	-8.170
F(ab')2+Sorbitol (SS)	-7.990	-7.767	-7.900
F(ab')2+Glucose (Sgu)	-7.797	-7.786	-7.534
F(ab')2+Fructose (SF)	-8.099	-7.740	-7.784

Table 7 present CD ellipticity values at 217 nm fpr native F(ab')2 and F(ab')2 mixtures with various 2% w/v stabilizers from each freezing cycle. Range of ellipticity values for each freezing pattern are -8.267 to -7.797 mdeg, -8.080 to -6.990 mdeg and -8.323 to -7.534 mdeg

for Slow, Rapid1 and Rapid3 freezing pattern, respectively. While the same sample when frozen with different freezing patterns had a different ellipticity values at 217 nm. Thus freezing patterns and stabilizers impacted on the secondary structures of F(ab')2.

CD ellipticity values in Table 7 are normalized by the CD ellipticity value of native F(ab')2 (-8.060 mdeg for lot no. 09002TA) by the following equation

$$\text{Normalized CD ellipticity value} = \frac{\text{(CD ellipticity of products)}}{\text{(CD ellipticity of native F(ab')2)}} \times 100 \quad \text{.....(1)}$$

$$= \frac{\text{(CD ellipticity of products)}}{\text{(-8.060 mdeg)}} \times 100 \quad \text{.....(2)}$$

The normalized CD values obtained are plotted as a function of freezing patterns and are shown in Figure 34.

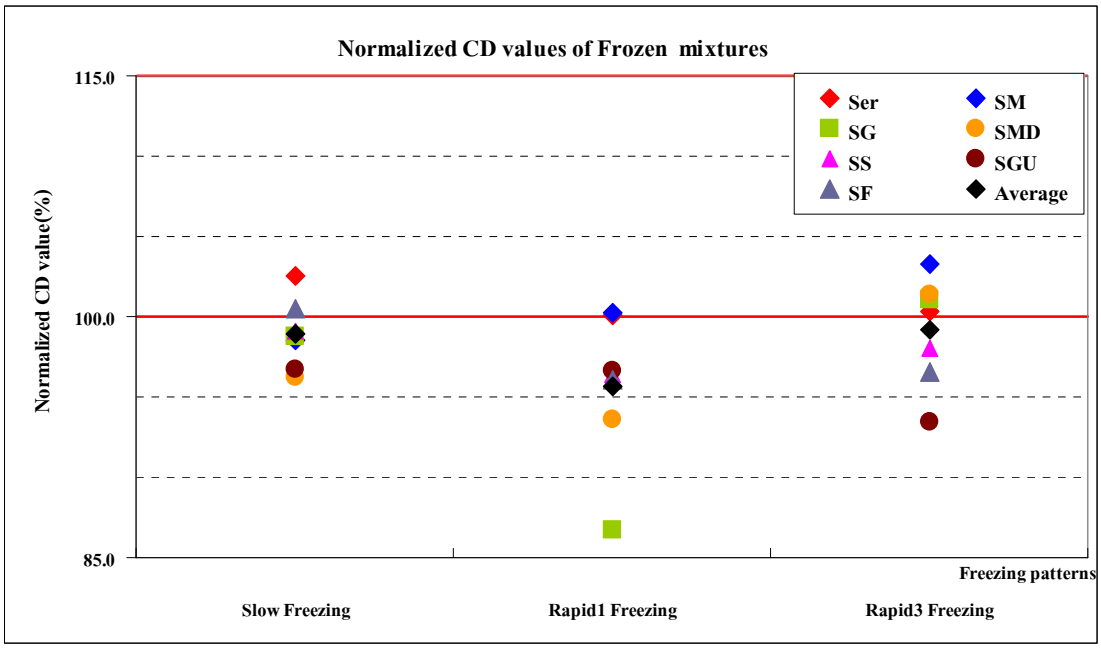


Figure 34 Normalized CD values of F(ab')2 mixtures with various stabilizers (2% w/v) after frozen with Slow, Rapid1 or Rapid3 patterns compared to native F(ab')2

Figure 34 displays normalized CD values of F(ab')2 mixtures by various freezing patterns. In most cases, normalized CD values were in the range 95 -105 %. Average normalized CD value of each freezing patterns were evaluated. The average normalized CD value of products obtained by Rapid3 freezing are closer to 100% compared to other freezing patterns. Thus, Rapid3 freezing pattern produced frozen F(ab')2 mixtures most similar to native F(ab')2.

Normalized CD values, F(ab')2 frozen were similar to 100%. Other formulations, mixture of F(ab')2 with stabilizers exhibit farther than 100%. Stabilizers may interact with the structure of F(ab')2 and impact the secondary structure of F(ab')2. However, previous studies have found that stabilizers such as, sugar had protein stabilizer effect and protected the secondary structure from freeze dry process (Allison et al., 2000; Garzon-Rodriguez et al., 2004; Liao et al., 2002 and Cleland et al., 2001). From our data, average normalized CD values show that Rapid3 freezing process exhibits same to 100%.

Preliminary of freezing process results summarized to

1. Freezing point determination. Freezing point of mixtures F(ab')2 with stabilizers are analyzed by DSC thermogram. The ranges of freezing point of all samples are -15 °C to -23°C. The freezing points were set for freezing temperature of lyophilization process. Our study used the freezing temperatures of lyophilization process are -40°C which could transform every sample from liquid to solid state.

2. The effect of freezing patterns on secondary structure of F(ab')2 .

The freezing patterns were studied such as Slow, Rapid1 and Rapid3 freezing patterns. The CD spectra of frozen mixtures F(ab')2 with various stabilizers were evaluted. The freezing process and stabilizers impact on secondary structure of F(ab')2. Every sample exhibited mainly β-sheetc conformation but different in magnitude. Rapid3 freezing pattern produced frozen mixtures which are closer to native F(ab')2 compared to other patterns.

4.3 Lyophilization Process Development

4.3.1 Freezing Process Evaluation

Samples were lyophilized by varying freezing steps as previously described (Slow, Rapid1 and Rapid3) then was dried with same standard in the primary and secondary drying steps(part 3.3.1). When lyophilization process was finished, lyophilized products were evaluated.

4.3.1.1 Appearance of Lyophilized Product

Table 8 Appearance of lyophilized products from lyophilization process

Formula	Freezing cycle used in Lyophilization process		
	Slow	Rapid1	Rapid3
Ser lyophilized product			
SG lyophilized product			
SM lyophilized product			
SMd lyophilized product			
SGU lyophilized product			

Table 8 Appearance of lyophilized products from lyophilization process (continue)

Formula	Freezing cycle used in Lyophilization process		
	Slow	Slow	Slow
SS lyophilized product			
SF lyophilized product			

Table 8 displays appearances of lyophilized products from lyophilization process which used different freezing steps (Slow, Rapid1 and Rapid3). The appearances of lyophilized products were classified to 3 groups.

1. Good appearance: SG and SM lyophilized products show elegant cake, the volume of cake was slightly lower than the filled volume and texture of cake did not collapse or melt.
2. Reduced cake volume: Cakes of Ser and SMd lyophilized products are reduced around the bottle. The texture of cakes almost perfect but volumes of cakes are lower than the volume of filling.
3. Collapsed products: SGU, SF and SS lyophilized products collapsed, no satisfied cakes are observed. The appearances are not acceptable as products from lyophilization process.

The study show that the same sample mixture when freeze dried with different processes resulted in the same product characters. For example SM lyophilized products had the same good appearance cakes after slow, Rapid1, Rapid3 freezing cycle were applied in the lyophilization process.

Lyophilized products which composed of glucose, sorbitol and fructose are collapsed. In the previous study found that glass transition temperatures of those agents are below -40 °C (Kadoya et al., 2010).Thus when samples are dried with the temperature not lower than -40°C and

or lower than their Tg. When proceed to primary drying, the samples will evaporate (instead of sublimate) causing a poor, unpredictable cake structures and eventaully collapsed.

4.3.1.2 Secondary Structure of Lyophilized Products

4.3.1.2.1 Initial Lyophilized products (DAY0)

Secondary structure of F(ab')2 : CD spectra at Day0

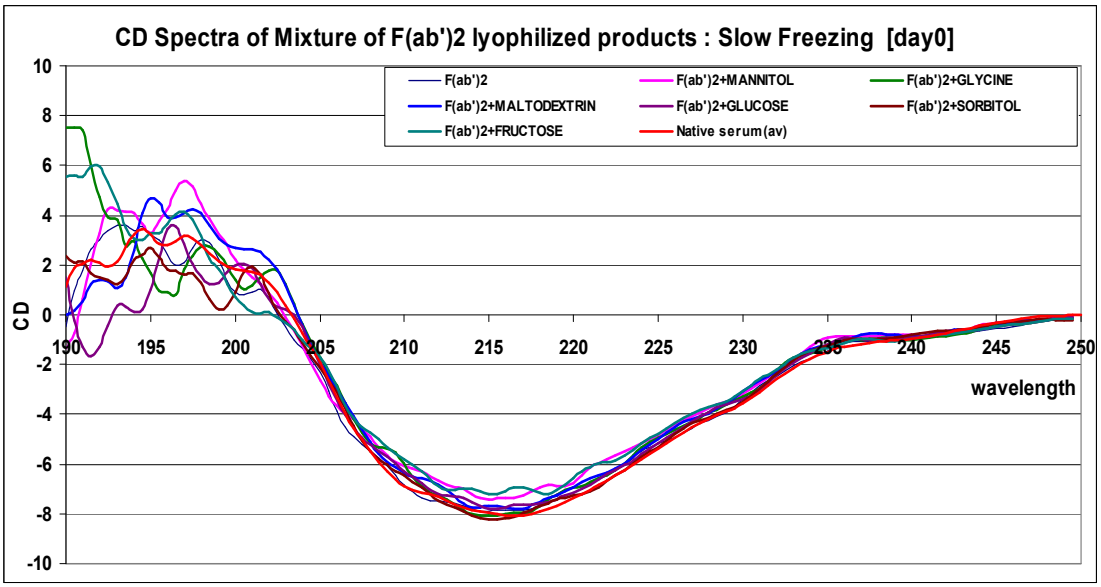


Figure 35 CD spectra of mixtures of F(ab')2 with 2% stabilizers after slow freezing lyophilization processes.

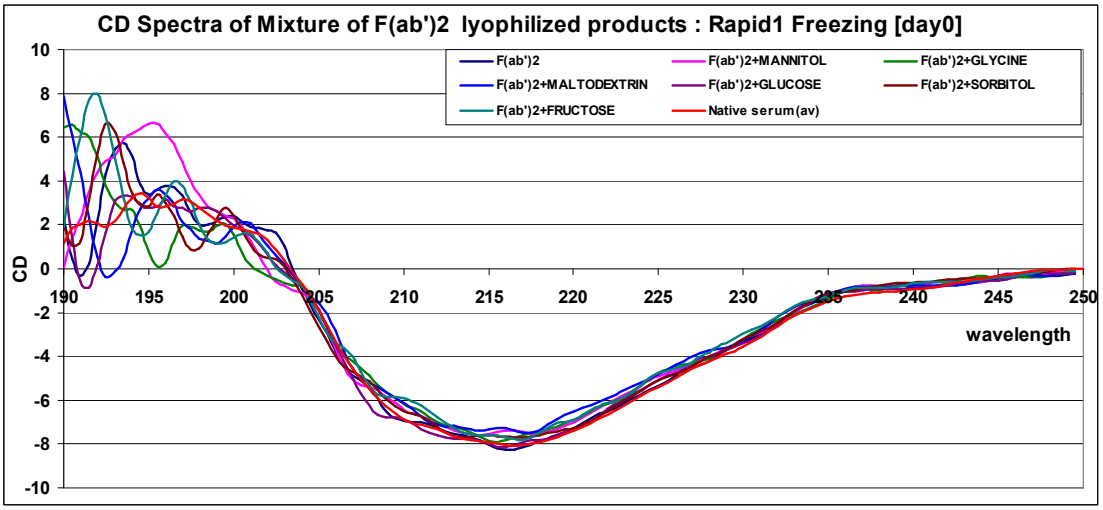


Figure 36 CD spectra of mixtures of F(ab')2 with 2% stabilizers after Rapid1 freezing lyophilization processes.

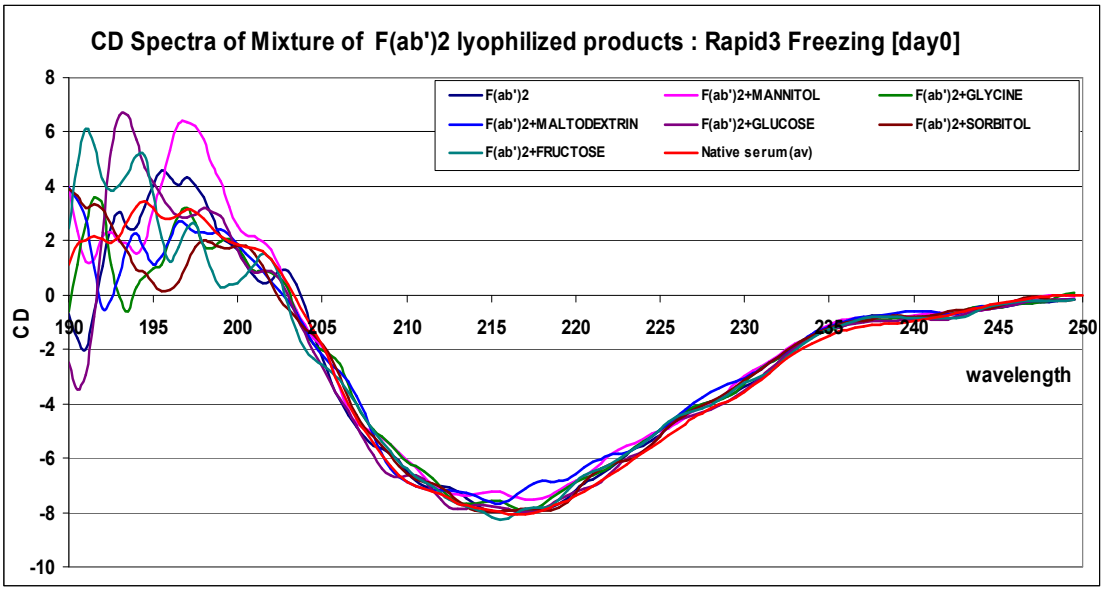


Figure 37 CD spectra of mixtures of F(ab')2 with 2% stabilizers after Rapid3 freezing lyophilization processes.

Figures 35-37 show CD spectra of mixtures of F(ab')2 with 2% stabilizers that was freeze dried with different freezing steps (Slow, Rapid1 and Rapid3 lyophilization processes). Data show secondary structure of protein mainly exhibit β-sheet conformation. These results are consistent with other studies that secondary conformational structure of F(ab')2 is β-sheet and the mimima occurs at 217 nm. (Rodriguez-Silva et al., 1997 and Kanavage, 2006)

Table 9 CD ellipticity value at 217 nm of of lyophilized mixtures of F(ab')2 with various stabilizers after Slow, Rapid1, Rapid3 lyophilization processes. [day0]

Sample	CD ellipticity value at 217 nm of lyophlized products		
	Slow freezing	Rapid1 freezing	Rapid3 freezing
$F(ab')_2$ (Ser)	-7.850	-8.158	-7.950
F(ab')2 + Mannitol (SM)	-7.254	-7.456	-7.520
F(ab')2+Glycine (SG)	-7.966	-7.605	-7.986
F(ab')2+Maltodextrin (SMd)	-7.787	-7.467	-7.120
F(ab')2+Glucose (SGu)	-7.626	-7.914	-7.965
F(ab')2+Sorbitol (SS)	-7.967	-7.707	-7.897
F(ab')2+Fructose (SF)	-6.931	-7.826	-7.884

Table 9 show CD ellipticity values at 217 nm of mixtures of F(ab')2 and stabilizers lyophilized products. The range of CD ellipticity values at 217 nm of all lyophilized products are -6.931 to -7.967 mdeg for Slow , -7.456 to -8.158 mdeg for Rapid1 and -7.120 to -7.967 mdeg for Rapid3 freeing process. Our study found that the same samples when freeze dried with different freezing patterns show slight differences in CD ellipticity values at 217 nm. So, the freezing step during lyophilization impact the values of CD spectra of the product. However, the main secondary structure of F(ab')2 in the products remain mainly β-sheet structure.

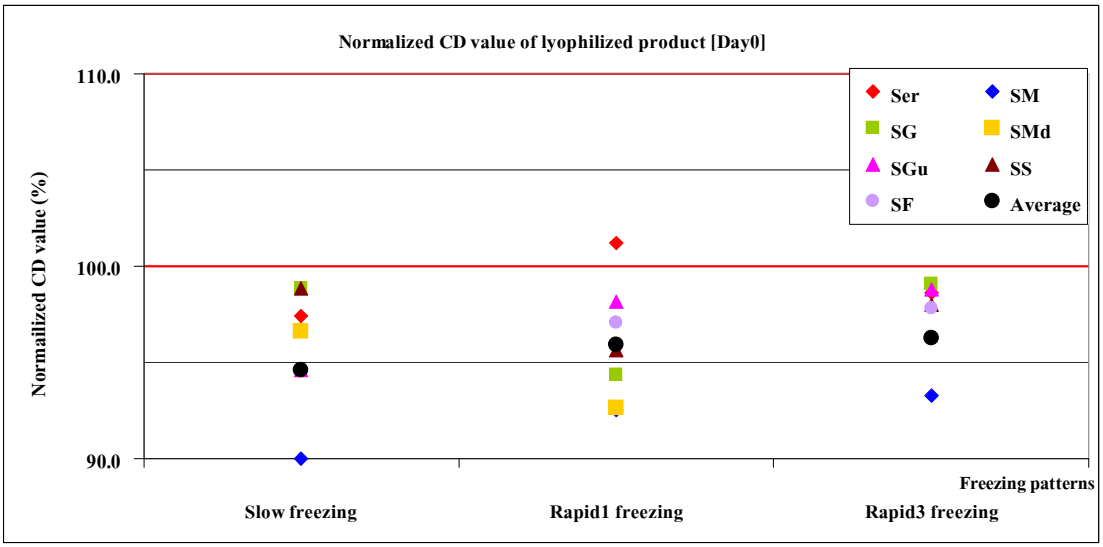


Figure 38 Normalized CD values of lyophilized mixtures of F(ab')2 with various stabilizers after Slow, Rapid1, Rapid3 lyophilization processes.

The normalized CD values of lyophilized products are within the range of 90 -110 %. The Normalized CD values should be close to 100% which means that CD ellipticity value is similar to native F(ab')2 . Average normalized CD value of Rapid3 freezing is the closest to 100%. Thus, Rapid3 seems to be an appropriate freezing condition for F(ab')2 Green pit viper antivenin products. This finding is in good correlation with the conclusion in previous part 4.2.2.

4.3.1.2.2 Lyophilized Products after Storage for 3 months

Secondary Structure of F(ab')2 : CD spectra at 3 months

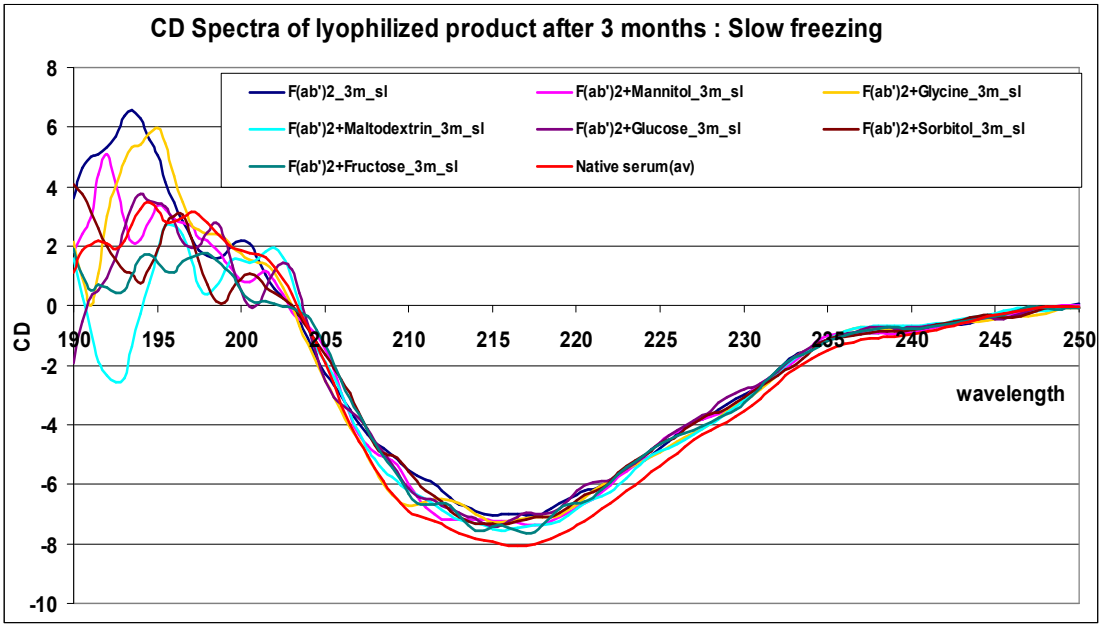


Figure 39 CD spectra of mixtures of F(ab')2 with 2% stabilizers after Slow freezing lyophilization process (stored 3 months at 2-8 °C).

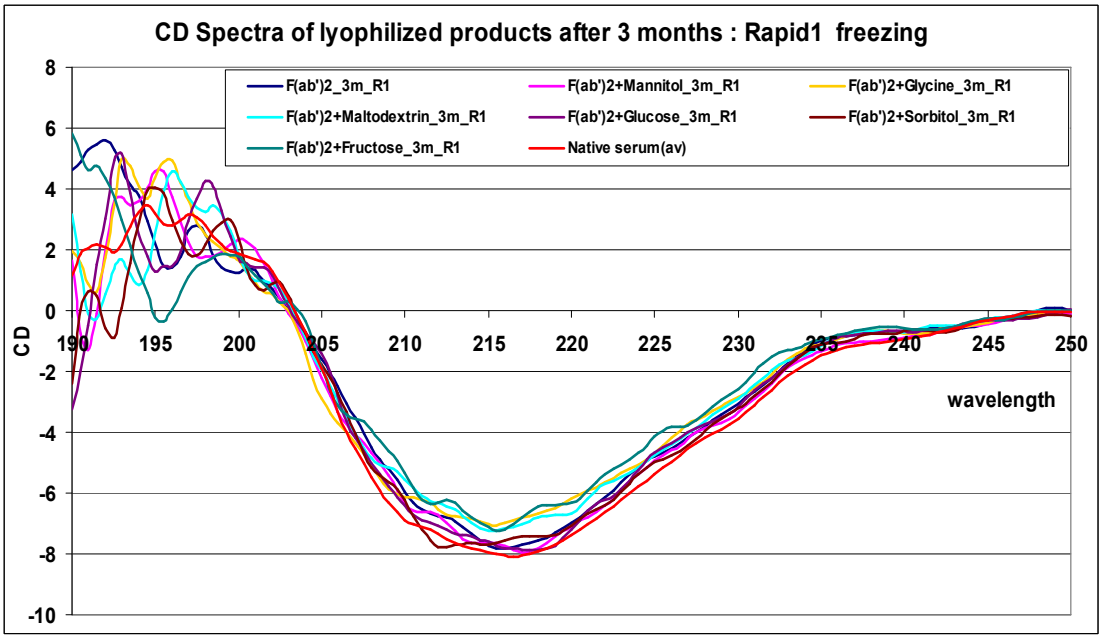


Figure 40 CD spectra of mixtures of F(ab')2 with 2% stabilizers after Rapid1 freezing lyophilization process (stored 3 months at 2-8 °C).

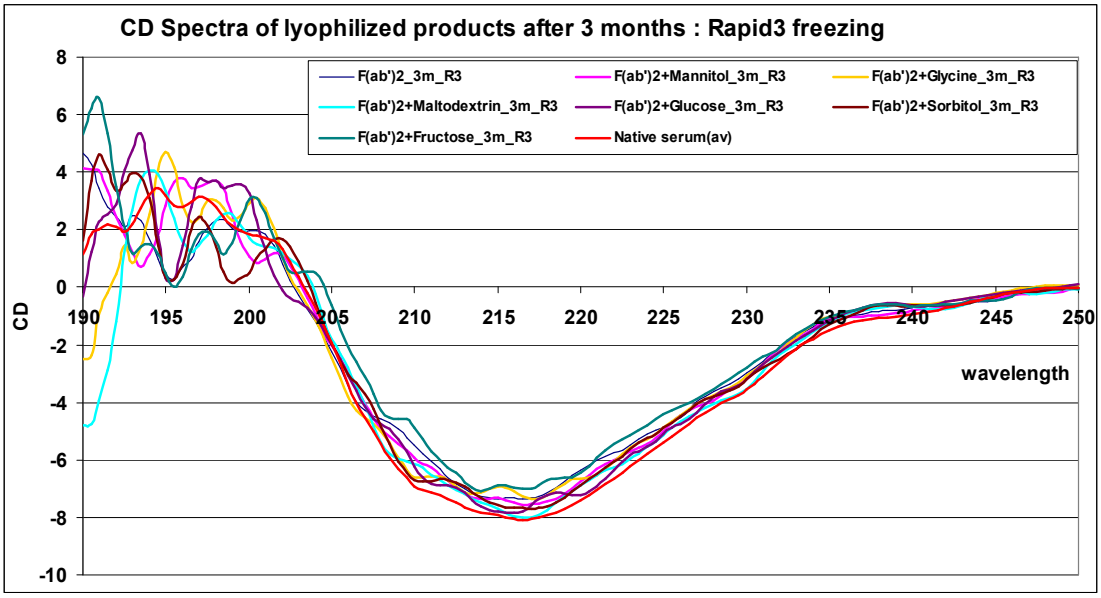


Figure 41 CD spectra of mixtures of F(ab')2 with 2% stabilizers after Rapid3 freezing lyophilization process (stored 3 months at 2-8 °C).

Figures 38-41 display CD spectra of lyophilized products obtained from Slow, Rapid1 and Rapid3 lyophilization process after storage at 2-8 °C for 3 months. These studies agreed well with previous studies that the main CD patterns are of β-sheet, but each formula and process had slight difference in spectral magnitude. They are the effect of formula with different ingredients and different freezing processes.

Table 10 shows CD ellipticity values at 217 nm of lyophilized products which were stored for 3 months at 2-8 °C. The range of CD ellipticity values at 217 nm of lyophilized products when storage for 3 months are -6.954 to -7.647 mdeg for Slow freezing , -6.798 to -7.937 mdeg for Rapid1 freezing and -7.303 to -7.995 mdeg for Rapid3 freezing. Our study found that when lyophilized products are stored for 3 months at 2-8 °C, the CD ellipticity values are close to CD ellipticity values of day0 and native F(ab')2. In addition, the main secondary structure of F(ab')2 is β-sheet structure.

Table 10 CD ellipticity values at 217 nm of of lyophilized mixtures of F(ab')2 with various stabilizers after lyophilized with Slow, Rapid1, Rapid3 lyophilization processes. (Stored 3 months at 2-8 °C)

Sample	CD ellipticity value at 217 nm of lyophilized product		
	Slow Freezing	Rapid1 Freezing	Rapid3 Freezing
$F(ab')_2$ (Ser)	-7.062	-7.690	-7.303
F(ab')2 + Mannitol (SM)	-7.346	-7.937	-7.547
F(ab')2+Glycine (SG)	-7.127	-6.798	-7.355
F(ab')2+Maltodextrin (SMd)	-7.421	-7.038	-7.995
F(ab')2+Glucose (SGu)	-6.954	-7.849	-7.587
F(ab')2+Sorbitol (SS)	-7.173	-7.423	-7.687
F(ab')2+Fructose (SF)	-7.647	-6.807	-7.006

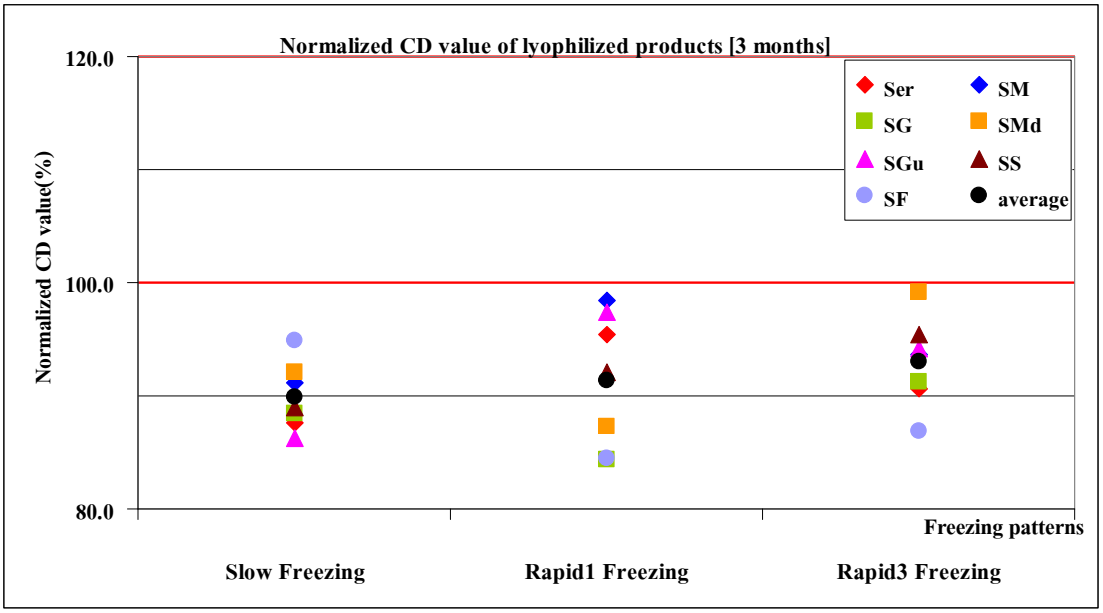


Figure 42 Normalized CD values of lyophilized mixtures of F(ab')2 with 2 % stabilizer when stored at 2-8 °C for 3 months compared to native F(ab')2.

Figure 42 presents Normalized CD values of lyophilized products that were stored at 2-8°C for 3 months. The ranges of normalized CD values of storaged lyophilized products are between 80 to 120%. Average normalized CD values of each freezing patterns are arranged to

from the closest to 100% : Rapid3, Rapid1 and Slow freezing. Lyophilized products when stored for 3 month, Rapid3 freezing shows similar pattern to native F(ab')2 .

From circular dichroism study, it can be concluded that Rapid3 freezing process produced lyophilized products with most stable secondary conformation structure, even after storage for 3 months. In addition, another benefit of Rapid3 freezing process is that it requires the shortest and most energy efficient freezing pattern compare to others.

4.3.1.3. Thermal Analysis Study

Thermal analysis of lyophilized product

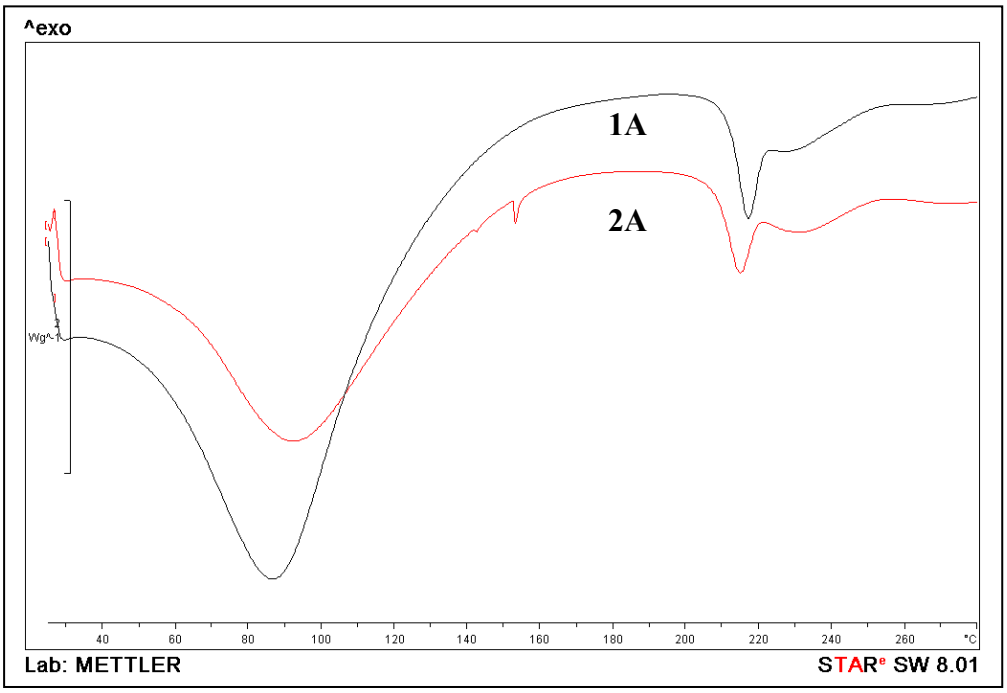


Figure 43 DSC thermogram of Ser lyophilized product obtained from slow freezing process(1A) and rapid3 freezing process(2A).

Figure 43 shows similar DSC thermograms of Ser lyophilized products obtained from Rapid3 and Slow freeezing processes. Starting at 50 °C to 140 °C of endothermic dehydration event. Endothermic peak at 210°C - 220 °C may be due to melting of the products then slight endothermic peak at 230-240°C due to decomposition. In general, thermograms of Ser lyophilized products from Slow freezing and Rapid3 freezing process are the same.

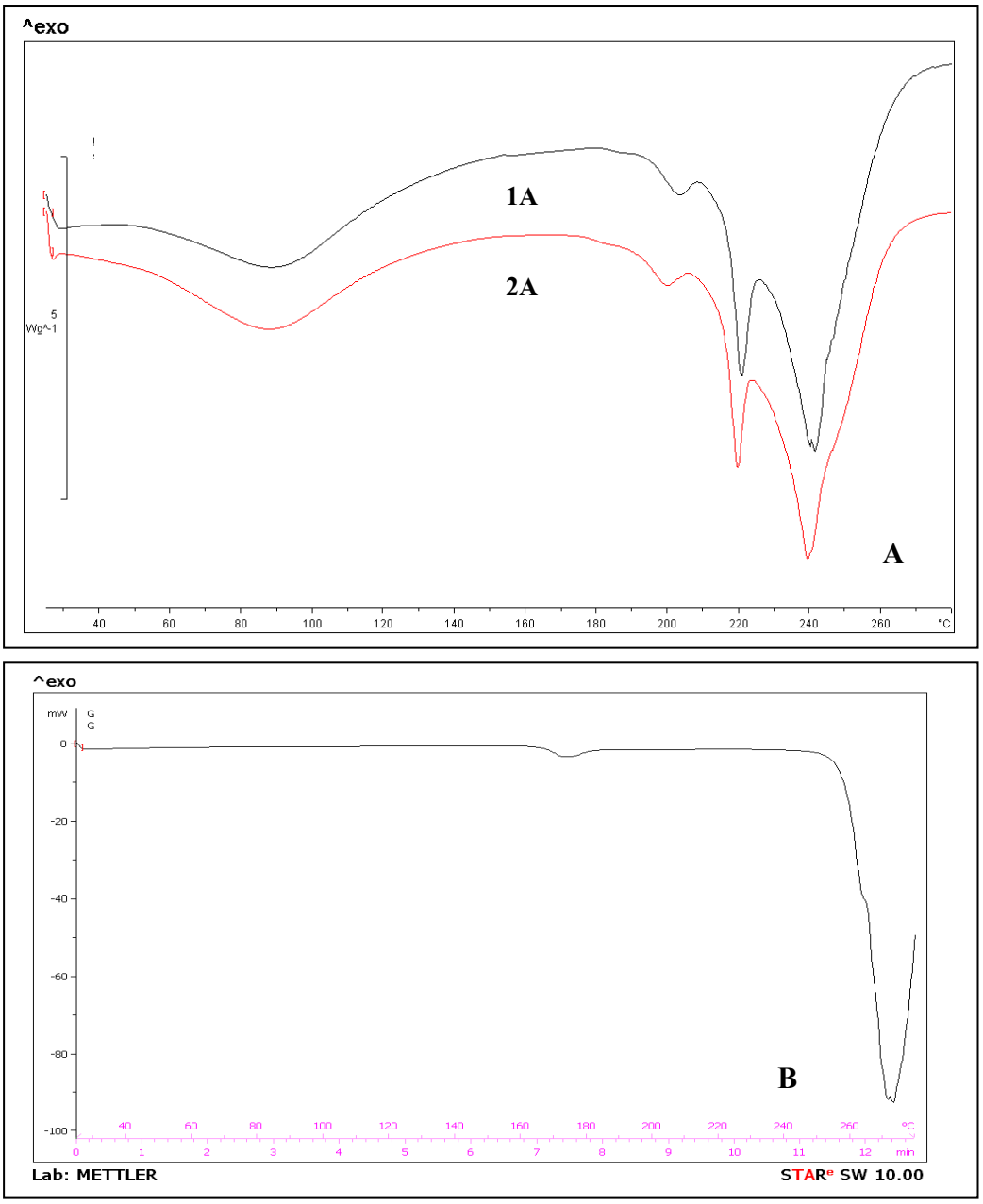
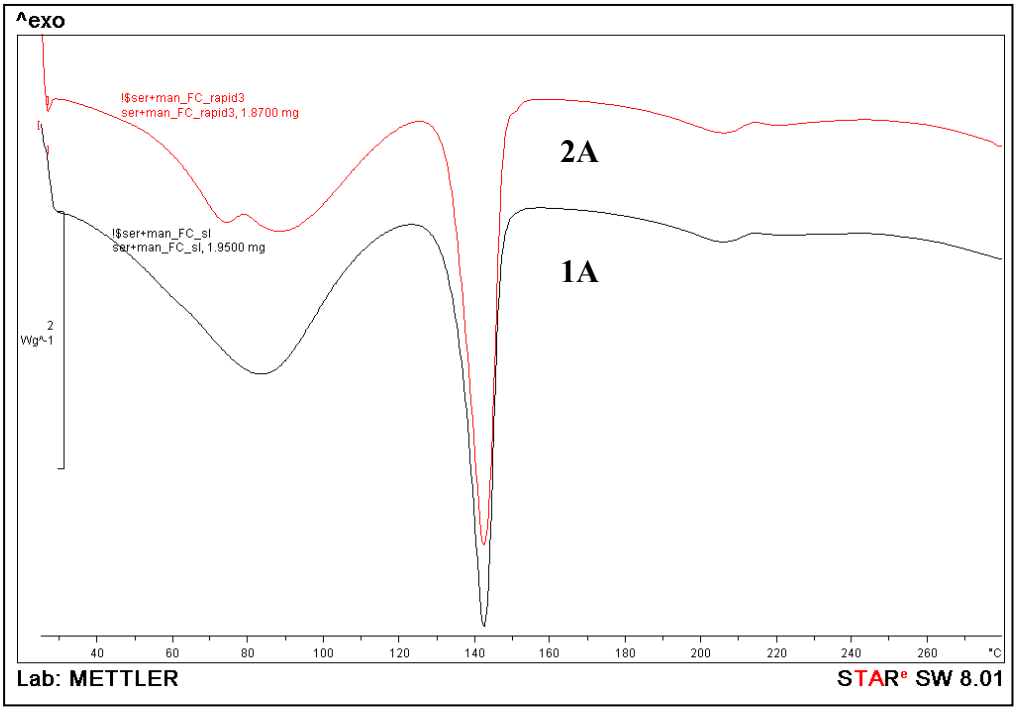


Figure 44 DSC thermograms of lyophilized products obtained from mixture of F(ab')2 and 2% glycine(SG) by slow freezing(1A), Rapid3 freezing process (2A) and pure glycine (B).

Figure 44 shows DSC thermograms of lyophilized products obtained from mixture of F(ab')2 with 2% glycine(SG). The Slow freezing and Rapid3 freezing processes resulted in the same DSC thermograms. Endothermic dehydration occurred at 60°C and Endothermic peaks at about 200, 220°C were melting of glycine and F(ab')2, respectively. Endothermic peaks at 230 - 260 °C are added decompositions of F(ab')2 and glycine. Thus, DSC thermogram of SG

lyophilized products show glycine decomposition peak at temperature slightly lower than pure glycine.



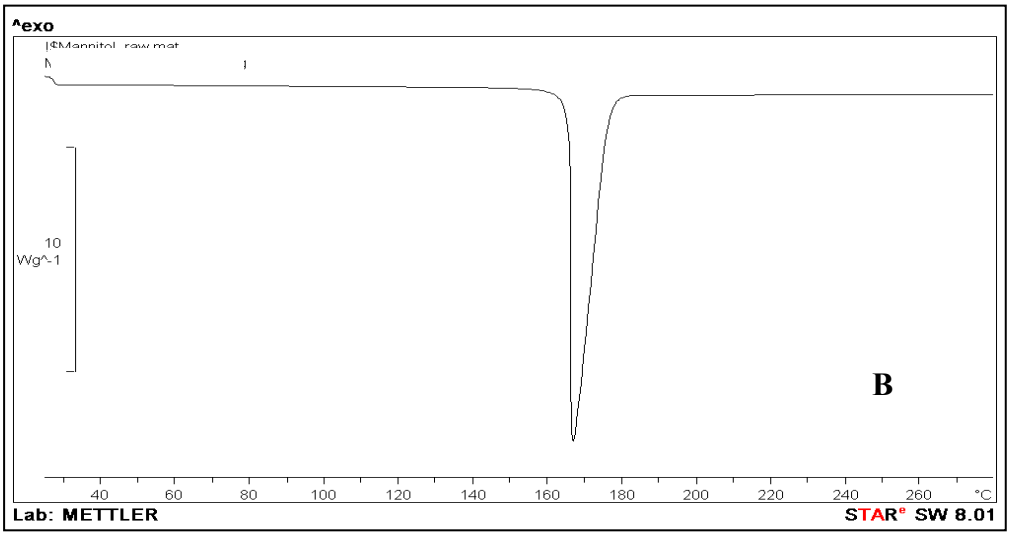


Figure 45 DSC thermogram of lyophilized product mixture of F(ab')2 and 2% mannitol (SM) obtained by Slow freezing (1A), Rapid3 freezing (2A) and pure mannitol (B).

Figure 45 shows DSC thermogram of lyophilized products of mixture of F(ab')2 with 2% mannitol(SM). The DSC thermogram of Slow freezing process resembles that of Rapid3 freezing process. Endothermic dehydration peaks occurr at 40 to 120°C. Endothermic peaks at 140 and 220°C are melting temperatures of mannitol and F(ab')2, respectively. F(ab')2 degrades at approximately 230°C.

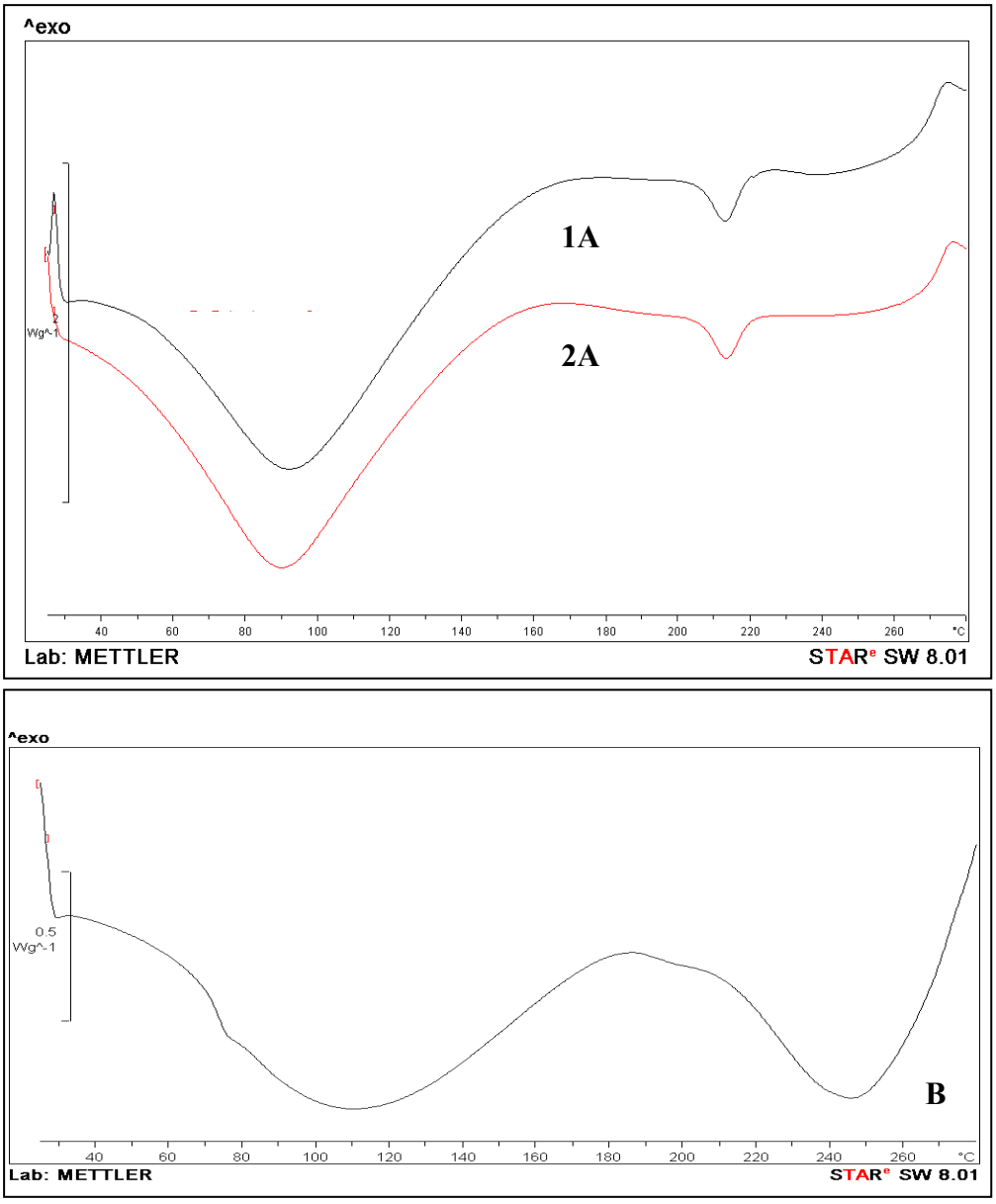


Figure 46 DSC thermogram of lyophilized product mixture of F(ab')2 and 2% maltodextrin (SMd) form Slow freezing(1A) and Rapid3 freezing(2A) and pure maltodextrin (B) .

Figure 46 shows DSC thermograms of lyophilized products of mixture of F(ab')2 with 2% maltodextrin(SMd). The DSC thermogram of Slow freezing lyophilization is similar to Rapid3 thermogram. Endothermic peak at 90°C is used for water evaporation from the product. Endothermic peak at 220°C is melting of F(ab')2. A broad endothermic event from 230-260°C are integrated decompositions of F(ab')2 and maltodextrin.

All DSC thermograms of lyophilized products from Slow freezing are similar to DSC thermograms of lyophilized products obtained by Rapid3 freezing process. The differences in DSC thermograms are due to the different stabilizers used in the formulas. From DSC studies, it can be concluded that various freezing processes used during lyophilization do not have significant impact on the thermal behaviors of the samples.

4.3.1.4 Crystallinity Study

XRPD patterns of lyophilized products

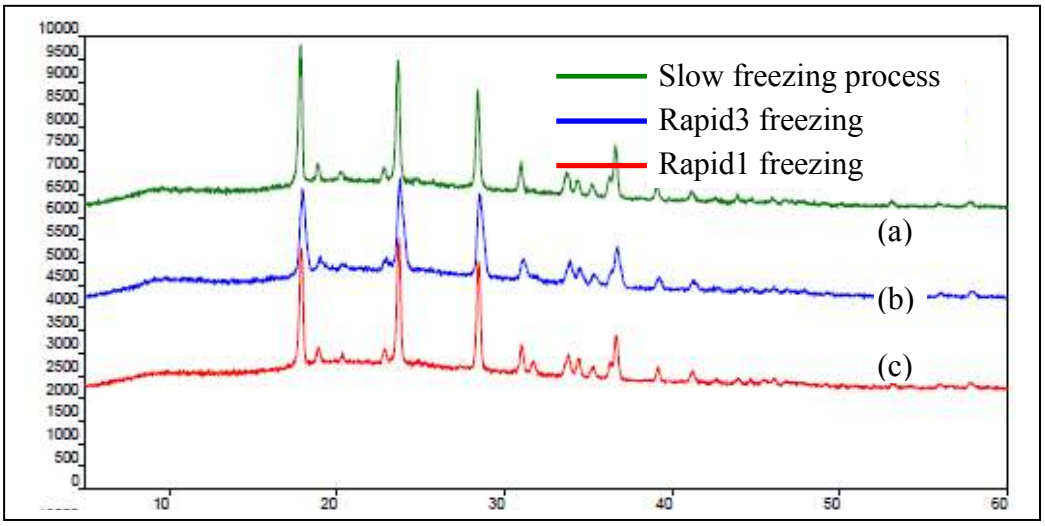


Figure 47 XRPD pattern of lyophilized products of F(ab')2 with 2% glycine mixtures(SG) after Slow, Rapid1 , Rapid3 freezing processes.

Figure 47 shows XRPD diffraction patterns of lyophilized products of F(ab')2 with 2% glycine mixtures (SG). X-ray powder diffraction patterns of every products obtained by various freezing processes are similar with dominant β-glycine peaks at about 18.1, 23.6, 28.6 ° 2θ. These results are consistent with Pyne and Suryanarayanan, (2001); Varshney, (2007) in that

lyophilized glycine obtained from processes as described in Figure 34 (average cooling rate of less than 2 °C/min) led to the formation of β–glycine. In agreement with Chongprasert (2001), which found that freeze drying of glycine solution with slowly freezing (0.1°C/min) produced mainly β–glycine form.

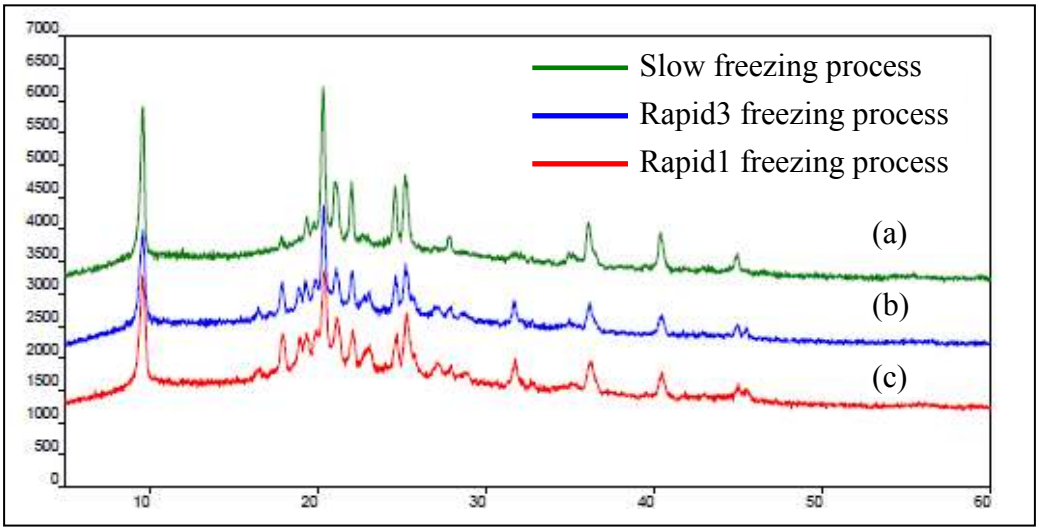


Figure 48 XRPD pattern of lyophilized products of F(ab')2 with 2% mannitol mixtures (SM) after Slow (a), Rapid1 (c) and Rapid3 (b) freezing processes.

Figure 48 shows XRPD patterns of lyophilized products of mixture F(ab')2 and 2% mannitol mixtures(SM). XRPD patterns of every lyophilized product were the same. With dominant peaks at 9.7° and 20.4° 2θ of δ-mannitol. These results showing δ-mannitol characteristics are similar to results obtained by other investigators (Liao, Krishnamurthy and Suryanarayanan, 2007; Hawe and Frieb, 2006; and Torrado and Torrado, 2002).

Figure 49 shows XRPD patterns of lyophilized products F(ab')2 and 2% maltodextrin mixtures(SMd). All of products obtained from lyophilization process did not show signs of crystallinity. This study found that lyophilized product of F(ab')2 and 2% maltodextrin mixtures (SMd) are highly amorphous.This is due to the fact that F(ab')2 are classified as amorphous glycoprotein in nature and maltodextrin which is also amorphous material. Maltodextrin also suppresses crystallinity formation of sodium chloride in the dried products.

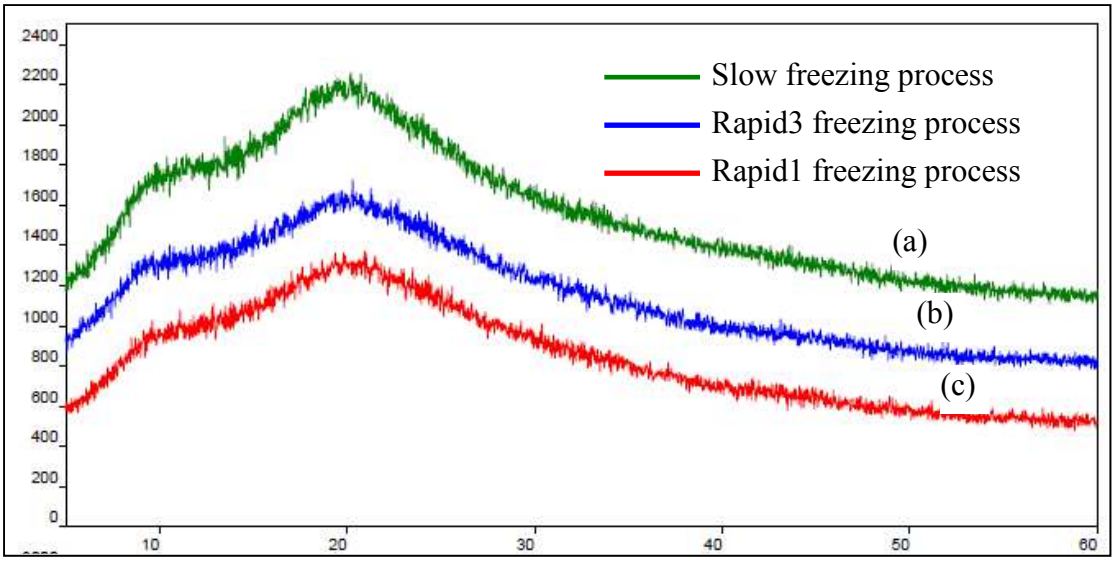


Figure 49 XRPD patterns of lyophilized products of F(ab')2 and 2% maltodextrin mixtures (SMd) after Slow(a) , Rapid1(c) and Rapid3 (b) freezing processes.

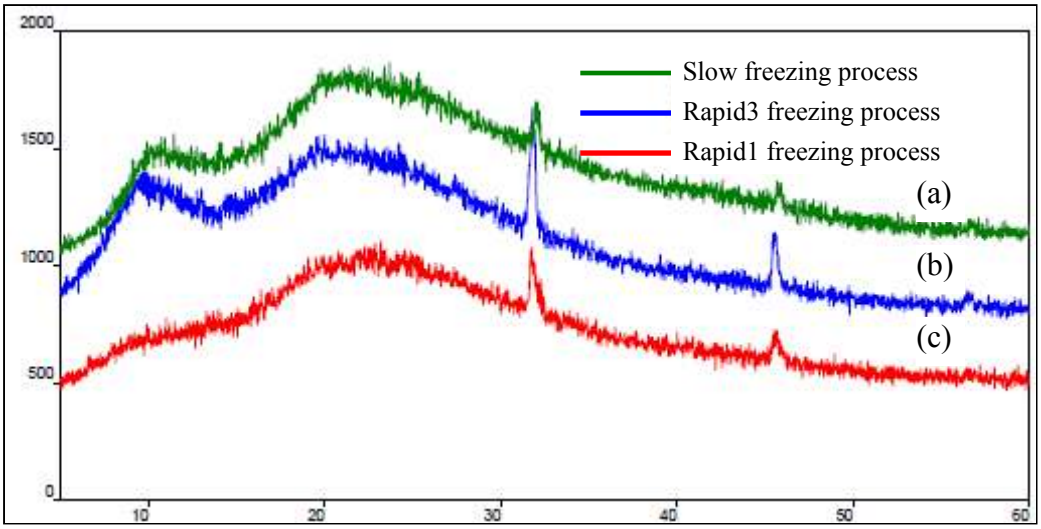


Figure 50 XRPD patterns of lyophilized product of F(ab')2 after Slow(a), Rapid1 (c) and Rapid3 (b) freezing processes.

Figure 50 shows XRPD patterns of lyophilized products of F(ab')2. The XRPD paterns of lyophilized products are mainly amorphous but exhibited crystalline peaks at about 32° 2θ and 45 ° 2θ. F(ab')2 is classified as glycoprotein which characterized as amorphous solid. However, XRPD patterns of lyophilized products show peaks of crystalline character which may be due to sodium chloride in F(ab')2 bulk concentrate (Dixon et al. 2008, Hawe and Frieb, 2006).

Lyophilization Development Process : Freezing Process Evaluation

Physical appearance: The lyophilized products of mixtures F(ab')2 with 2% stabilizers classified 3 groups: Good appearance(SM and SG lyophilized products), Reduced cake volume (Ser and SMd lyophilized products) and Collapsed products(SGu, SF and SS lyophilizedd products). The same products show same appearance when freeze dried different process. Because All lyophilization process set the same primary and secondary drying step. SM and SG lyophilized products had a good appearance because mannitol and glycine in formulas are crystalline material which produced elegant and rigid cake. SGu, SF and SS lyophilized products composed of glucose, fructose and sorbitol, respectively. The glass transition temperature of glucose, fructose and sorbitol are lower than -40°C (Hatley and Franks, 1991 and Kadoya, et al. 2006). Drying temperature of all lyophilization process is higher than the glass transition temperature then products melt and collapsed.

Secondary structure of F(ab')2 . The CD spectra of all lyophilized product display mainly β-sheet conformation. But them showed slightly different in CD ellipticiy value. The best lyophilization cycle which show the lowest of the difference in CD value is Rapid3 freezing. For lyophilized when stored for 3 months, the CD spectra of sample are similar to initial products (Day0). Both 2 condition, the best lyophilzation is Rapid3 freezing.

Thermal properties: DSC thermograms of lyophilized products were analyzed such as SM, SG, SMd and Ser. The same product when freeze dried with different process but show the same thermogram. The DSC thermogram shows peaks of active ingredient (F(ab')2) and composition of each formula.

Crystallinity: The XRPD pattern of same products when freezd dreid with different process are similar. SM lyophilized products show crystalline peaks of δ-mannitol and SG lyophilized products display crystalline peak of β-glycine. While all of lyophilized products show amorphous baseline of F(ab')2. SMd lyophilized products show amorphous base line of both maltodextrin and F(ab')2. Ser lyophilized products display mainly amorphous and exhibit crystalline peak which may be peak of sodium chloride (Hawe and Frieb, 2006 and Dixon, 2008).

4.3.2 Primary and Secondary Drying Process Evaluation.

Table 11 Optimization of Primary drying cycle during the lyophilization process of F(ab')2 Green Pit Viper antivenin.

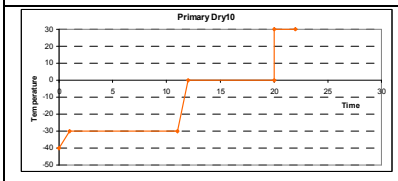
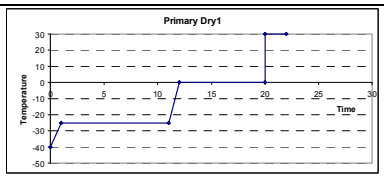
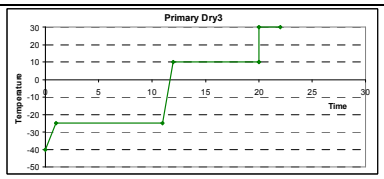
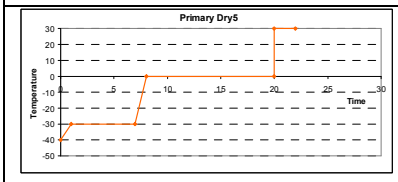
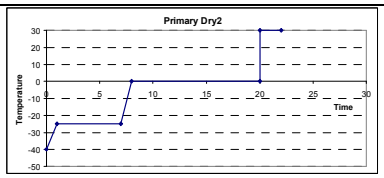
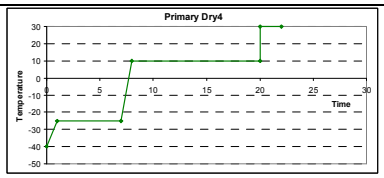
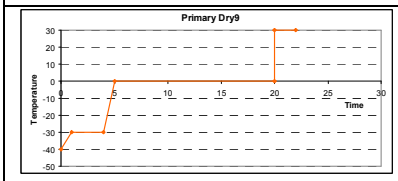
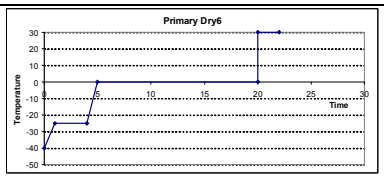
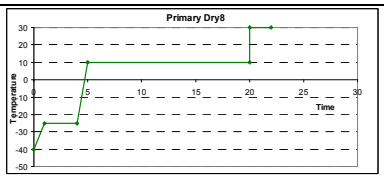
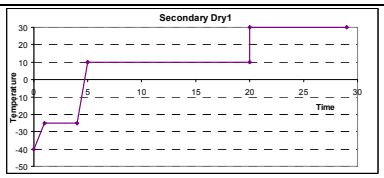
Optimizing of Primary drying cycle		
Primary dry temperature :-30 °C, 0 °C	**Primary dry temperature :-25 °C, 0 °C**	**Primary dry temperature :-25 °C, 10 °C**
 PrimaryDry10	 PrimaryDry1	 PrimaryDry3
 PrimaryDry5	 PrimaryDry2	 PrimaryDry4
 PrimaryDry9	 PrimaryDry6	 PrimaryDry8
		 SecondaryDry1

Table 11 shows various primary drying cycles to find the optimized lyophilization process. Rapid3 freezing was used in freezing step in all the lyophilization cycles. Lyophilization cycles were divided into 3 groups depending on the drying temperature steps selected. Lyophilization cycle group1 (PrimaryDry1, PrimaryDry2 and PrimaryDry6) controlled the temperature at -25 °C and 0 °C with varying drying duration. Lyophilization cycle group2 (PrimaryDry3, PrimaryDry4 and PrimaryDry8) controlled the primary drying temperature at -25 °C and 10°C with various drying duration. Lyophilization cycle group3 (PrimaryDry10, Primary

Dry5 and PrimaryDry9) controlled the temperature for primary drying at -30 °C and 0 °C. SecondaryDry1 (30 °C for 9 hours, pressure 100 mTorr) was used in the lyophilization process after Primary8 was done. The duration of secondary dry1 is 9 hours compared to the secondary drying duration done in all other cycles of only 2 hours (30 °C for 2 hours, pressure 100 mTorr).

4.3.2.1 Appearance of Lyophilized Product

Table 12 Pictures of F(ab')2 lyophilized products obtained by various primary drying cylces during the lyophilization process

Lyophilization process		Lyophilized products			
		SG	SM	SMd	Ser
Lyophilization cycle group 1	PrimaryDry1				
	PrimaryDry2				
	PrimaryDry6				
Lyophilization cycle group 2	PrimaryDry3				
	PrimaryDry4				
	PrimaryDry8				

Table 12 Pictures of F(ab')2 lyophilized products obtained by various primary drying cylces during the lyophilization process (continue).

Lyophilization process		Lyophilized products			
		SG	SM	SMd	Ser
Lyophilization cycle group3	PrimaryDry10				
	PrimaryDry5				
	PrimaryDry9				
Secondary1					

From Table 12, Lyophilized products of mixture of F(ab')2 with glycine and mixture of F(ab')2 with mannitol (SG, SM) had a good structured cakes. The volumes of cakes were the same volume of filling. When assessment from percent of good lyophilized products (Figure 51), lyophilized of SG and SM had 100 % of good lyphilized products

Lyophilized products of mixture of F(ab')2 with maltodextrin (SMd) were classified reduced cake and collapsed cake which dependent on the conditions of lyophilization cycle. Lyophilized products of SMd shows reduced volume of cakes compared to filling volume and evidence of shrunk cake around the side of the bottles. Collapsed lyophilized products is found with mixture of F(ab')2 with maltodextrin (SMd).

Lyophilized products of F(ab')2 solution (Ser) are classified as havin shrunk cakes and collapsed cakes which dependent on the condition of lyophilization cycle.Texture and appearance of Ser are similar to SMd but collapse to more severe degree.

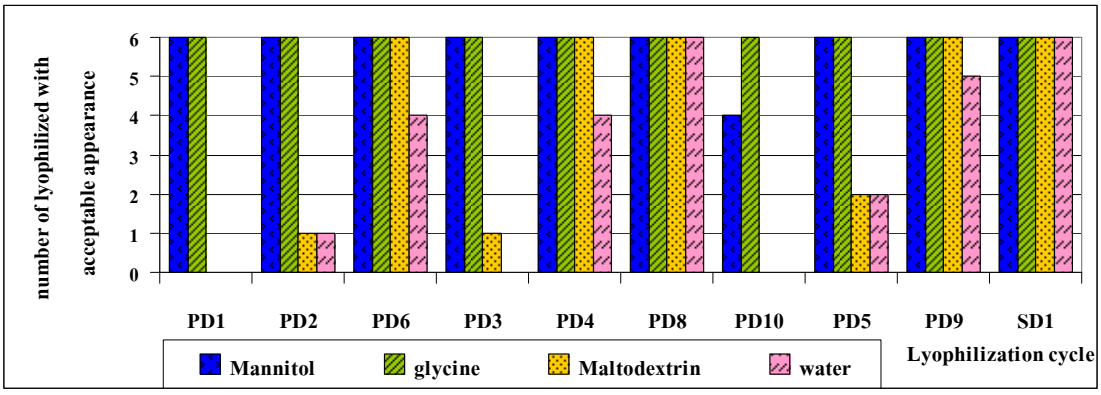


Figure 51 Histograms member of acceptance of lyophilized products obtained by various primary drying cycles (n = 6)

Table 13 Condition of lyphilization cycle

Grop1					**Group2**					**Group3**				
DT1	Time(hr)	DT2	Time(hr)	**Cycle**	DT1	Time(hr)	DT2	Time(hr)	**Cycle**	DT1	Time(hr)	DT2	Time(hr)	**Cycle**
	10		8	**PD10**		10		8	**PD1**		10		8	**PD3**
-30	6	**0**	12	**PD5**	**-25**	6	**0**	12	**PD2**	**-25**	6	**10**	12	**PD4**
	3		15	**PD9**		3		15	**PD6**		3		15	**PD8**

DT1 = Drying temperature step1 DT2 = Drying temperature step2

According to Table 13 and 11, The lyophilization cycles were classified into 3 groups by primary drying temperatures. The drying temperatures were divided further to 2 steps, drying temperature1 (DT1) and drying temperature2 (DT2). While each group has the total duration of drying of 18 hours. Figure 56 show number of lyophilized products with acceptable appearance from every lyophilization cycles. Comparing Table 10, 11 and Figure 56 it is obvious that

primary dying “duration” has more pronounced effect on the physical appearance of the products than primary drying “temperatures” used. However, significant results were mainly due to duration of exposure to drying temperature in step2 (DT2). Acceptable appearances of products were achieved with the longest drying duration (PD9, PD6 and PD8) using drying temperatures in step2 (DT2). Lyophilization of all products should use PD8 or SD1 primary drying cycles to obtain acceptable lyophilized products. The lowest energy used for drying, PD10 lyophilization cycle, produced the lowest percentage of good lyophilized products. While the highest energy used for drying, PD8 lyophilization cycle, produced the highest percentage of good lyophilized products. The physical stability of products depend mostly on the effective removal of residual moisture. Lowest energy drying may not be sufficient to effectively remove all the residual water resulting in product collapse. On the other hand, highest energy drying may be highly effective in the removal of water and produce a very stable rigid structure. From this study, PD8 and SD1 lyophilization cycle are proved to be optimal for F(ab’)2 lyophilization.

4.3.2.2 Secondary Structure of Initial (Day0) F(ab’)2 Lyophilized Products

4.3.2.2.1 CD spectra of initial Ser lyophilized products (day0)

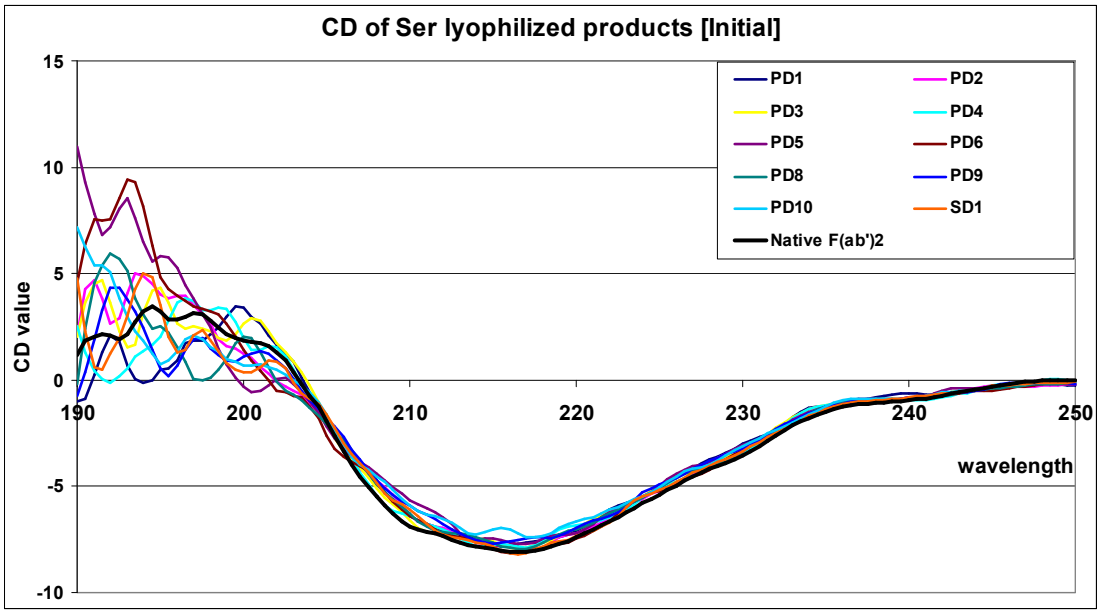


Figure 52 CD spectra of Ser lyophilized products obtained by using various primary drying cycles

4.3.2.2.2 CD spectra of initial SM lyophilized products (day0)

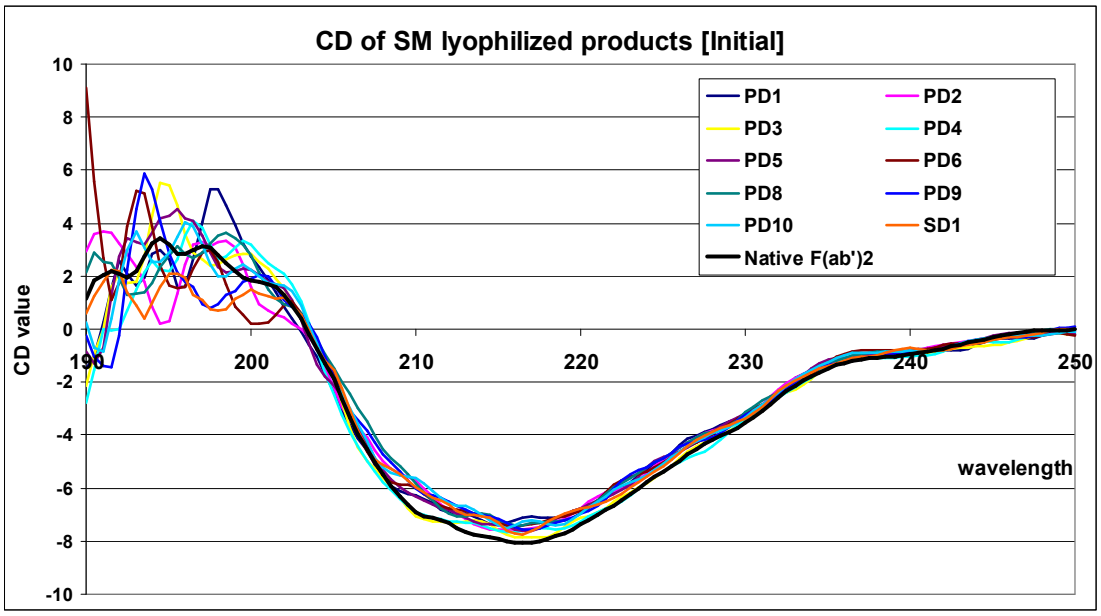


Figure 53 CD spectra of SM lyophilized product obtained by using various primary drying cycles.

4.3.2.2.3 CD spectra of initial SG lyophilized products (day0)

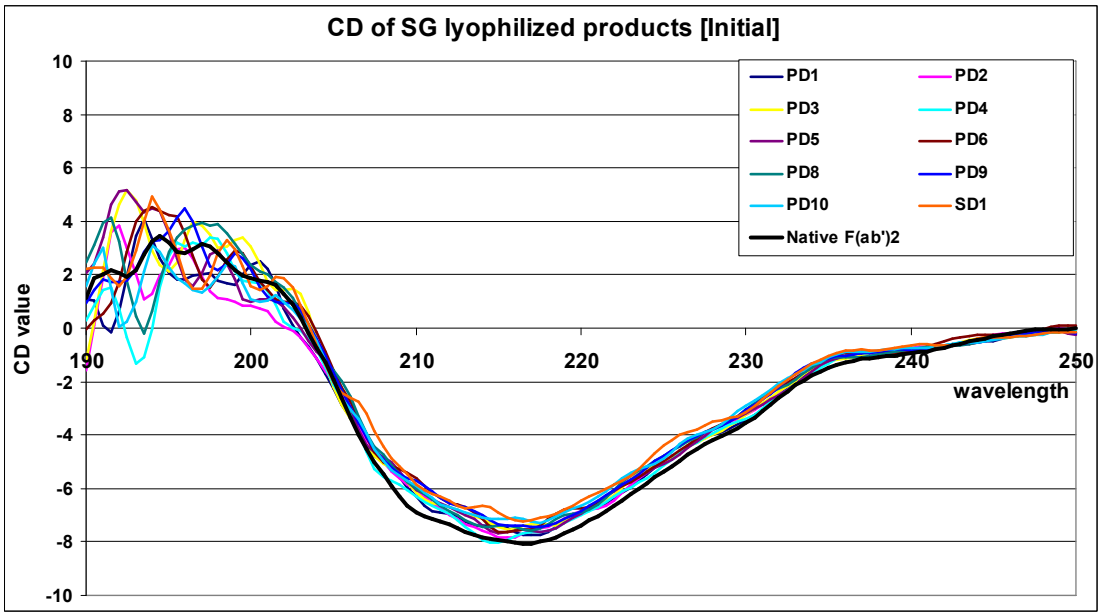


Figure 54 CD spectra of SG lyophilized products obtained by using various primary drying cycles

4.3.2.2.4 CD spectra of initial SMd lyophilized products (day0)

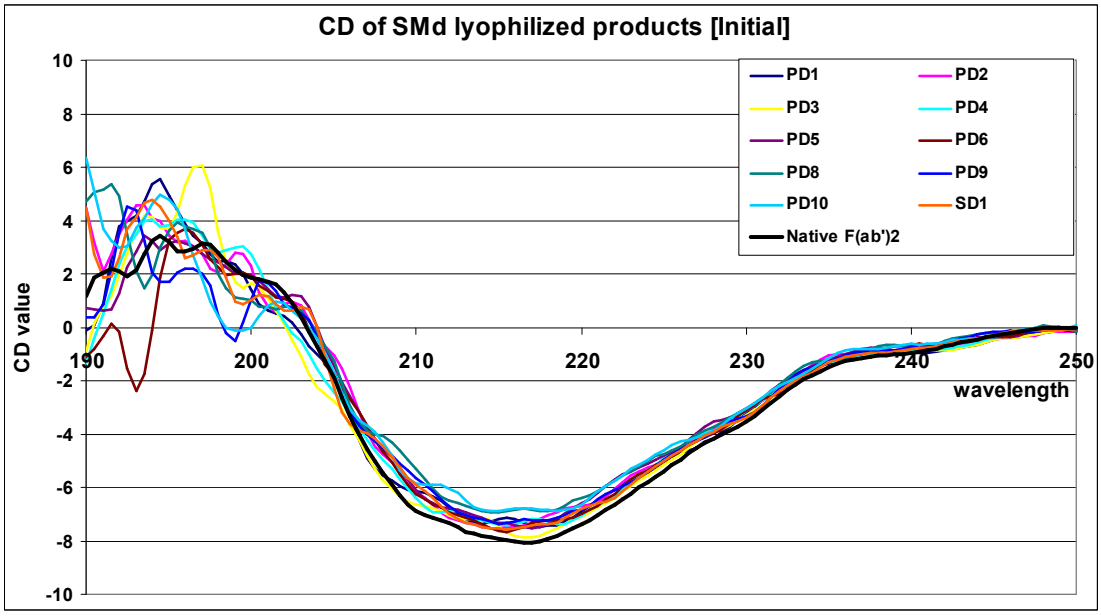


Figure 55 CD spectra of SMd lyophilized products obtained by various primary drying cycles.

Figure 52-55 present CD spectra of Ser, SM, SG and SMd lyophilized products using various primary dying cycles. The conformation mainly displays β-sheet. However, these lyophilized product show minor differences in CD values when compared to native F(ab')2.

Table 14 CD ellipticity values at 217 nm of lyophilized products obtained by various primary drying cycles.

Program	CD ellipticity value at 217 nm of lyophilized products			
	Ser	SM	SG	SMd
PrimaryDry1	-7.646	-7.067	-7.754	-7.424
PrimaryDry2	-7.997	-7.518	-7.589	-7.316
PrimaryDry3	-7.892	-7.851	-7.348	-7.860
PrimaryDry4	-7.883	-7.545	-7.658	-7.197
PrimaryDry5	-7.730	-7.622	-7.607	-7.518
PrimaryDry6	-8.039	-7.356	-7.533	-7.346

Table 14 CD ellipticity values at 217 nm of lyophilized products obtained by various primary drying cycles. (continue)

Program	CD ellipticity value at 217 nm of lyophilized products			
	Ser	SM	SG	SMd
PrimaryDry8	**-7.945**	**-7.327**	**-7.597**	**-6.848**
PrimaryDry9	**-7.428**	**-7.570**	**-7.434**	**-7.210**
PrimaryDry10	**-7.379**	**-7.199**	**-7.218**	**-6.788**
SecondaryDry1	**-8.132**	**-7.624**	**-7.168**	**-7.425**
Native F(ab')2	**-8.060**			

CD ellipticity values in Table 13 are normalized by the CD ellipticity value of native F(ab')2 (-8.060 mdeg for lot no. 09002TA) by equation (1)

$$\text{Normalized CD ellipticity value} = \frac{\text{(CD ellipticity of products)}}{(-8.060)} \times 100 \quad \ldots\ldots\ldots\ldots (2)$$

The normalized CD values obtained are plotted as a function of process and shown in Figure 56.

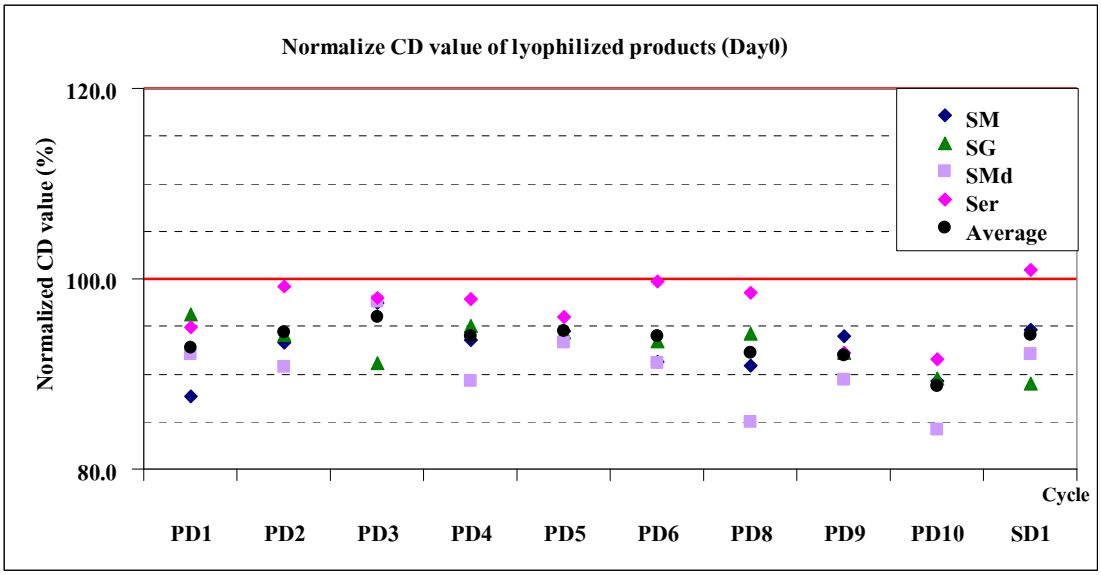


Figure 56 Normalized CD values of lyophilized products from various primary drying cycles.

Normalized CD values of each lyophilization cycles were determined. The average normalized CD values are in the range of not less than 90% (except PD10 cycle). Average of normalized CD value, PD3 cycle is closest to 100%. Ser lyophilized products from several cycle had normalized CD value are similar to 100%. Stabilizers may interact with the structure of F(ab')2 and impact the secondary structure of F(ab')2.

When comparing normalized CD values using criteria that normalized CD value must be within the range 100 ± 10% (90 – 110%). PD2, PD3, PD5, PD6 cycle passed the above criteria. Normalized CD value can be arranged as the following order PD3, PD5, PD2 and PD6. This conclusion is from the conformation of protein conformation alone. To selected optimal lyphilization process, one must determine other important factors involving quality of lyophilized products such as physical appearance and stability, etc.

4.3.2.3 Secondaty Structure of Lyophilized Products Storage for 3 months.

4.3.2.3.1 CD spectra of lyophilized products of Ser after storaged for 3 months

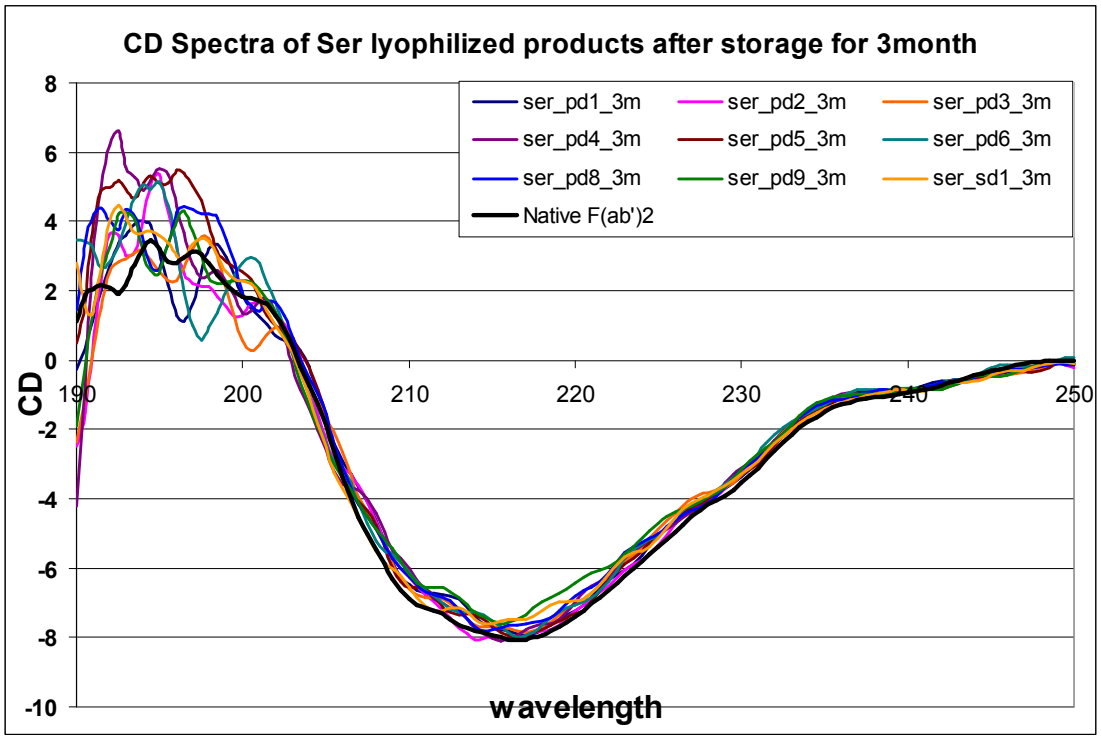


Figure 57 CD spectra of Ser lyophilized products when storage at cold condition for 3 months.

Figure 57 presents CD spectra of Ser lyophilized product after each primary drying cycle when stored at cold condition for 3 months. The conformation mainly displayed β-sheet. However, the lyophilized products show minor differences in the magnitude of CD ellipticity value compared to native F(ab')2.

4.3.2.3.2 CD spectra of lyophilized products of SM when storaged for 3 months

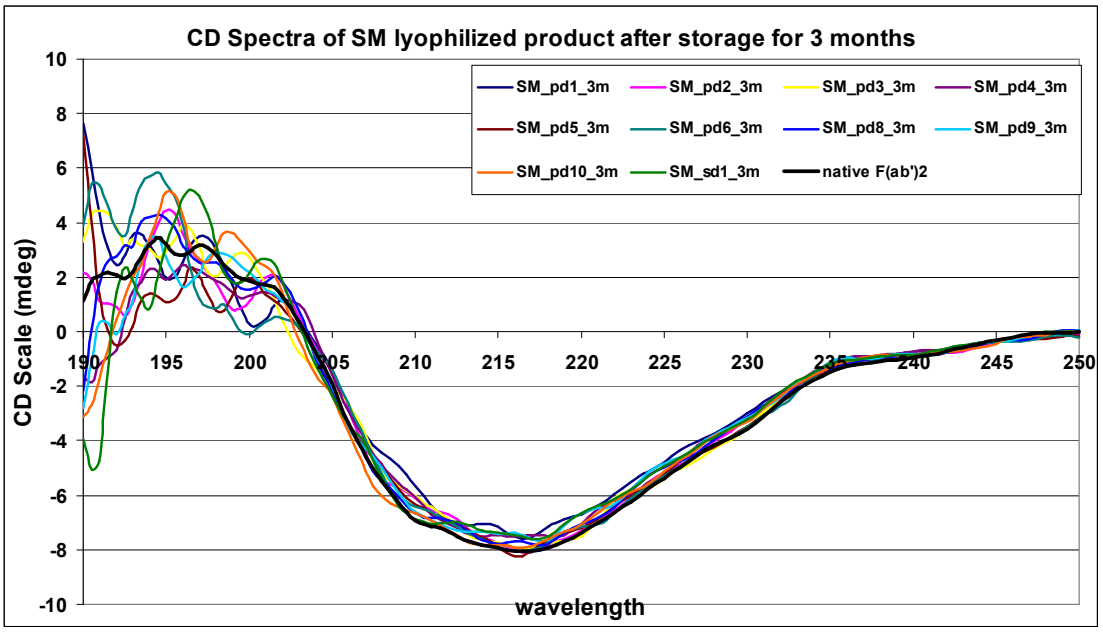


Figure 58 CD spectra of SM lyophilized product after storage at cold condition for 3 months.

Figure 58 presents CD spectra of SM lyophilized product when stored at cold condtion for 3 months. The products display mainly β-sheet conformation. However, minor differnce in magnitude of CD ellipticity values are seen when compare to native F(ab')2.

4.3.2.3.3 CD spectra of lyophilized products of SG when storaged for 3 months

Figure 59 presents CD spectra of SG lyophilized product after stored at cold condition for 3 months. The conformation remained mainly β-sheet structure. However, lyophilized product show minor differences in magnitude of CD values when compared to native F(ab')2.

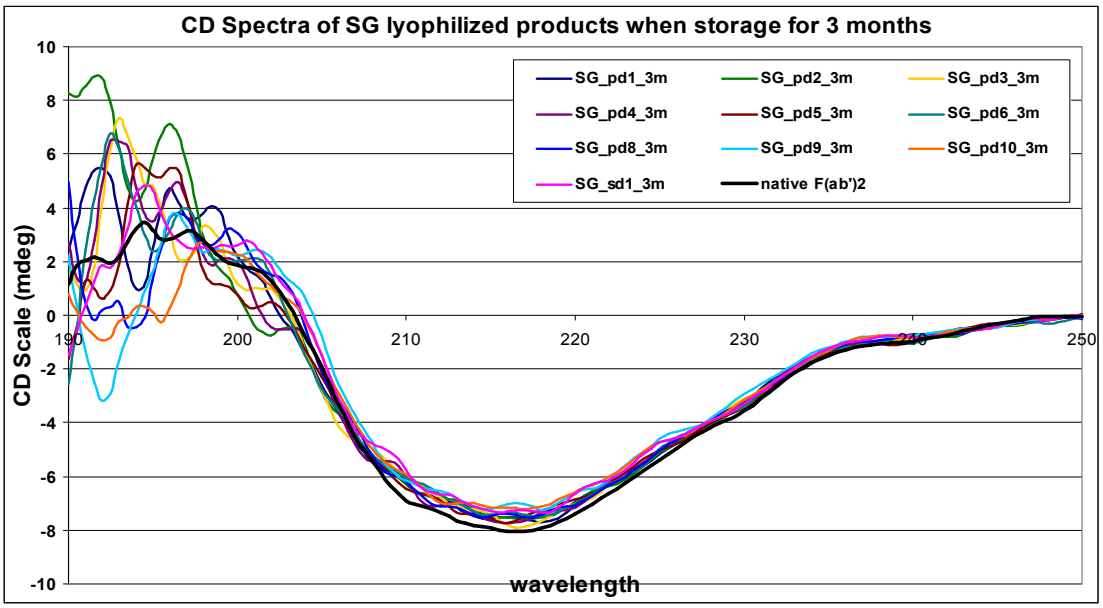


Figure 59 CD spectra of SG lyophilized product after storage at cold condition for 3 months

4.3.2.3.4 CD spectra of lyophilized products of SMd when storaged for 3 months

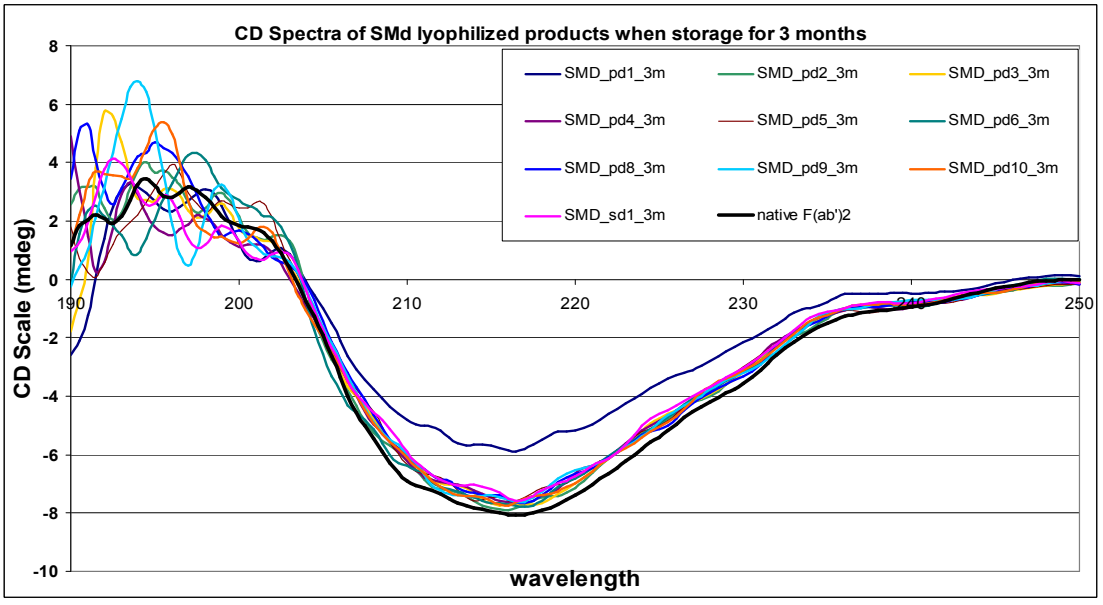


Figure 60 CD spectra of SMd lyophilized products after storage at cold condition for 3 months

Figure 60 presents CD spectra of SMd lyophilized product after stored at cold condition for 3 months. The conformation remained mainly β-sheet. However, lyophilized product show minor differences in CD values when compared to native F(ab')2, except for product obtained by PD1.

Table 15 CD ellipticity values at 217 nm of lyophilized products after storage at cold condition for 3 months.

Program	CD ellipticity value at 217 nm of lyophilized products			
	Ser	SM	SG	SMd
PrimaryDry1	**-7.923**	**-7.547**	**-7.601**	**-5.797**
PrimaryDry2	**-7.965**	**-8.087**	**-7.599**	**-7.731**
PrimaryDry3	**-7.844**	**-7.921**	**-7.891**	**-7.719**
PrimaryDry4	**-7.791**	**-7.475**	**-7.656**	**-7.504**
PrimaryDry5	**-7.943**	**-8.065**	**-7.497**	**-7.476**
PrimaryDry6	**-7.961**	**-8.013**	**-7.435**	**-7.787**
PrimaryDry8	**-7.588**	**-7.785**	**-7.537**	**-7.658**
PrimaryDry9	**-7.357**	**-7.622**	**-7.055**	**-7.606**
PrimaryDry10	**-**	**-7.882**	**-7.201**	**-7.498**
SecondaryDry1	**-7.490**	**-7.608**	**-7.263**	**-7.540**
Native F(ab')2	**-8.060**			

CD ellipticity values in Table 13 are normalized by the CD ellipticity value for native F(ab')2 (-8.060 mdeg for lot no. 09002TA) by equation (1) and finalized in equation (2)

$$\text{Normalized CD ellipticity value} = \frac{\text{(CD ellipticity of products)} \times 100}{(-8.060)} \quad \ldots\ldots\ldots\ldots(2)$$

The normalized CD values obtained are plotted as a function of primary drying process and shown in Figure 61.

Figure 61 show normalized CD value of lyophilized products of F(ab')2 mixtures with stabilizers (2% w/v) after storage at cold condition for 3 months. The average normalized CD values that are within the range of 95-105% include PD2, PD3, PD5 and PD6. However, both initial samples and storaged samples should be considered along with other chemical and physical properties of biological parenteral products.

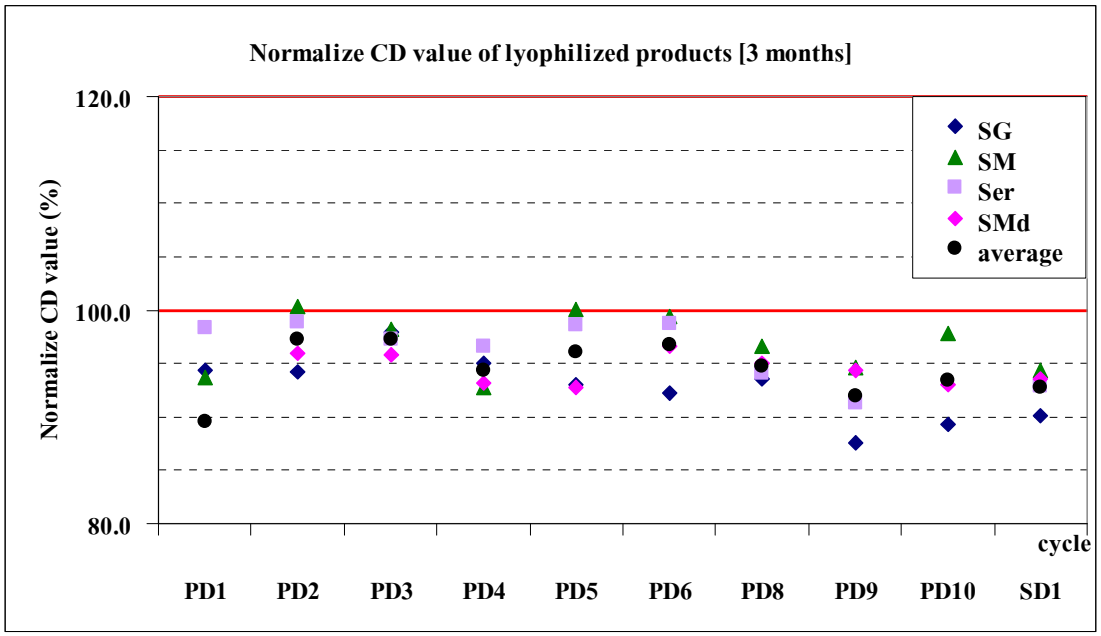


Figure 61 Normalized CD values of F(ab')2 mixtures with stabilizers (2% w/v) lyophilized products after storage at cold condition for 3 months.

The Evaluation of results from Circular dichroism

Because lyophilized products were analyzed for both initial (Day0) and stored sample (3 months). Thus, the secondary structures are determined with selection criteria for the appropiate drying cycle as follow.

1. The average normalized CD value from each cycle must be within the range of 90 -110 %.
2. None of the individual are out of 90 -110% range.

Initial lyophilized products (day0) found that PD2, PD3, PD5 and PD6 met the requirements. The stored lyophilized products (3 months) found that PD2, PD3, PD4, PD5, PD6 and PD8 passed the criteria.

The best lyophilization process that passed both criterion are PD2, PD3, PD5 and PD6. However, toselect the optimal lyophilization cycle, must determine other important factors involve in the quality of lyophilized products in the next section.

4.3.2.4 Moisture Content s

4.3.2.4.1 Lyophilized products: initial condition (day0)

A. Thermogravimetry method

Table 16 Moisture contents of lyophilized products obtained from various primary drying cycle by thermogravimetry

Lyophilization cycle	Lyophilized products :			
	Ser	SM	SG	SMd
PrimaryDry1	na	2.477	2.481	5.468
PrimaryDry2	6.312	3.551	4.936	5.191
PrimaryDry3	5.076	1.945	3.157	3.091
PrimaryDry4	5.605	2.267	3.695	7.455
PrimaryDry5	9.390	4.162	2.913	6.845
PrimaryDry6	5.053	2.131	3.193	4.441
PrimaryDry8	4.309	5.143	3.287	4.880
PrimaryDry9	5.748	3.731	4.163	8.114
PrimaryDry10	Na	2.155	4.761	4.385
SecondaryDry1	6.066	3.417	4.080	3.012

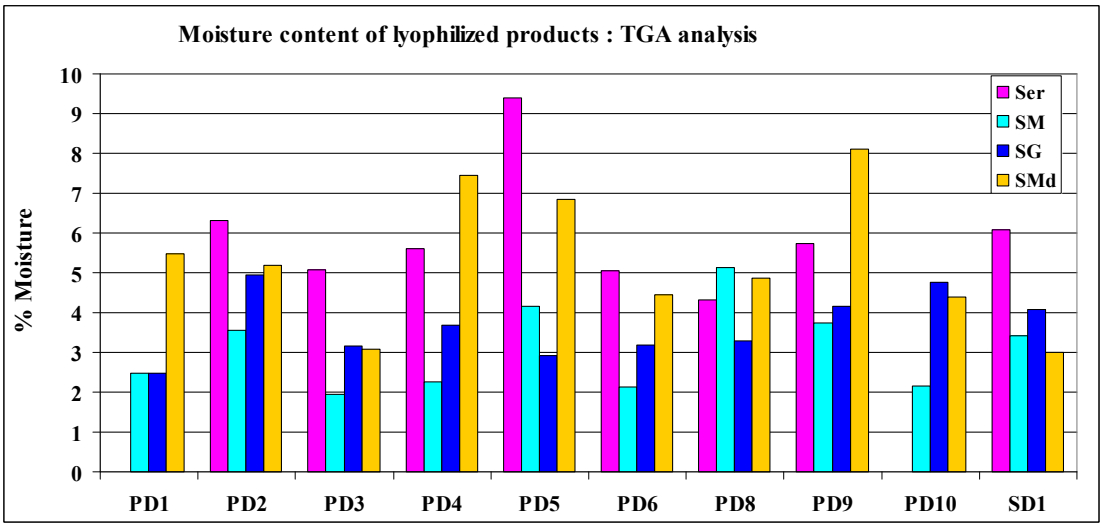


Figure 62 Graphs of moisture contents of lyophilized products obtained from various primary drying cycle by thermogravimetry

Table 16 and figure 62 show moisture content of lyophilized products by TGA analysis. Ser lyophilized products obtained by various primary drying cycles had highest average moisture contents and range of moisture contents of 4.309 – 9.390 %. However, Ser obtained by PD1 and PD10 can not be analyzed since the textures of the products are not dried completely and became highly viscous. The range of moisture contents of lyophilized products can be arranged as follow: Ser > SMd > SM > SG. Generally, moisture content of lyophilized products should not be more than 3.0 % which for SM and SG lyophilized product had a slightly more than criteria. Limitation in study is that the lyophilizer does not cap the vials in vacuum condition automatically when the lyophilization cycle is finished, air was released in the chamber and vials were closed manully. Because lyophilizer does not close vials under vacuum condition, moisture from the environment may have contact the lyophilized products before the vials were sealed, resulting in diverse moisture contents.

B. Karl Fischer method

Table 17 Moisture contents of lyophilized products obtained from various primary drying cycles by Karl Fischer method.

Lyophilization cycle	Lyophilized products :			
	Ser	SM	SG	SMd
PrimaryDry1	**na**	**2.440**	**1.358**	**9.200**
PrimaryDry2	**12.512**	**4.087**	**1.117**	**3.740**
PrimaryDry3	**na**	**2.714**	**1.625**	**5.818**
PrimaryDry4	**3.256**	**2.789**	**1.491**	**2.822**
PrimaryDry5	**9.576**	**4.429**	**4.449**	**6.603**
PrimaryDry6	**4.828**	**5.171**	**1.169**	**2.148**
PrimaryDry8	**4.493**	**2.896**	**1.818**	**2.808**
PrimaryDry9	**2.985**	**3.440**	**2.377**	**2.081**
PrimaryDry10	**na**	**2.549**	**1.395**	**Na**
SecondaryDry1	**4.828**	**5.171**	**1.169**	**2.148**

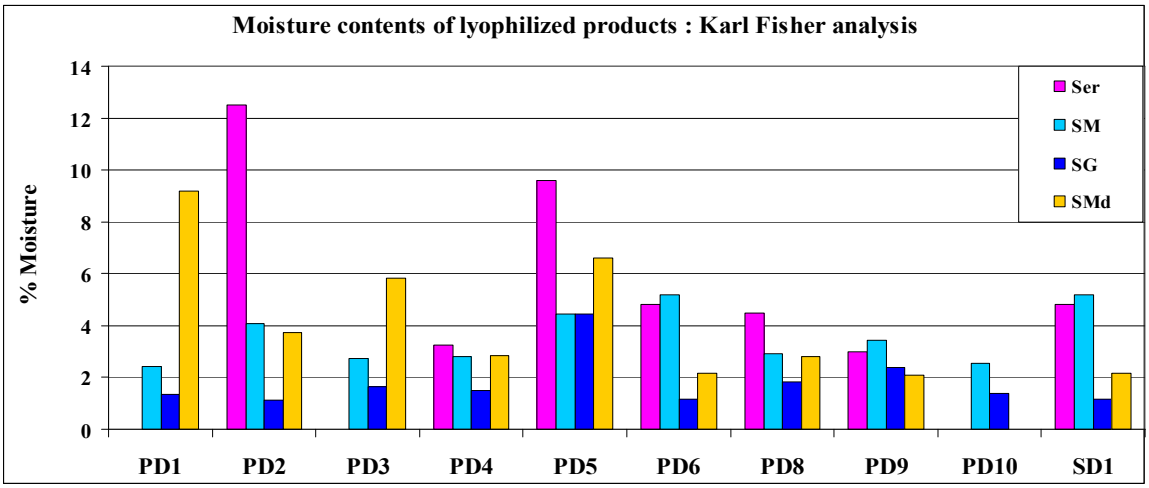


Figure 63 Graphs of moisture contents of lyophilized product obtained from various primary drying cycles by Karl Fisher method.

Table 17 and figure 63 display moisture contents of lyophilzed products obtained from various primary drying cycles by Karl Fisher method. Ser lyophilized products has the highest moisture content. Range of moisture contents are 2.985 – more than 12 %, 2.440 – 5.171 %, 1.117 – 2.377 % and 2.081 – 9.20 % for Ser, SM, SG, and SMd lyophilized products, respectively. Level of moisture contents of products are arranged as follow: Ser > SMd > SM > SG. Our data, moisture content of SM and SG lyophilized products are slightly higher than criteria. Because lyophilizer do not property closed the vial under vacuum condition, so the moisture may be increased within the vieals from the environment.

4.3.2.4.2 Moisture contents : lyophilized products were stored for 3 months

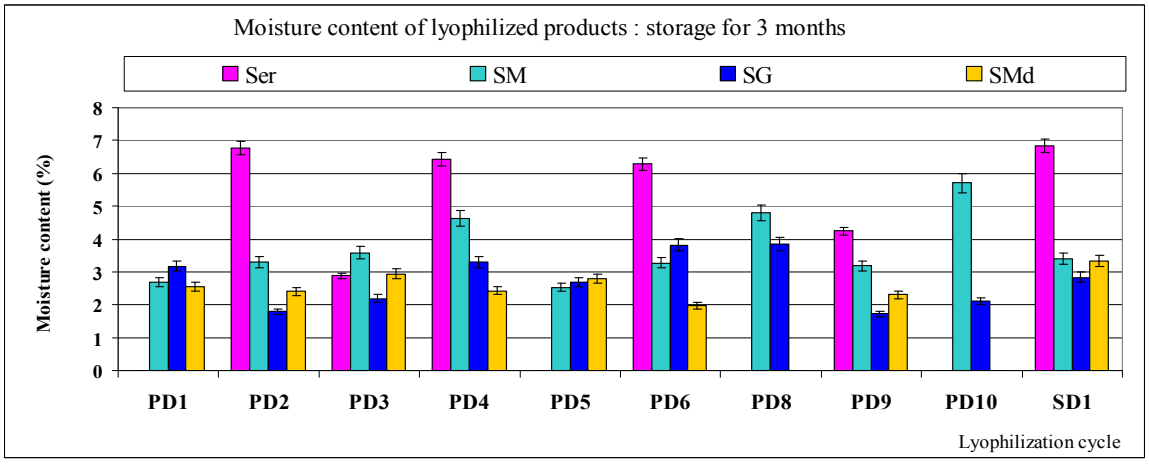


Figure 64 Graphs moisture contents of lyophilized products when storage for 3 months by thermogravimetry method

Table 18 Moisture contents of lyophilized products when were storaged for 3 months by thermogravimetry method

Lyophilization cycle	Lyophilized products :			
	Ser	**SM**	**SG**	**SMd**
PrimaryDry1	na.	2.680	3.183	2.555
PrimaryDry2	6.771	3.298	1.795	2.410
PrimaryDry3	2.879	3.590	2.190	2.934
PrimaryDry4	6.434	4.632	3.309	2.429
PrimaryDry5	na.	2.534	2.704	2.783
PrimaryDry6	6.295	3.280	3.817	1.980
PrimaryDry8	na.	4.813	3.845	na.
PrimaryDry9	4.246	3.190	1.725	2.304
PrimaryDry10	na.	5.710	2.099	Na
SecondaryDry1	6.828	3.394	2.838	3.350

Figure 64 and Table 18 display moisture content of lyophilzed products which were storaged at cold condition (2-8 °C) for 3 months by thermogravimetry method. Ser lyophilized products had the highest average moisture content. Range of moisture contents are 2.879 – 6.828%, 2.534 – 5.710%, 1.725 – 3.845% and 1.980 – 3.350% for Ser, SM, SG, and SMd lyophilized products, respectively. However the changes of moisture contents are dramatic compared to initial products. While the moisture contents of SM and SG lyophilized products remain slightly higher than criteria (3% w/w).

Moisture contents of lyophilized products are similar to moisture content criteria of 3% w/w which SM and SG lyophilized products are most preferable. Conclusion can not be drawn to explain the dramatic differences in moisture contents of initial and storaged products which may be due to uncontrollable vial closure procedure as metioned in the previous section.

4.3.2.5 Crystallinity Properties

4.3.2.5.1 XRPD of lyophilized products: initial condition (day0)

SG lyophilized products

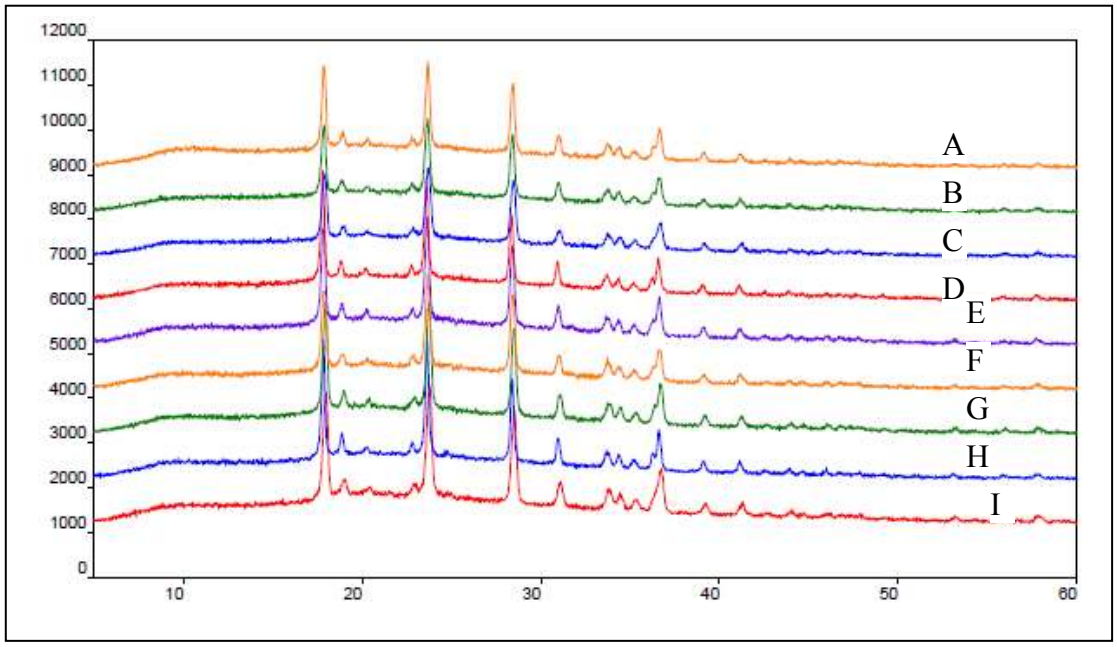


Figure 65 XRPD patterns of SG mixture lyophilized products after various primary drying cycles. secondaryDry1 (A), PrimaryDry10 (B), PrimaryDry8 (C), PrimaryDry6 (D), PrimaryDry5 (E), PrimaryDry4 (F), PrimaryDry3 (G), PrimaryDry2 (H) and PrimaryDry1 (I).

Figure 65 shows XRPD patterns of SG lyophilized product from various primary drying cycles. Every XRPD patterns of SG lyophilized products had the same XRPD patterns with peaks showing similar positions. Main peaks of SG lyophilized products are at 17.9, 23.6, 28.4 ° 2Θ and those positions were determined to be due to glycine in β–glycine form. While the main component, F(ab')2 , are shown as amorphous baseline shift after freeze drying no matter which primary drying cycle were used.

SM lyophilized products

Figure 66 shows XRPD patterns of SM lyophilized products obtained from various primary drying cycles. Every XRPD patterns of SM lyophilized products had the same XRPD patterns. The major peak of SM lyophilized products are at 9.6, 20.3, 21.1, 24.6 and 25.2° 2Θ. Positions of peaks were identified as crystalline δ-mannitol for all primary drying cycles used.

Main component, F(ab')2, resulted in amorphous structure after freeze drying no matter which primary drying cycles were used.

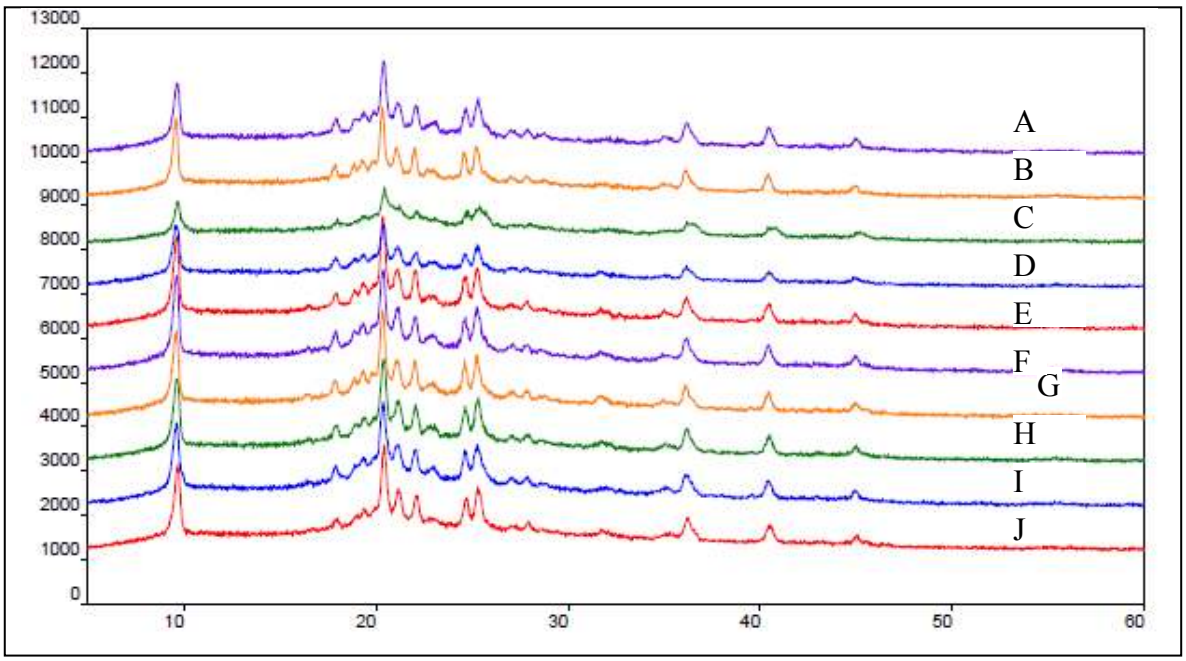


Figure 66 XRPD patterns of SM lyophilized products after various primary drying cycles. SecondaryDry1 (A), PrimaryDry10 (B), PrimaryDry9 (C), PrimaryDry8 (D), PrimaryDry6 (E), PrimaryDry5 (F), PrimaryDry4 (G), PrimaryDry3 (H), PrimaryDry2 (I) and PrimaryDry1 (J).

SMd lyophilized products

Figure 67 displays XRPD patterns of SMd lyophilized products with various primary drying cycles. Every XRPD paterns show amorphous structures and did not present any crystalline peak.Thus, both F(ab')2 and maltodextrin are amorphous after freeze drying and maltodextrin also suppress the crystal formation of sodium chloride in the dried matrix.

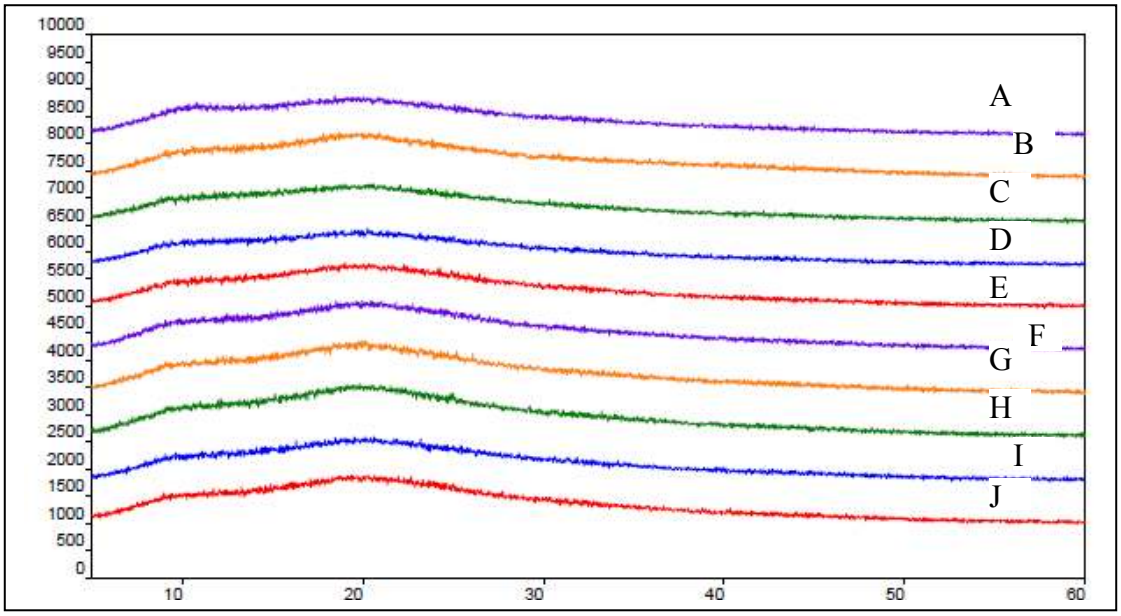


Figure 67 XRPD pattern of SMd lyophilized products after various primary drying cycles. Where SecondaryDry1 (A), PrimaryDry10 (B), PrimaryDry9 (C), PrimaryDry8 (D), PrimaryDry6 (E), PrimaryDry5 (F), PrimaryDry4 (G), PrimaryDry3 (H), PrimaryDry2 (I) and PrimaryDry1 (J).

Ser lyophilized products

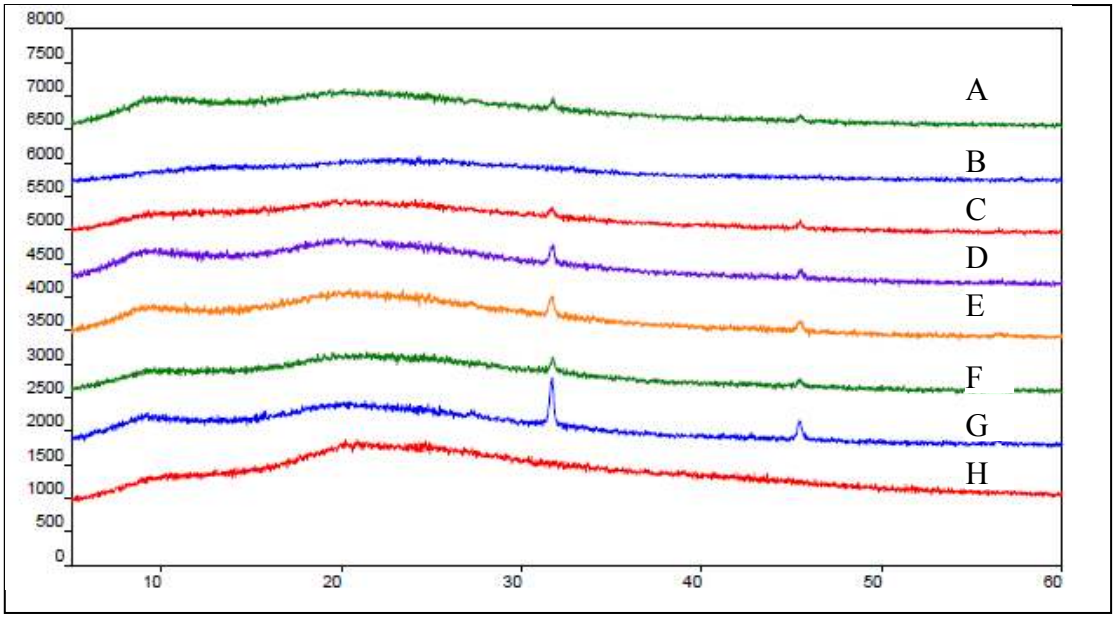


Figure 68 XRPD patterns of Ser lyophilized product after various primary drying cycles, When SecondaryDry1 (A), PrimaryDry9 (B), PrimaryDry8 (C), PrimaryDry6 (D), PrimaryDry5 (E), PrimaryDry4 (F), PrimaryDry3 (G), PrimaryDry2 (H).

Figure 68 presents XRPD patterns of Ser lyohilized products from various primary drying cycles. XPRD patterns of lyophilized products using PD2 and PD9 cycles resulted in amorphous patterns. But when other primary drying cycles were used (PD3, PD4, PD5, PD6, PD8 and SD1) a dominant crystalline peak is present at about 31.7 $^{\circ}$ 2θ and was characterized as sodium chloride (Dixon et al. 2008, Hawe and Frieb, 2006). Sodium chloride is a normal component found in concentrated bulk of F(ab')2.

The XRPD of SG lyophilized products mainly show β–glycine with every primary drying cycles used. SM lyophilized products from every cycles mainly contained δ-mannitol. SMd remain amorphous no matterwhich cycle were used for lyophilization.

When stored lyophilized product at cold condition for 3 months, the XRPD patterns remained unchanged from initial. (Appendix E) So, lyophilized products remain stable without any modification from their initial solid morphology when storaged at cold condition (2-8 °C) for 3 months.

Lyophilization Development Process : Primary and Secondary Drying Evaluation

Physical appearance: SM and SG lyophilzed products are good appearance for all lyophilzation process. Because mannitol and glycine are crystalline excipients which produced elegant cake of lyophilized products.(Passot et al., 2005 and Meyer, et al., 2009) SMd and Ser lyophilized products produced good appearance in some lyophilization cycle depend on condition of lyophilization process. PrimaryDry8 and SecondaryDry1 are the best lyophilization process because produced the maximum of number of acceptance products.

Secondary structure: All of CD spectra exhibit predominanly β-sheet which had slightly in CD ellipticity value. CD spectra of initial product (Day0) and storaged lyophilized products (3 months) of lyophilized products were evaluated and decide for the best of lyophilization process. The criteria were set up follow as: the average of normalized CD value from each cycle are not more than 100 $\pm$ 10% and normalized CD value of all lyophilized products must be within range 100 $\pm$ 10% , these criterias were evaluated both initial and storaged lyophilized products. So lyophilization cycle were passed criteria specified such as PD2, PD3, PD5 and PD6 lyophilization process.

Moisture contents: moisture contents were analyzed from Karl fishcher tiration and Thermogravimetry method. The most of SM and SG lyophilized products had moisture contents about 3% (limit for lyophilized products). Comparison moisture contents of initial products (Day0) and stored lyophilized products (3 months) had a difference in moisture contents which may be lyophilizer could not close vial under vacuum condition and moisture from environment entran in vial.

Crystallinity : All SM lyophilized products from various lyophilization show the same XRPD patterns and All SG lyophilized products from variuous lyophilization show same XRPD patterns together. SM lyophilized products display crystalline peaks of δ-mannitol and SG lyophilized products show crystalline peaks of β-glycine. While SM and SG lyophilized produts exhibit amorphus baseline of F(ab')2. SMd lyophilized product show amorphous baseline of maltodextrin and F(ab')2. Ser lyphilized product show mainly amorphous baseline but Ser lyophilized product from PD3, PD4, PD5, PD6, PD8 and SD1 show crystalline peaks of sodium chloride at about 31.8° 2 Θ (Hawe and Frieb, 2006 and Dixon, 2008).

The results of lyophilized products used for selected optimal lyophilization process. From circular dichroism data, the optimal lyophilization cycle such as PD2, PD3, PD5 and PD6. Moisture content of lyophilized products may be not evaluated from limited of lyophilizer. XRPD pattern of SG, SM, SMd from all cycle were similar. However, Ser lyophilized products show difference of XRPD pattern. Lyophilized products from PD2 and PD9 cycle show amorphous peak which were desired products for our products (pure active ingredients). So CD data and XRPD data were evaluted and selected optimal lyophilization process. The optimal lyophilization process is PD2 lyophilization process which was used for lyophilization in next section.

4.4 Formulation Development of Green Pit Viper Antivenin Lyophilized Products

For this study, 2 lots of F(ab')2 concentrated bulk were used.

- Lot no. 09002TA : was used in process adjustment in parts 3.2 -3.3
- Lot no. 10002TA : was use in formulation part 3.4

Table 19 Green Pit Viper antivenin F(ab')2 concentrated bulks and their properties

Lot no. F(ab')2 Concentrated bulk	Potency (mg/ml)	Protein content (%)	pH	Osmolarity (Osm/kg)
09002TA	2.64	8.78	6.50	296
10002TA	2.76	7.90	6.63	302

Table 19 shows important properties of two lots of F(ab')2 concentrated bulk, such as potency, protein content, pH and osmolarity. Potency, pH and osmolarity of lot no.10002TA were slightly higher than lot no.09002TA. However, protein content of lot no. 09002TA was slightly higher than lot no. 10002TA. These small differences in properties of the F(ab')2 concentrated bulk lot no. 09002TA and lot no. 10002 TA did not affect their freeze drying behaviors.

Formulation was done under the assumption of products to be used parenterally. Osmolarity of each formulation was controlled at about 300 mOsm/kg. $F(ab')_2$ was formulated with stabilizers that produced good lyophilized cake(glycine and mannitol). Formulations of green pit viper antivenin are as follow

1. 2SG = mixture of F(ab')2 (0.7 mg/ml) with 2% glycine
2. 4SM = mixture of F(ab')2 (0.7 mg/ml) with 4 % mannitol
3. SGM = mixture of F(ab')2 (0.7 mg/ml) with 1 % glycine and 1.5% mannitol
4. SGc = mixture of F(ab')2 (1.4 mg/ml) with 4% glycine
5. SMc = mixture of F(ab')2 (1.4 mg/ml) with 8 % mannitol

Table 20 also shows osmolarity and pH of 5 formulation freeze drying. Osmolarities were divided into two groups. First group show 335, 296 and 299 mOsm/kg for 2SG, 4SM and SGM, respectively. For SGc and SMc formula had very high osmolarity due to the concentrations of solutes. After lyophilization, the SGc and SMc are reconstituted with two fold (4 ml) of

purified water. Generally, osmolarity of parenteral dosage forms should be formulated to obtain 300 mOsm/kg. In this study, all of the formulations passed the above criteria.

Table 20 Osmolarity and pH of Green pit viper antivenin formula

Formula	Total volume produced (ml)	Osmolarity (Osm/kg)		pH
		Before lyophilization	After lyophilization *	
2SG	4	335	321	6.24
4SM	4	296	295	6.28
SGM	4	299	292	6.26
SGc*	2	645	318	6.26
SMc*	2	600	290	6.32

* Volume of reconstitution after lyophilization is 4 ml

Reconstitution with 4 ml of purified water for adjustment to 0.7 mg/ml of F(ab')2. Thus, initial osmolarity of SGc was 322.5 mOsm/kg and SMc was 300 mOsm/kg.

4.5 Evaluation of Lyophilized Green Pit Viper Antivenin Products

Green pit viper antivenin formulations were lyophilized using PrimaryDry 2 (PD2) cycle. When the cycles are terminated, lyophilized products were analyzed according to the following.

4.5.1. Physical Appearance

Appearances and textures of lyophilized products are presented in Table 27. The cakes of all formulas appear good and stable due to crytalline structure of mannitol and glycine stabilizers. 2SG, SGc, 4SM and SMc resulted in lyophilized products of similar dried volume to initial volume of filling. Lyophilized SGM had slightly shrinked structurefrom the initial volume. From this data, appearance, all products met specification of having good cake or texture and with product volume similar to the volume of filling.

Table 21 Appearances of lyophilized products using Primarydry2 lyophilization cycle

Formula	**Appearance**
2SG	
4SM	
SGM	
SGc	
SMc	

4.5.2 Protein Conformation Evaluation

CD spectra of formula from lyophilization

Figure 69 shows CD spectra of every formulations which were lyophilized and reconstituted for analysis. The pattern of each CD spectrum for each formula is similar to native F(ab')2 which show negative minima at wavelength of 217 nm and zero intensity at 206 nm, representing mainly of β-sheet. As in previous studies (Arpad Szenszi, 2006; Arnoldus W.P.

Vermeer, 2000; Anthony D. kanavage, 2006; Stefanie Schule, 2007; Andrea Hawe,2009), the secondary structure of IgG consisted mainly of β-sheet when observed with CD.

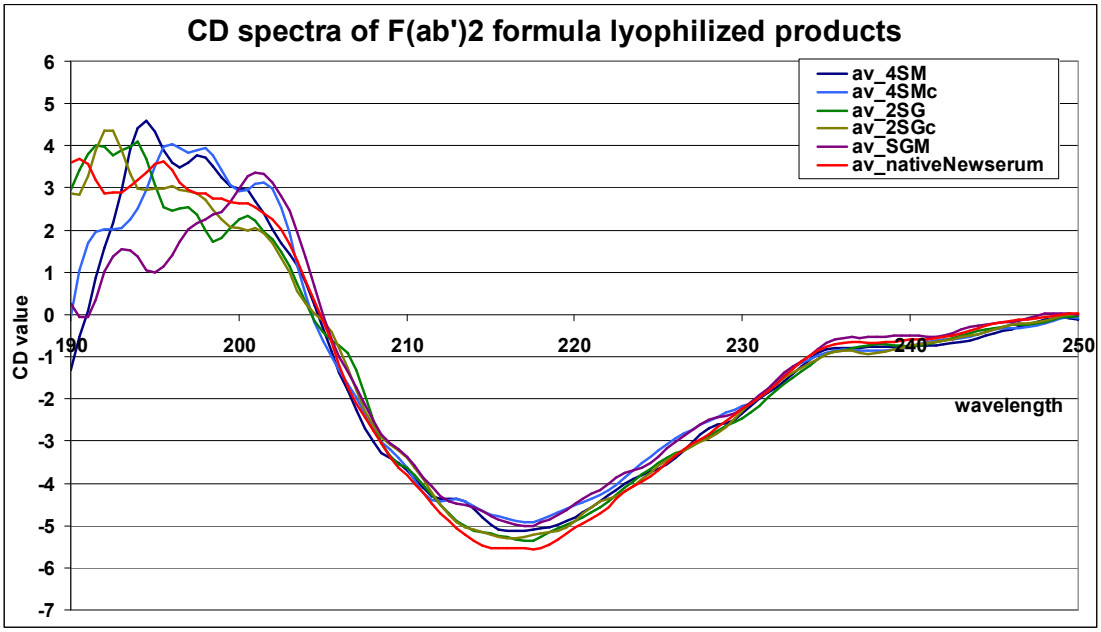


Figure 69 CD spectra of lyophilized products of Green pit viper antivenin formulation using PrimaryDry2 cycle. [day0]

Table 22 CD ellipticity values at 217 nm of lyophilized Green pit viper antivenin formulations.

Formula	CD ellipticity values at 217nm (mdeg)
4SM	-5.116
SMc	-4.920
2SG	-5.373
SGc	-5.271
SGM	-5.012
Native F(ab')2	-5.543

CD ellipticity values in Table 22 are normalized by the CD ellipticity value for native F(ab')2 (-5.543 mdeg for lot no. 10002TA) by following equation number (1) which resulted in equation number (3)

$$\text{Normalized CD ellipticity value} = \frac{\text{(CD ellipticity of products)}}{(-5.543)} \times 100 \quad \ldots\ldots\ldots\ldots(3)$$

The normalized CD values obtained are plotted as a function of formulations developed and shown in Figure 70.

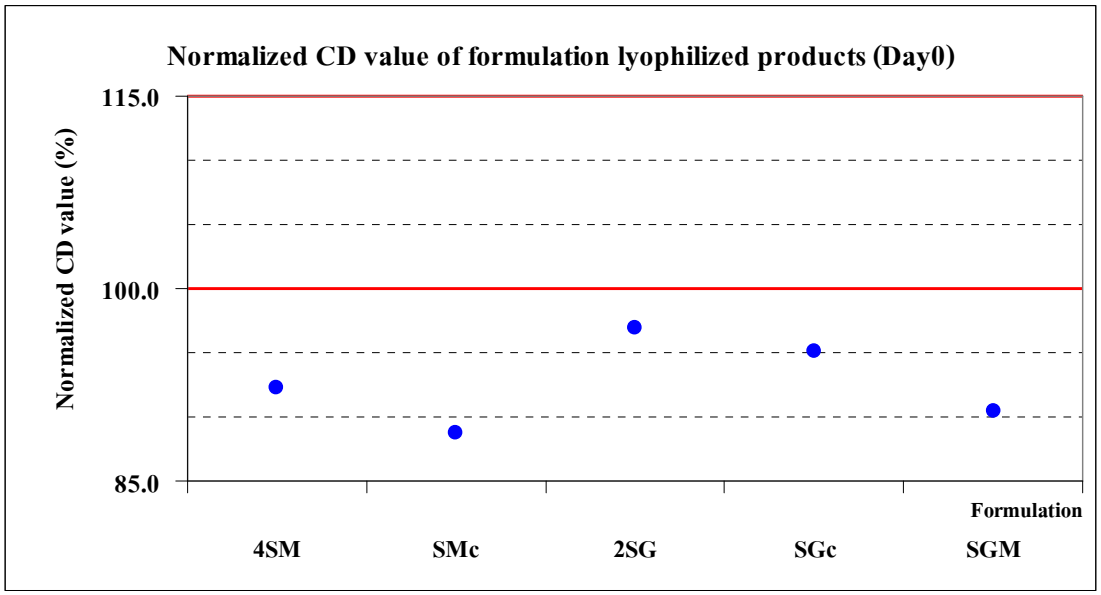


Figure 70 Normalized CD values of lyophilized product of Green pit viper antivenin formulas and native F(ab')2 [day0]

Table 22 and Figure 70 present CD ellipticity values at 217 nm and normalized CD values. 2SG formula had CD value at 217 nm most similar to CD ellipticity value of native F(ab')2. Normalized CD values were arranged from the closest to native F(ab')2 to the most different as 2SG, SGc, 4SM, SMG and SMc. In support of previous research (Meyer et al., 2009), this study found that glycine formula more stabilize antibody than mannitol formula.

SMc and SGc are more concentrated than 4SM and 2SG, hence, molecules of F(ab')2 and the additive in SMc and SGc can interacted more frequently than 4SM and 2SG

formulas. As a result, concentrated formulas show normalized CD values further away from 100% than 4SM and 2SG.

Normalized CD value of SGM is more than 4SM or 2SG due to the fact that it has 2 stabilizers in the formula (1% glycine and 1.5% mannitol). Two stabilizers may have higher interaction between molecules than single stabilizer formulas (4SM or 2SG).

4.5.3 Crystallinity Property

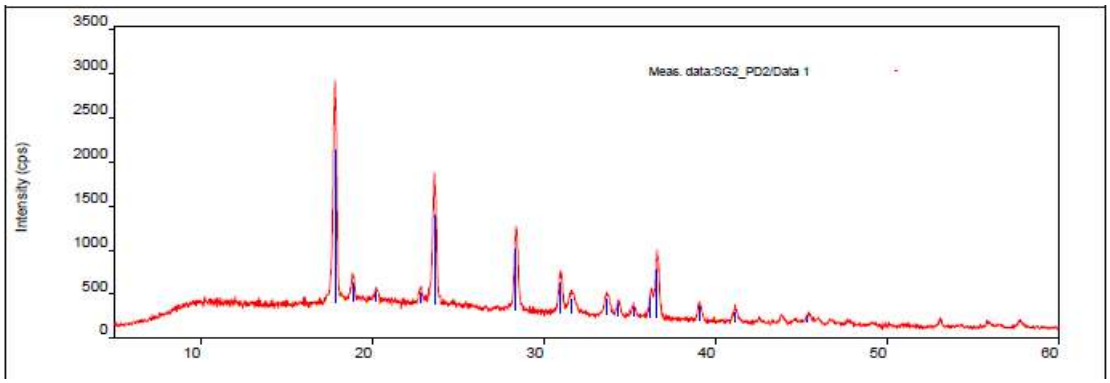


Figure 71 XRPD pattern of 2SG lyophilized product.

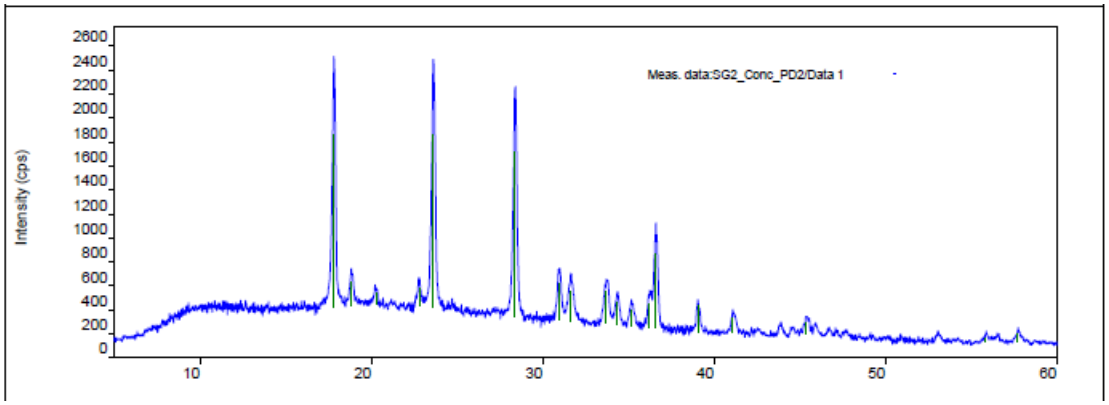


Figure 72 XRPD pattern of SGc lyophilized product.

Figure 71 and figure 72 show XRPD patterns of 2SG and SGc lyophilized product. X-ray powder diffractogram patterns of 2SG and SGc formulas are similar which the major peaks are presented at 18.1, 23.6, 28.6 ° 2θ. XRPD patterns were compared with previous studies, glycine is mainly β–glycine form. These results are consistent with Pyne (2001), Varshney (2007) in that lyophilized glycine with slow freezing rate led product to β–glycine. In agreement with

Chongprasert(2001), which found that freeze drying of glycine solution with slow freezing rate produced mainly of β–glycine structure.

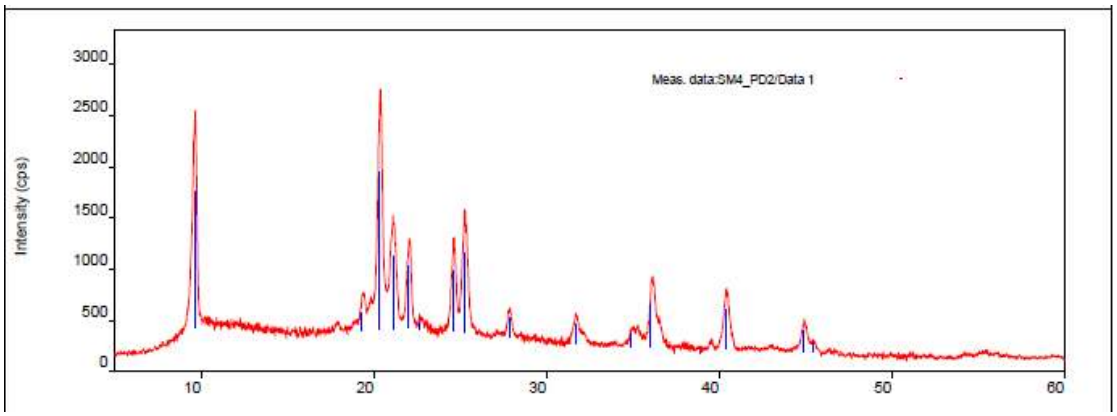


Figure 73 XRPD pattern of 4SM lyophilized product.

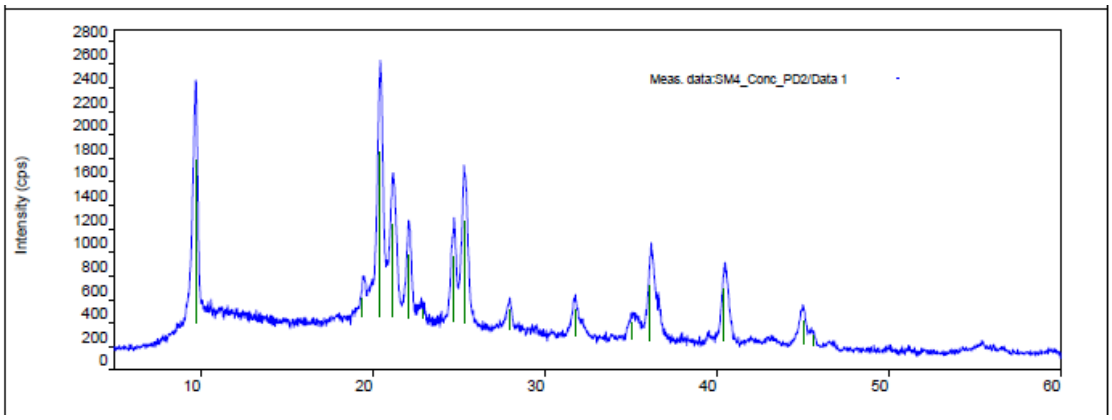


Figure 74 XRPD pattern of SMc lyophilized product.

Figure 73 and 74 show XRPD patterns of 4SM and SMc lyophilized product. XRPD pattern of 4SM lyophilized product was similar to SMc lyophilized product. XRPD pattern of 4SM lyophylized product show crystalline peaks at 9.682° and 20.351° 2θ. XRPD pattern of SMc lyophylized product show peak at 9.726° and 20.427° 2θ. From our data, XRPD pattern of 4SM is the same as SMc which is characteristic of δ-mannitol. These results that mannitol lyophilized has characterize to δ-mannitol is similar to result from previous investigations by Xiangmin Liao in Pharmaceutical Research(2007), Andrea Hawe in European Journal of Pharmaceutical Sciences(2006) and Susana Torrado in Chem. Pharm. Bull(2002).

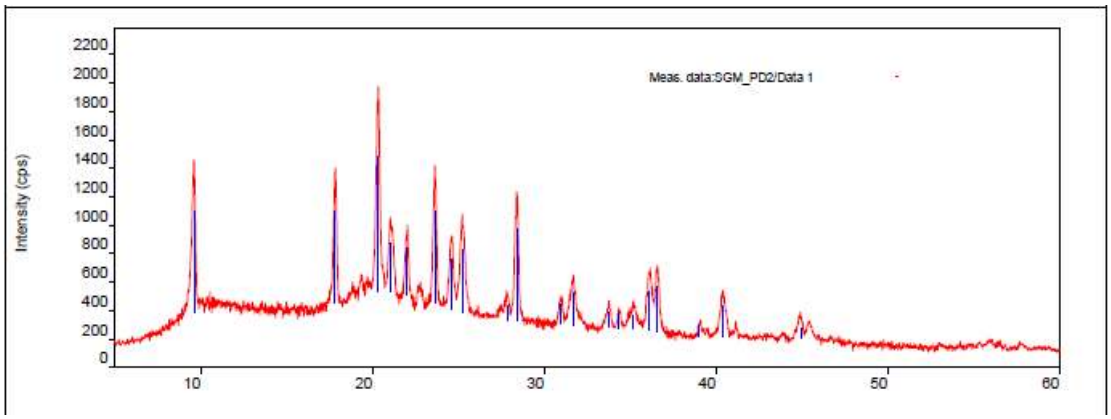


Figure 75 XRPD pattern of SGM lyophilized product.

XRPD pattern of SGM formula in Figure 77 present additional crystalline peaks of β–glycine and δ-mannitol with no foreign peaks present.

4.5.4 Moisture Content

Table 23 Moisture contents of lyophilized product of green pit viper antivenin formulations obtained by TGA

Formulation	Moisture contents (%)
2SG	5.689
4SM	3.562
SGM	1.481
SGc	4.353
SMc	2.708

Table 23 presents moisture contents of lyophilized products obtained by Thermogravimetric method (TGA). Moistures are 5.689, 3.562, 1.481, 4.353 and 2.708 % w/w for 2SG, 4SM, SGM, SGc and SMc, respectively. Usually moisture of lyophilized products should not be more than 3% w/w but some formulas shown in this study are higher which depend on each active ingredient.

Table 24 present moisture contents of lyophilized product from Karl Fischer Titration. Moistures are 3.359, 1.211, 2.326, 3.318 and 1.119 % for 2SG, 4SM, SGM, SGc and SMc, respectively. As observed, the moisture values obtained by each method are differenct due to

technique differences. However, when compared relation to each other, 2SG with the higest moisture content. No other trends were observed due to the freeze dryer does not have apparatus which closes the caps under vacuum automatically. So moistures present in the environment may enter the vial and resulted in varied moisture levels.

Table 24 Moisture contents obtained by Karl Fischer Titration

Formulation	Moisture contents (%)
2SG	3.263
4SM	2.715
SGM	2.952
SGC	2.271
SMc	2.210

4.5.6. Protein Contents

Table 25 Protein contents of lyophilized product

Formulation	**Protein contents (%)**	
	Theoretical	**After lyophilization**
4SM	2.0145	1.211
SMc	2.0145	1.119
2SG	5.0145	3.359
SGc	4.0145	3.318
SGM	3.0145	2.326

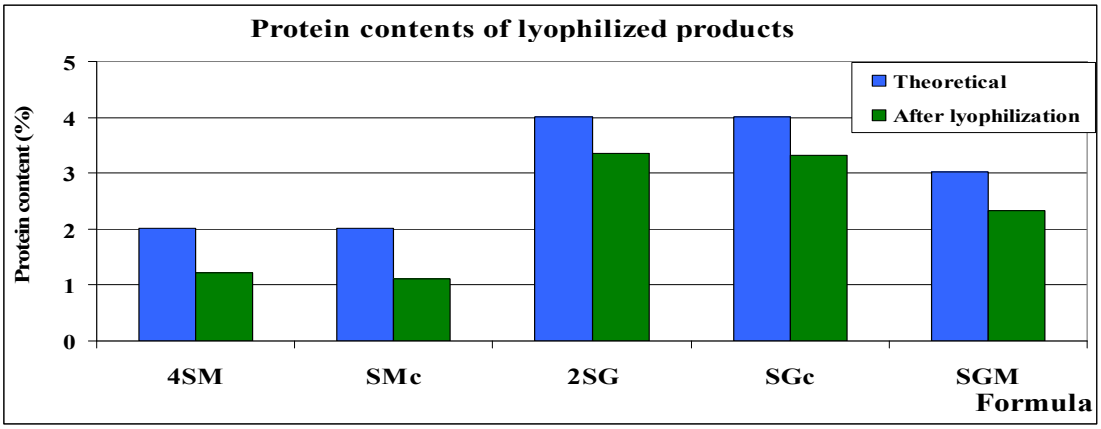


Figure 76 Protein contents of lyophilized formulation theoretical and after lyophilization.

Table 25 and Figure 76 present protein contents of lyophilized formulations by Kjeldahl method. Protein content was 1.211, 1.119, 3.359, 3.316 and 2.236 % for 4SM, SMc, 2SG, SGc and SGM formula. Protein contents cam be arranged from low to high as SMc, SM, SGM, SGc and 2SG. 2SG and SGc formulas had protein contents higher than 4SM, SMc and SGM formulas because 2SG and SGc formula composed of 2%glycine which is additional amino acid.So protein contents of 2SG and SGc are about 3 % and more than other formulations.While SGM had protein content higher than 4SM and SMc because SGM composed of glycine 1% in the formula. 4SM and SMc did not have glycine in the components and protein contents of them are about 1%. Protein contents of 4SM and SMc were from F(ab')2 in the formula and did not result from other additives.

4.5.7 Purity of Product

Green pit viper antivenin Formulation from PrimaryDry2 lyophilization program => day0

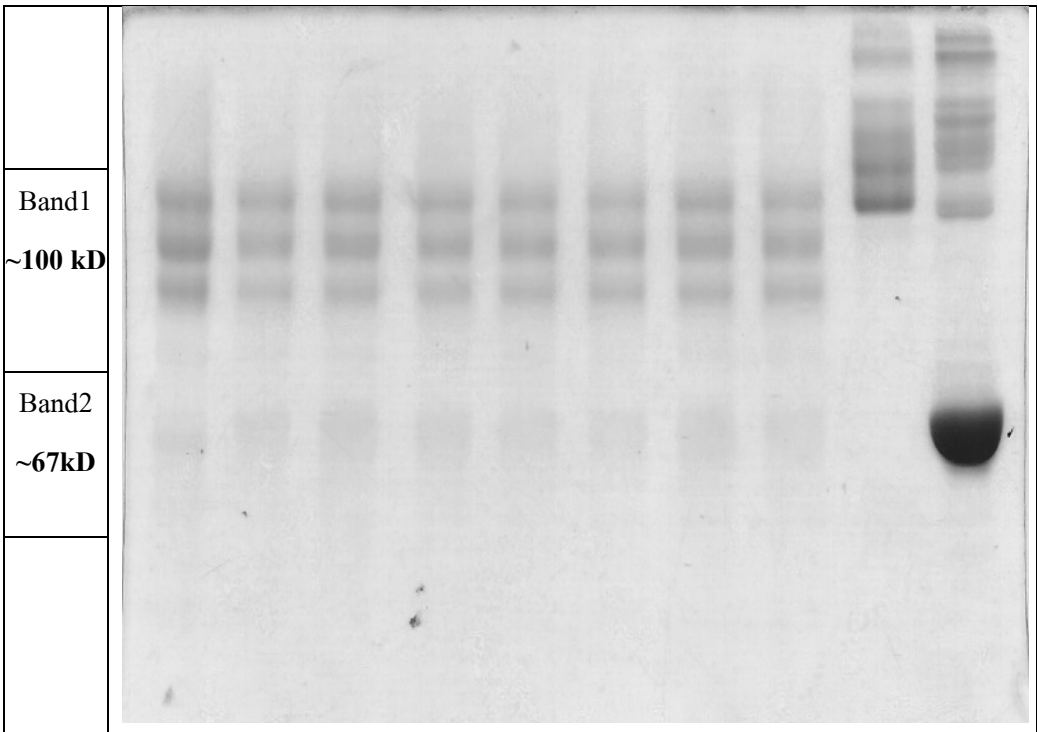


Figure 77 SDS-PAGE band of lyophilized product from SDS-PAGE

Figure 77 shows bands of SDS-PAGE for the lyophilized products, standard IgG, standard albumin and standard F(ab')2. Every lyophilized products show bands of SDS-PAGE coresponding to standard F(ab')2[lane 1]. The main bands of lyophilized products in lane 2, 3, 4, 5, 6, 7 and 8 contained mainly of F(ab')2 These results from SDS-PAGE present lyophilization process did not degrade molecules of the advice protein or did not aggregrate protein. However, Intensities of bands may not be similar depending on the concentration of proteins in the formulations. From previous studies(Jones and Landon, 2002; 2003; Raweerith and Ratanabanangkoon, 2003), Molecular weights of F(ab')2 is about 100 kD, IgG is about 150 kD and albumin is about 67 kD. Bands of standard F(ab')2 from the table concentrate are most intense at 100 kD (main band) and least intense about 60 kD.

Table 26 SDS-PAGE Band intensities of Green Pit Viper antivenin lyophilized products determined by Densitometer

Sample	Band intensity (%)			
	Band1	**Band2**	**Band3**	**Band4**
std F(ab')2	82.7	17.3	-	-
4SM	81.01	18.99	-	-
SMc	80.68	19.32	-	-
2SG	81.93	18.07	-	-
SGc	81.71	18.29	-	-
SGM	82.67	17.33	-	-
4SM	81.75	18.25	-	-
2SG	81.08	18.92	-	-
IgG	-	-	89.9	10.1
Albumin	-	57.58	11.81	30.61

Table 26 shows Intensities of SDS-PAGE bands of lyophilized products, standard IgG, standard albumin and standard F(ab')2. For standard F(ab')2 and lyophilized products had 2 band follow as band1 : mainly band which upper of SDS-PAGE and band2 : below band of SDS-PAGE. Densitometry idenified and calculate intensity of bands and reported % intensity of bands

for each lane. Intensities of bands for lanes1-8 (standard F(ab')2 and lyophilized products) found that band were more than 80%

4.5.8. F(ab')2 Contents

Table 27 presents percentage content of F(ab')2 by densitometry from SDS-PAGE data. F(ab')2 contents found to be 81.63%, 80.58%, 82.56%, 81.03% and 80.71% for 2SG, SGc, 4SM, SMc and SGM, respectively. For criteria stated in European pharmacopoeia (2000), F(ab')2 content of the formula should not be less than 75 %. So every formula passed within the specified limit.

Table 27 F(ab')2 Contents of lyophilized products (n = 3)

Formulations	Content (%)	
	F(ab')2	other protein
4SM	**81.63**	**18.37**
2SG	**80.58**	**19.42**
SMc	**82.56**	**17.44**
SGc	**81.03**	**18.97**
SGM	**80.71**	**19.29**

4.5.9. Potency Determinations

Table 28 Number of survival mice after 48 hours when injected with mixtures of venom and antivenom.

Concentrations of venom (mg/ml)	Concentrations of antivenin (mg/ml)	Total mice used		Survival of mice	
		Controlled	Tested	Controlled group	Tested group
0.5	0.7	6	6	6	6
0.7	0.7	6	6	6	6
1.1	0.7	6	6	1	1
Potency : Probit analysis				**0.996**	**0.996**

Green pit viper antivenin product from QSMI lot no.TA00510: Potency (Certificate of Analysis) = 0.85 mg/ml

Controlled group: Green pit viper antivenin from Queen Saovabha Memorial Institute (Lot no. TA00510)

Tested group : F(ab')2 and 2% glycine mixture lyophilized product

Table 28 displays potency data of 2SG lyophilized product compared to Green Pit Viper Antivenin product from QSMI as controlled group. The potency neutralizing test was done in mice. Mice for this were divided into 2 groups, Controlled group (Green pit viper antivenin from QSMI) and Test group(2SG lyophilized product). Each group are divided further to 3 subgroups according to the concentrations of snake venom. The concentration of snake venom in this test were 0.5, 0.7 and 1.1 mg/ml. Every samples were reconstituted then mixed with snake venom solution according to Table 43 and incubated at 37 C for 30 min. The solution were injected into the tail of mice by intravenously. Observed for 48 hours, then record the number of survival of mice for each group. Concentration of venom, total mice uesed for each group and mice survival were used to calculate by routine statistical method called Probit Analysis. The potency of 2SG formula or the tested group is found to be 0.996 mg/ml which is equivalent to the controlled group. Thus, this lyophilization process and this formulation were able to produce lyophilized products with preserved activity of F(ab')2 active ingredient above the cut-off criteria of 0.7 mg/ml.

The results of all lyophilized products, the appearances were rigid and good cake structure. Because all formulas used crystalline bulking agents (mannitol, glycine) which formed good network in the cakes. Protein conformation analysis was done by spectropolarimeter. CD spectrum are similar to native F(ab')2 which show mainly β-sheet structure. However, the ellipiticity values of spectra show minor difference. The CD values at 217 nm were selected to determine the secondary structure of F(ab')2 Green pit viper antivenin. 2SG lyophilized product show pattern and CD value most identical to native F(ab')2. Purity of the product was tested using SDS-PAGE analysis. Every lyophilized products show identical bands as standard F(ab')2 with molecular weight of 100 kDa. SDS-PAGE results were analyzed further with Densitometry. F(ab')2 contents of all formulations value of over 75 % which are the criteria of product. The

protein contents of products were analyzed with Kjeldahl Method. Each formula had different protein contents which were due to the effects of formulation and concentrations, especially; excipients.Glycine is amino acid in nature and eventually have an impact on protein content of the formula. The formulations composed of glycine show higher protein contents than previously expected.Thermal analysis using DSCshow differences in DSC patterns. Generally, endothermic peak is formed at 100 °C for water evaporation and some other endothermic peaks depending on the formulations. Moisture contents were analyzed with Karl Fischer method and all results show approximately 3% moisture content and lower. Thus, results remained from the above studies indicate that the best formulation is found to be 2SG formula. The 2SG lyophilized product was analyzed for potency using mouse nutralizing test. The potency of 2SG is identical to the potency of controlled group of 0.996 mg/ml. The potency of 2SG is within the limit and met the requirement.

4.6 Stability Study of Lyophilized Green Pit Viper Antivenin Products

Lyophilized products were stored at various conditions for 3 months. Specific conditions for this stability study are as follow (table 29)

Table 29 Condition of stability study conditions.

Stability storage Conditions	Temperature (°C)	Relative humidity(%RH)	Code
Accelerated	45 ± 2 °C	75 ± 5 % RH	AC
Ambient	30 ± 2 °C	-	MB
Cold	2 – 8 °C	-	cold

After 3 months, lyophilized products were analyzed and evaluated to determine the most suitable formulation for the selected lyophilization process (PrimaryDry2).

4.6.1 Physical Appearance

Table 30 Appearances of lyophilized products from stability studies after storaged for 3 months.

Formula	Accelerated	Ambient	Cold
4SM			
SMc			
2SG			
SGc			
SGM			

Table 30 presents pictures of lyophilized products after stability studies (accelerated, ambient and cold condition). The product appearances from each of the 3 conditions are similar. In addition, a comparison was made between initial lyophilized products and lyophlized products after stability studies and was found to be identical in appearance. In summary, the appearances of every lyophilized product are stable after stored under various stability conditions.

4.6.2 Osmolarity of Stability Products

The lyophilized products were reconstituted with purified water then analyzed by osmometer.

Table 31 Osmolarity of lyophilized products from stability study.

Formulation	Osmolarity (mOsm/kg)			
	Acceleration	Ambient	Cold	Initial
4SM	272.5	270.5	272	296
SMc	264.5	269.5	273	300
2SG	308.5	309.5	306.5	335
SGc	305.5	302	306.5	322.5
SGM	270	264.5	271.5	299

Table 31 shows osmolarity of lyophilized products after storage under various stability study conditions for 3 months. Range of osmolarity of lyophilized products were 270.5-272.5, 264.5-273, 306.5-309.5, 302-306.5 and 264.5-271.5 mOsm/kg for 4SM, SMc, 2SG, SGc and SGM , respectively. The criteria of osmolarity for injectable drugs are approximately 300 mOsm/kg to ensure its isotonicity. (European Pharmacopoiea, 2008). 2SG and SGc formulations have osmolarity nearest to 300 mOsm/kg for all 3 conditions used in the stability studies. On other hand, Osmolarity of 4SM, SMc and SGM are only about 270 mOsm/kg. Thus when lyophilized products were stored under vaious stability studies condition, only 2SG and SGc products are acceptable within the specified.

4.6.3 Secondary Structure of F(ab')2

The lyophilized products after stability study were reconstitued with purified water, diluted 100 folds and analyzed with spectropolarimeter. CD spectra of the products were evaluated and compared.

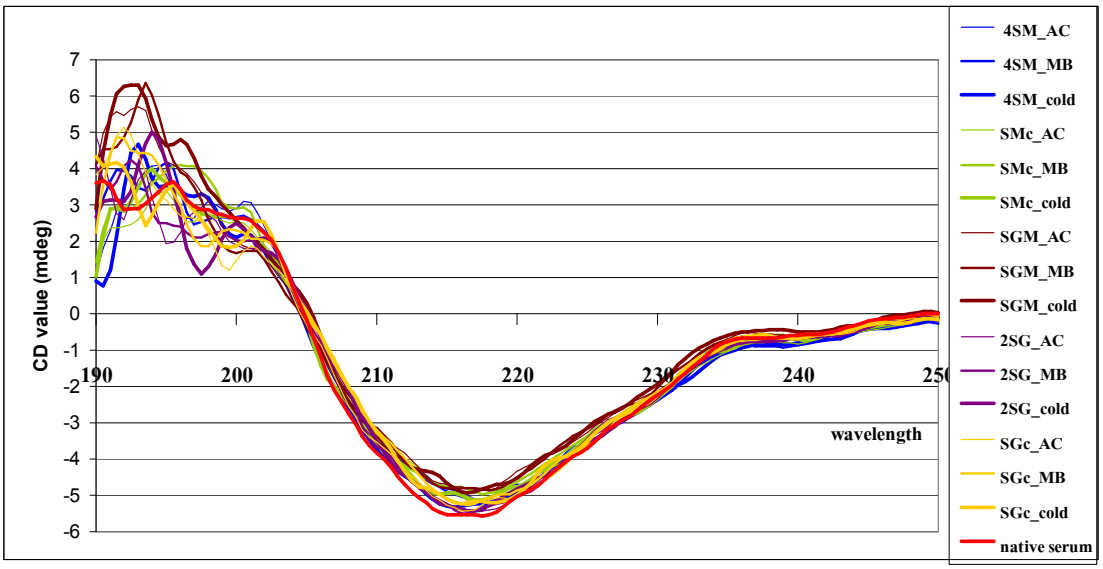


Figure 78 CD spectra of green pit viper antivenin lyophilized products after stored under various stability conditions for 3 months.

Figure 78 shows CD spectra of lyophilized products after storage under various stability conditions for 3 months and reconstituted for analysis. The patterns of CD spectra of every formulations are the same as native F(ab')2 which shows minima at wavelength of 217 nm and a zero intensity wavelength at 206 nm representing mainly of β-sheet. As in previous studies (Szenszi, 2006; Vermeer, 2000; Kanavage, 2006; Schule, 2007; Hawe, 2009) The secondary structure of IgG elucidated by CD mainly represents β-sheet conformation. However, the magnitude of elipiticity values of each sample are slightly different which may be due to the variation in ingredient in the formula.

Table 32 displays CD ellipticity values at 217 nm of lyophilized products after storage under various stability conditions and initial. The range of CD ellipticity values are [(-5.113) – (-5.248)], [(-4.898) – (-5.132)], [(-5.412) – (-5.431)], [(-5.160) – (-5.391)], [(-4.816) –(-4.909)] for 4SM, SMc, 2SG, SGc and SGM, respectively. When comparing CD ellipticity values at 217 nm of every formulations with initial, CD ellipticity values had slighly different values as 0.135 mdeg for 4SM, 0.234 mdeg for SMc, 0.019 mdeg for 2SG, 0.232 mdeg for SGc and 0.093 mdeg for SGM. When compared CD values after stability study condition with initial condition, 2SG and 4SM formula had CD value nearby initial condition.

Table 32 CD ellipticity values at 217 nm of lyophilized products after stability study compared with initial lyophilized products.

Formula	CD ellipticity value at 217 nm (mdeg)			
	Accelerated	Ambient	Cold	Initial
4SM	-5.113	-5.141	-5.248	-5.116
SMc	-5.103	-4.898	-5.132	-4.920
2SG	-5.412	-5.425	-5.431	-5.373
SGc	-5.391	-5.235	-5.160	-5.271
SGM	-4.835	-4.816	-4.909	-5.012
Native F(ab')2	-	-	-	-5.543

CD ellipticity values in Table 38 are normalized by the CD ellipticity value for native F(ab')2 (-5.543 mdeg for lot no. 10002TA) by following equation number (1) resulting in equation number (3).

$$\text{Normalized CD ellipticity value} = \frac{\text{(CD ellipticity of products)}}{\text{(-5.543)}} \times 100 \quad \ldots\ldots\ldots\ldots (3)$$

The normalized CD values obtained are plotted as a function of developed formulations and shown in Figure 79.

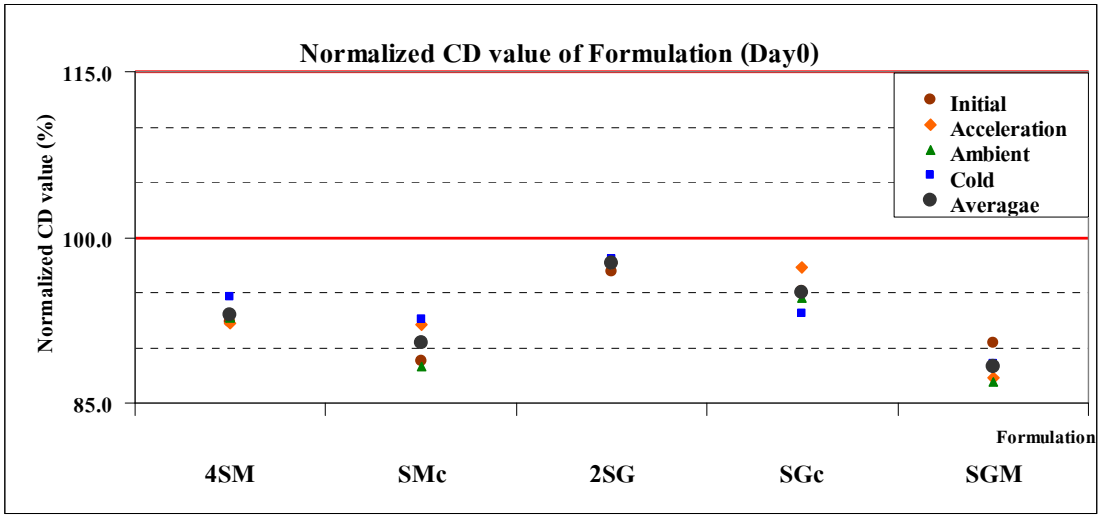


Figure 79 Normalized CD values of initial lyophilized products and lyophilized products stored under various stability condition for 3 months compared to native F(ab')2 .

Figure 79 shows normalized CD values of initial lyophilized products and lyophilized formulations stored under various stability conditions for 3 months compare to native F(ab')2. 2SG formula has the closest normalized CD value to native and could be postulated that this formula retained its native F(ab')2 structure.

This study found that SGc formula show normalized CD value slightly further away from 100% than 2SG formula. In addition, SMc formula had normalized CD value further away from 100% than 4SM formula. SGc and SMc formulas are more concentrated than 2SG and 4SM. This concentration increase may have the affect on the molecular rearrangement in proper secondary structures after reconstitution.

4.6.4 Crystalinity Properties of Lyophilized Products.

The lyophilized products after stability study were analyzed by XRPD and the diffraction patterns were

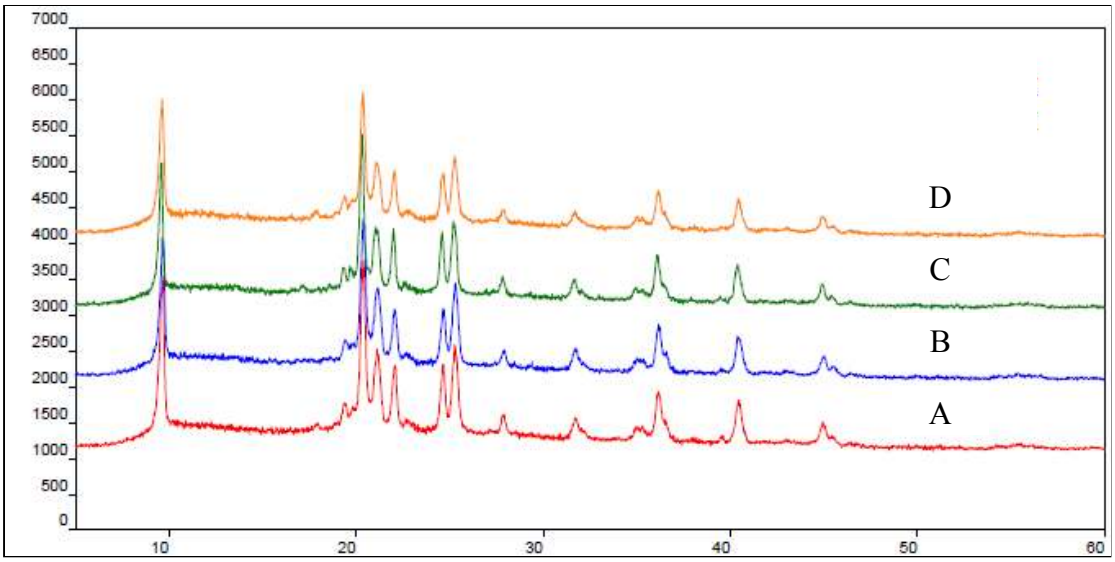


Figure 80 XRPD patterns of 4SM lyophilized products from Accelerated (A), Ambient (B), Cold (C) and Initial conditions (D).

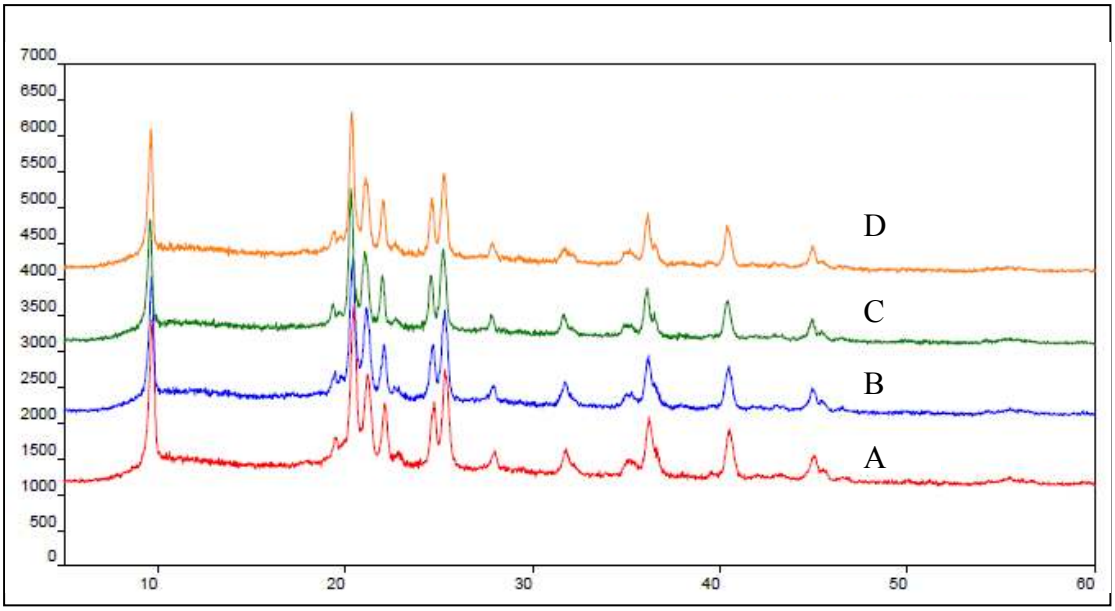


Figure 81 XRPD patterns of SMc lyophilized products from Accelerated (A), Ambient (B), Cold (C) and Initial conditions (D).

Figures 80 and 81 show XRPD patterns of 4SM and SMc lyophilized product after stability study. The XRPD pattern of 4SM and SMc lyophilized products show the same XRPD patterns as the initial condition product (day0). The main peaks of 4SM and SMc lyophilized products are crystalline peaks at 9.6° , 20.3° , 21.1° , 24.6° and 25.2° 2θ. These data indicated that the main form of mannitol are δ-mannitol. In addition, XRPD of initial sample (day0) for both 4SM and SMc lyophilized products were same as XRPD of products which were stored for 3 months under various stability conditions. So both 4SM and SMc lyophilized products remained crystalline property stable for 3months of stability test. The finding that XRPD of 4SM and SMc lyophilized products were stable for 3 months when were storage at stability study is similar to results from previous investigations by Hawa, A., and Frieb, W. in 2006.

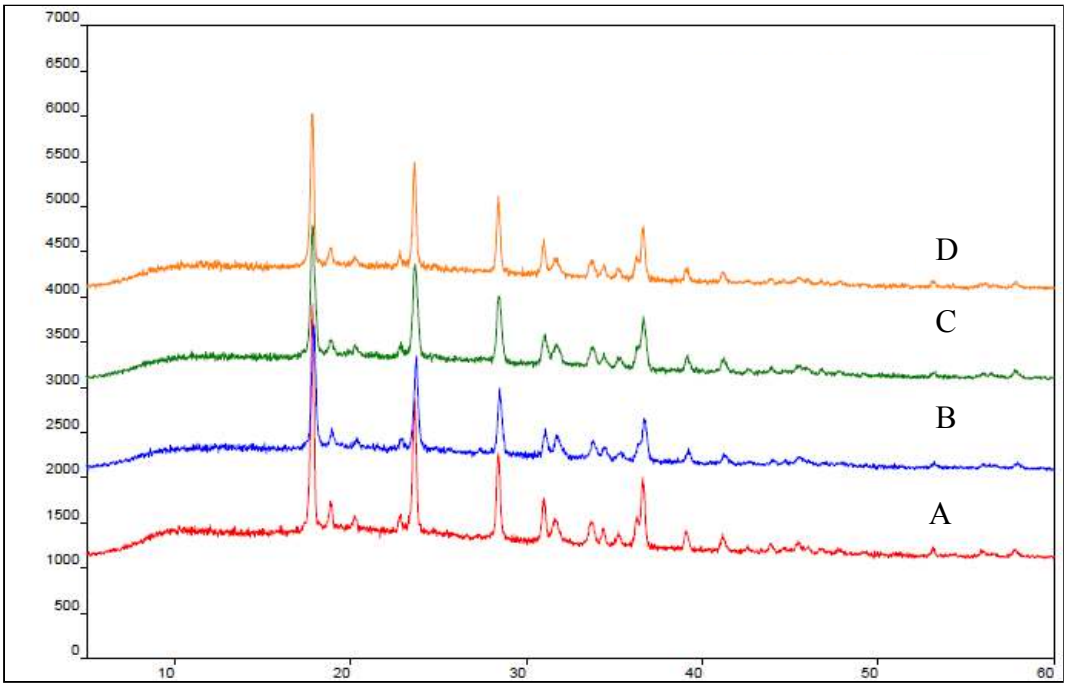


Figure 82 XRPD patterns of 2SG lyophilized products from Accelerated (A), Ambient (B), Cold (C) and Initial conditions (D).

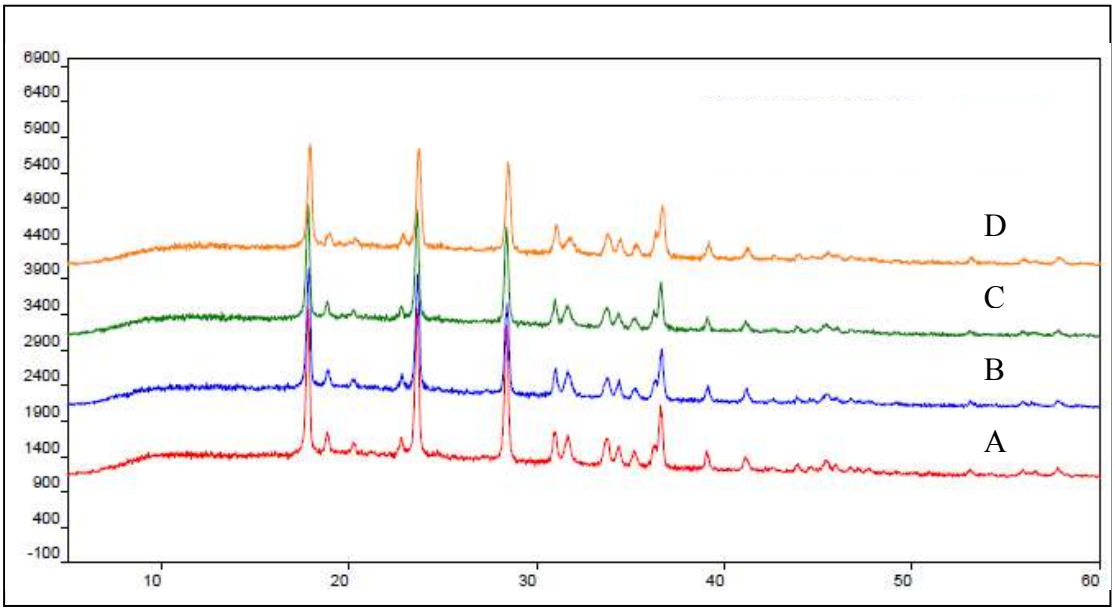


Figure 83 XRPD patterns of SGc lyophilized products from Accelerated (A), Ambient (B), Cold (C) and Initial conditions (D).

Figure 82 and 83 show XRPD of 2SG and SGc lyophilized product from stability study. The XRPD of each 2SM lyophilized products had a same XRPD pattern both initial condition (Day0) and stability study condition for 3 months. The peak of 2SG and SGc lyophilized products were about 17.9, 23.6, 28.4 ° 2θ and those position ware determined to β–glycine form.(main peak : 18.1, 23.6, 28.6 ° 2θ). The XRPD of SGc lyophilized products had same peak as 2SG lyophilized products which all XRPD of lyophilized products contained mainly β–glycinetogether. However, XRPD of initial sample (day0) of both 2SG and SGc lyophilized products were as same as XRPD of sample which were storaged for 3 months at stability studies. So both 2SG and SGc lyophilized products remained crystalline property stable for 3months of stability test.

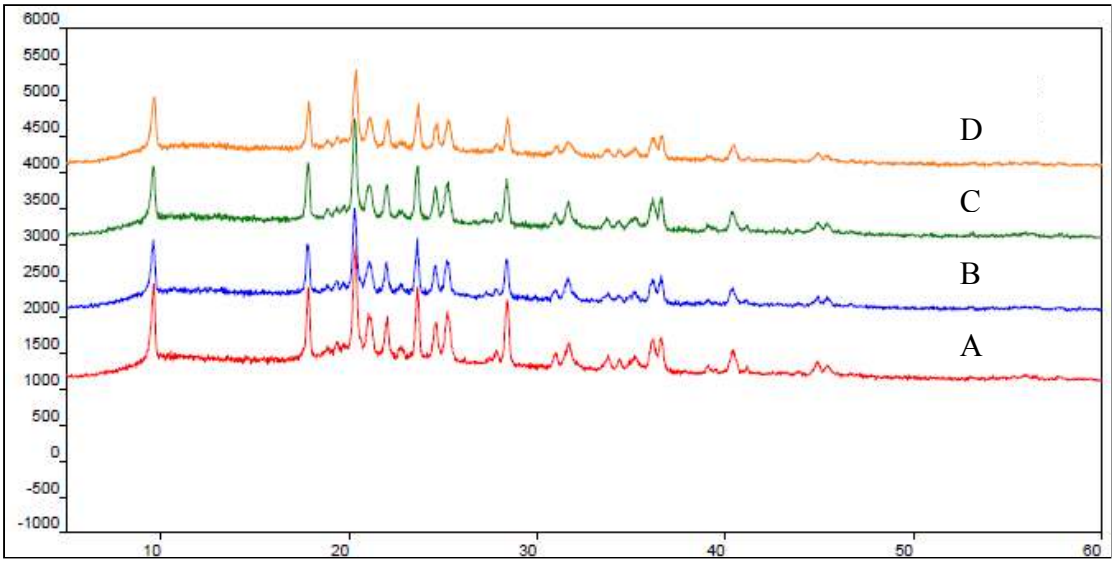


Figure 84 XRPD patterns of SGM lyophilized products from Accelerated (A), Ambient (B), Cold (C) and Initial conditions (D).

Figure 84 display XRPD pattern of SGM lyophilized product from initial(day0) and stability study for 3 months. All of XRPD were as same as and main peak was about 9.6°, 17.9°, 20.3°, 23.6° and 28.4° 2θ. The main peak of those lyophilized products were combined β–glycine(17.9°, 23.6° and 28.4° 2θ) and δ-mannitol(9.6° and 20.3° 2θ). When comparision between initial condition (day0) and stability study for 3 months, the XRPD of all samples were

similar. So SGM lyophilized products were remained crystallinity property stable for 3 months of each stability condition.

4.6.5 Moisture Contents

Table 33 Moisture contents of Green pit viper antivenin formulations and products after stability study obtained by thermogravimetric method

Formulations	Initial moisture	Moisture of products after stability study		
		Accelerated	Ambient	Cold
4SM	3.5615	0.8719	0.9547	2.3214
4Smconc	2.7077	1.1608	0.6154	1.6097
2SG	5.6891	1.1727	1.6983	4.0733
2Sgconc	4.3534	2.1178	1.2684	3.1578
SGM	1.4813	0.5822	1.8725	4.2571

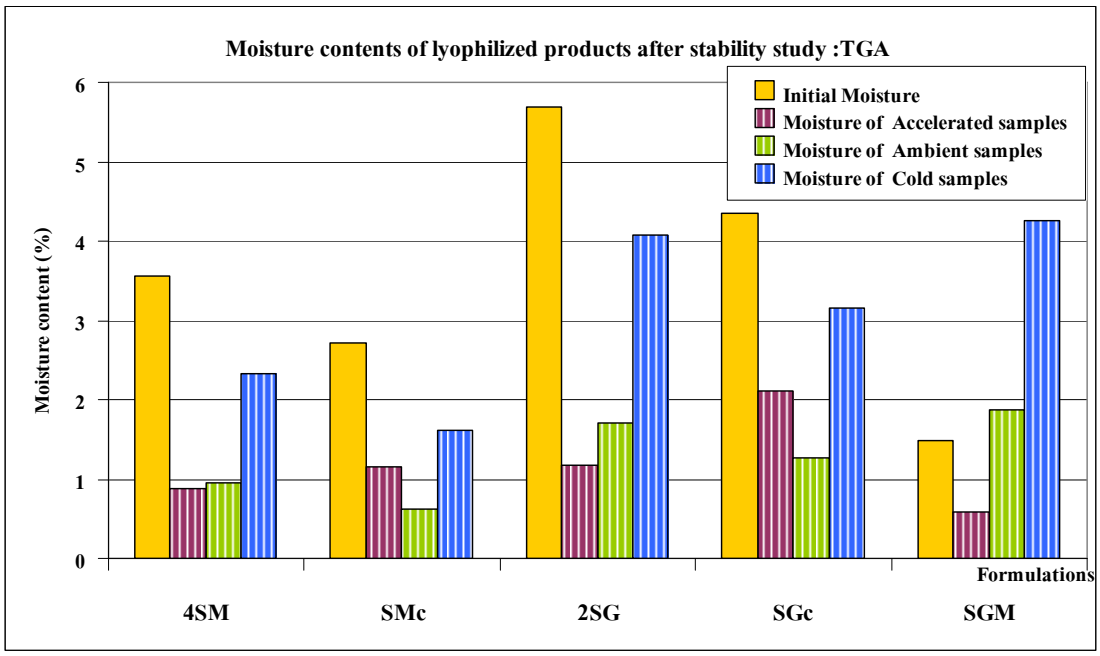


Figure 85 Moisture contents of lyophilized products after stability study.

Table 33 and Figure 85 show moisture contents of lyophilized products after stablity study and the initial products. Moisture contents of lyophilized products from accelerated and

ambient conditions were not more than 3.0 % w/w. Arrange moisture content from all condition were SMc ,4SM, SGc, SGM and 2SG, respectively. The SMc lyophilized products had a lowest moisture content when compared to all conditions and its value are not more than 3.0%. However, the lyophilizer was not able to automatically close the vial under vacuum condition, so moisture content may varied due to the environment encountered before vial closing.

4.6.6 Thermal Analysis

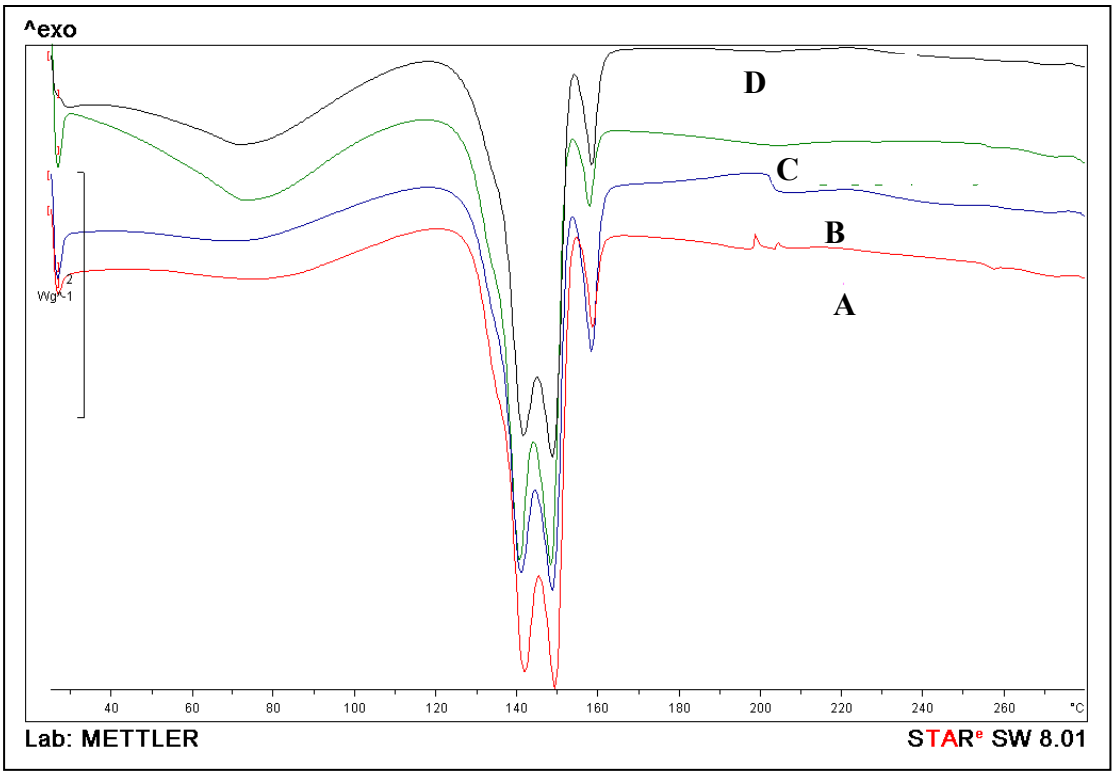


Figure 86 DSC thermograms of 4SM lyophilized product from accelerated (A), ambient(B), cold(C) and initial condition (D)

Figure 86 shows DSC thermograms of initial 4SM lyophilized products and from stability study.All of thermograms exhibit the same main peaks. Thermogram at about 80 °C showed slight endothermic event which was used for water evaporation. At about 140, 150 and 160 °C had dominant endothermic peaks which was used to product decomposition (mannitol) and showed exothermic peaks about 145 and 160 °C which product might recrystallization then release energy to system. The slight endothermic peaks about of 200°C which was F(ab')2

decomposition peak. (Compared to DSC thermogram of F(ab')2 lyophlized product [4.3.1.3]) The results of all DSC thermogram are similar and the products' chemical integrity after stability were intact.

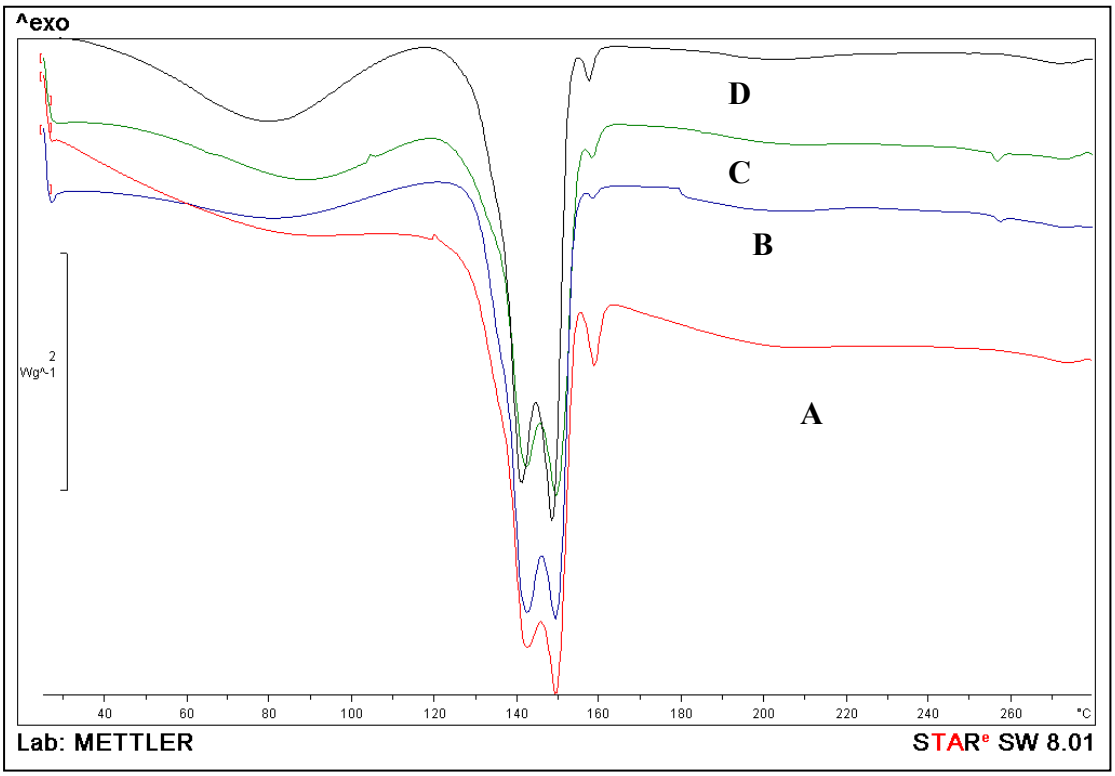


Figure 87 DSC thermograms of SMc lyophilized product from accelerated (D), ambient (C), cold (B) and Initial condition (A)

Figure 87 shows DSC thermogram of initial SMc lyophilized products and after stability study. All of the thermograms show the same main peaks of DSC thermogram. At about 60-100°C show slight endothermic event for water evaporation. At about 140, 150 and 160 °C had dominant endothermic peaks which was used to product decomposition (mannitol) and showed exothermic peaks about 145 and 155 °C which product might recrystallization then release energy to system. The slight endothermic peaks about of 200°C which was F(ab')2 decomposition peak. (Compared to DSC thermogram of F(ab')2 lyophlized product [4.3.1.3]) The results of all DSC thermogram are similar and the products' chemical integrity after stability were intact.

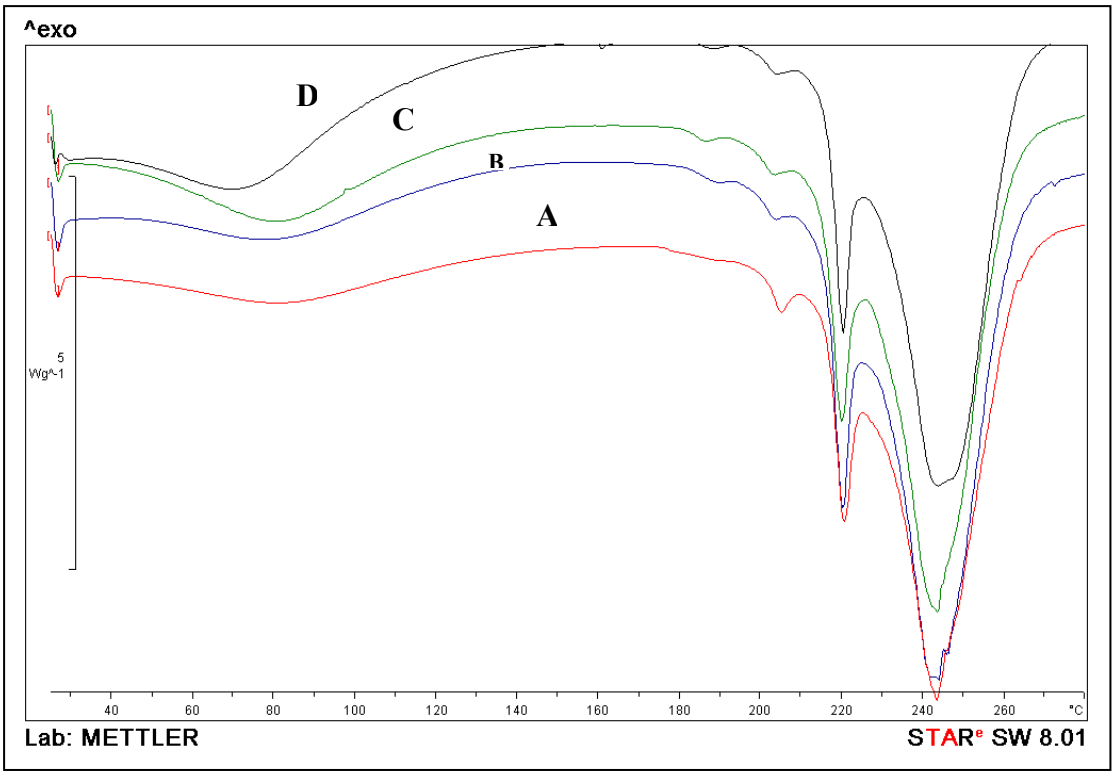


Figure 88 DSC thermograms of 2SG lyophilized product from acceleration (A), ambient (B), cold and initial condition (D).

Figure 88 shows DSC thermograms of initial 2SG lyophilized products and from stability study. All of DSC thermogram exhibit the same peak. Thermogram at about 80°C showed slight endothermic event which was used for water evaporation. The endothermic peak at about 200, 220 and 240 °C which were used to products composition. Endothermic peaks at 200 – 220°C was used for decomposition of F(ab')2 (from data 4.3.1.3) and endothermic peak at 240°C was used for decomposition of glycine. The exothermic peak at about 210 and 225 °C which were recrystallization of decomposed product. The results of all of DSC thermograms are similar and the products chemical integrity after stability was intact.

Figure 89 shows DSC thermograms of initial SGc lyophilized products and from stability study. All of DSC thermogram exhibit the same peaks. Thermogram at about 80 °C showed slight endothermic event which was used for water evaporation. The endothermic peaks at about 200, 220 and 240 °C which were high temperature, the energy was used to products composition.

Endothermic peaks at 200 – 220°C was used for decomposition of F(ab')2 (from data 4.3.1.3) and endothermic peak at 240°C was used for decomposition of glycine. And the exothermal peak at about 205 and 225 °C which were recrystallization of decomposed product and released energy to system. The results of all DSC thermograms are similar and the products chemical integrity after stability were intact.

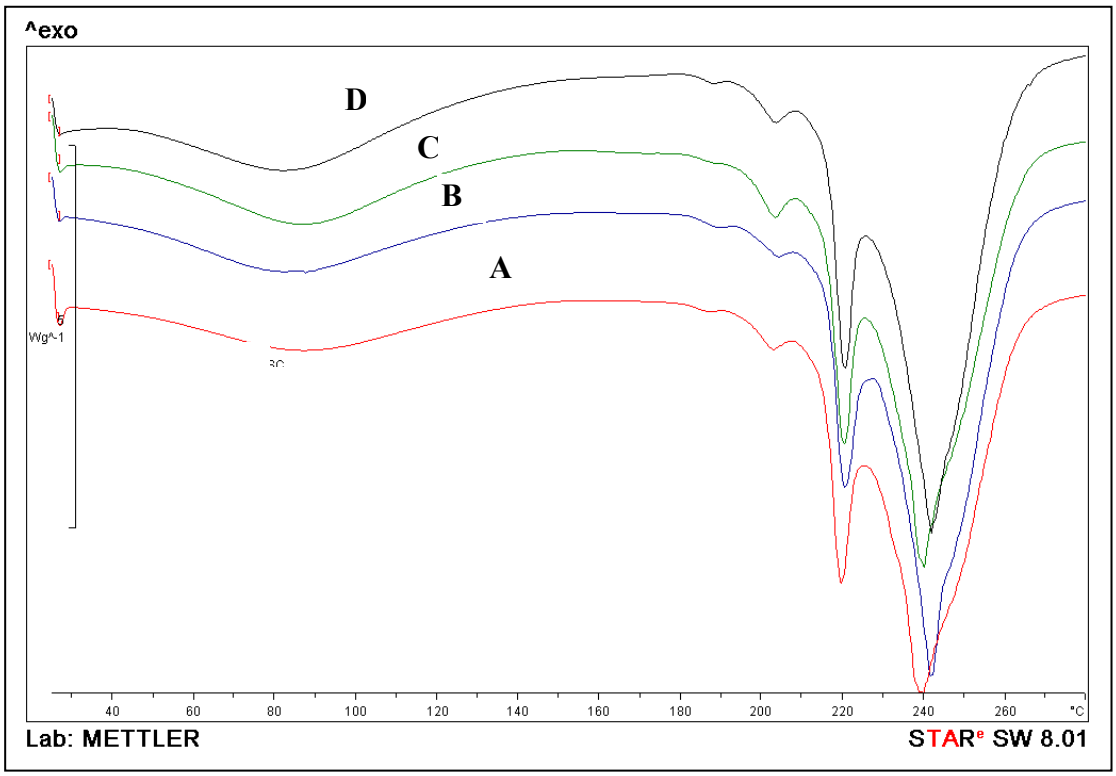


Figure 89 DSC thermograms of SGc lyophilized product from acceleration (A), ambient (B), cold (C) and initial condition (D).

Figure 90 shows DSC thermogram of initial SGM lyophilized products and from stability study. All of DSC thermogram shows the same peak of thermogram. At 60-110 °C show slight endothermic event for water evaporation. The endothermic peak at 130 °C for mannitol decomposition. The slight endothermic peak at 200°C was peak of F(ab')2 decomposition and 240°C which was used to glycine composition. The result of all DSC thermograms are similar and remain the chemical stability according to stability study time.

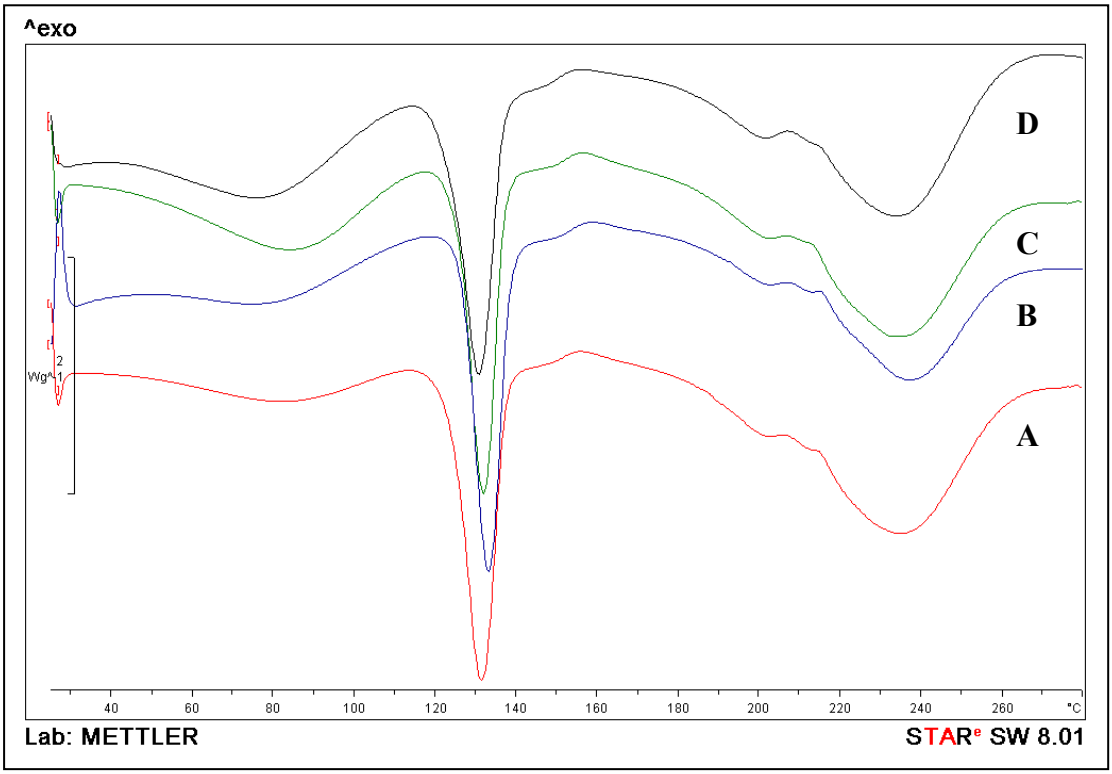


Figure 90 DSC thermograms of SGM lyophilized product from acceleration (A), ambient (B), cold(C) and initial condition (D).

DSC thermogram of all formulations both initial and stability study found that DSC thermogram show peak of each ingredeint in each formulations. DSC thermograms of initial sample and stability study samples are similar and remained chemical integrity during duration of stability study.

4.6.7 Protein Contents

Table 34 and figure 91 show protein contents of lyophilized products. Protein content from stability study was 1.029 – 1.276%, 1.039 – 1.198 %, 3.219 – 3.427%, 3.082 – 3.392%, and 1.998 – 2.293% for 4SM, SMc, 2SG, SGc and SGM respectively. The protein content of stability study products was nearby initial product of each formula. When lyophilized products were storaged at stability condition for 3 months, the protein content had a sligthly changed. So, protien contents of lyophilized product were unchanged when were storaged at stabilitystudy for 3 months.

Table 34 Protein content of Green pit viper antivenin formula and products from stability study

Formulartions	Initial	Protein contents after stability study		
		Accelerated	Ambient	Cold
4SM	1.211	1.276	1.196	1.029
SMc	1.119	1.198	1.108	1.039
2SG	3.359	3.427	3.416	3.219
SGc	3.318	3.392	3.082	3.118
SGM	2.326	2.293	1.998	2.054

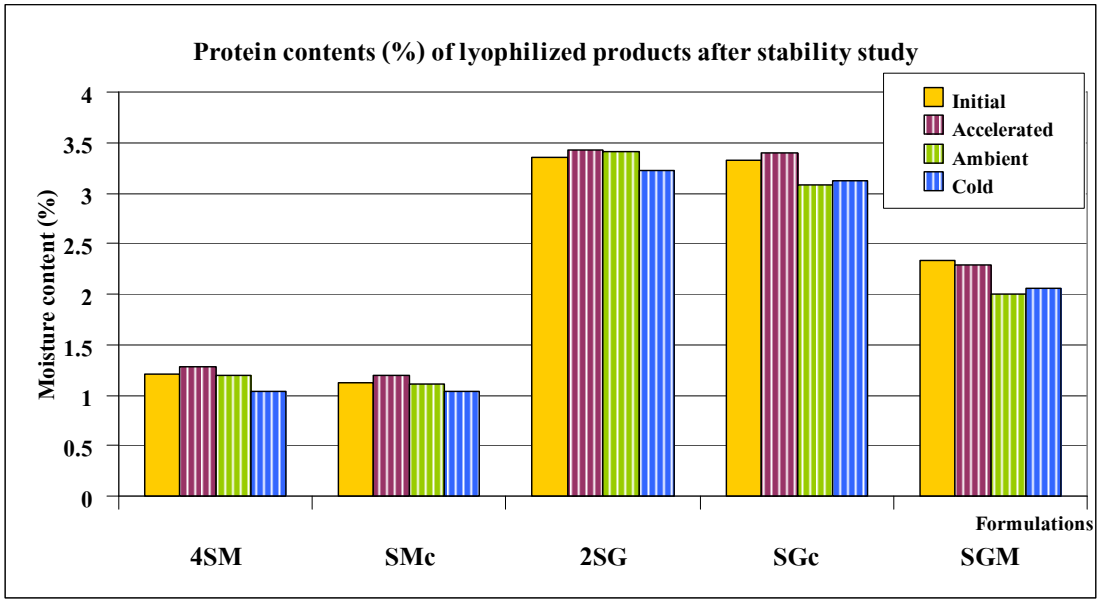


Figure 91 Graph protein contents of lyophilized products stability studiy from Kjeldahl method

4.6.8 Purity of Protein

The lyophilized products were analyzed by SDS-PAGE and were evaluated SDS-PAGE band.

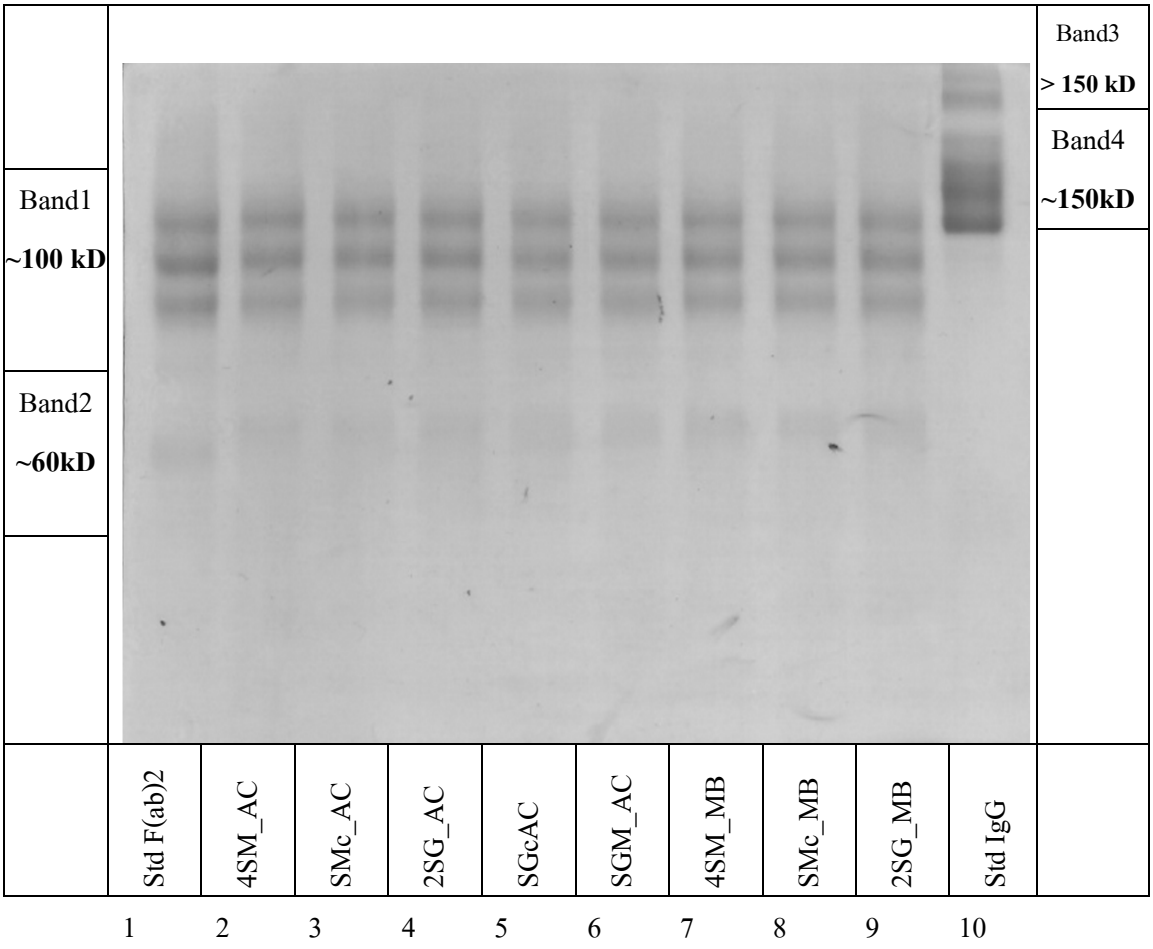


Figure 92 SDS-PAGE band of stability studied lyophilized products from SDS-PAGE (sample1)

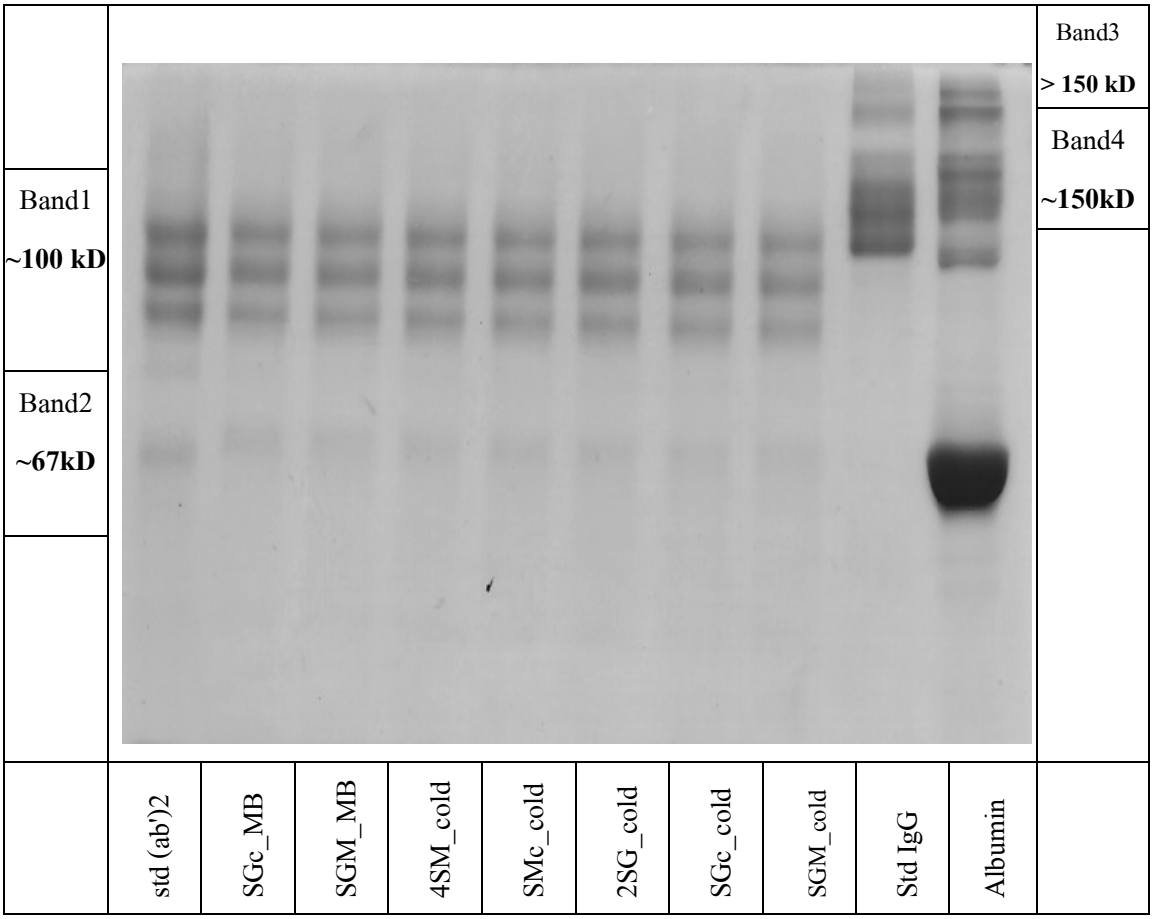


Figure 93 SDS-PAGE band of stability studied lyophilized products from SDS-PAGE (sample2)

Figure 92 and 93 show SDS-PAGE band of lyophilized products after stability study. Our data, it was found that the band of each sample was as same as standard F(ab')2 which was similar to initial lyophilized products. Every stability study lyophilized products show bands of SDS-PAGE coresponding to standard F(ab')2[lane 1]. The main bands of stability study lyophilized products in lane 2, 3, 4, 5, 6, 7, 8 and 9 (Figure 92) and line2, 3, 4, 5, 6, 7 and 8 (Figure 93) contained mainly of F(ab')2 These results of SDS-PAGE present under stability study condition did not degrade molecules of the advice protein or did not aggregrate protein. The stability study lyophilized products had a same purity with initial products and similar to standard F(ab')2 . So, the lyophilized products remained puritiy of product during duration of stability study test and did not impact from stability conditions, including accelerated condition. From

previous studies (Jones and Landon, 2002; 2003; Raweerith and Ratanabanangkoon, 2003), Molecular weights of F(ab')2 is about 100 kD, IgG is about 150 kD.

4.6.9 F(ab')2 Contents of Lyophilized Products

Table 35 F(ab')2 contents of Green Pit Viper antivenin formulations and after stability study products determined by densitometer

Formulations	Initial		Accelerated		Ambient		Cold	
	% F(ab')2	other protein	% F(ab')2	other protein	% F(ab')2	other protein	% F(ab')2	other protein
4SM	81.63	18.37	85.29	14.71	83.73	16.27	85.19	14.81
SMc	81.43	18.57	84.03	15.97	84.93	15.07	84.39	15.61
2SG	81.71	18.29	84.25	15.75	83.61	16.39	85.86	14.14
SGc	81.03	18.97	84.99	15.01	83.95	16.05	84.02	15.98
SGM	80.71	19.29	86.60	13.40	84.08	15.92	83.53	16.47

Table 35 shows percentage content of F(ab')2 of initial lyophilized products and after stability study from SDS-PAGE data. The F(ab')2 content of stability study lyophilized products was 83.73-85.29 %, 84.03-84.93%, 83.61-85.86 %, 83.95-84.99 % and 83.53-86.60 % for 4SM, SMc, 2SG, SGc and SGM, respectively. The criteria stated in European pharmacopoeia (2000), F(ab')2 contents of the formula should not be less than 75 %. All of lyophilized products from all condition of stability had F(ab')2 more than 80%. Thus, every formula and stability study lyophilized products passed within the specifeid limit. Although every formula remained the F(ab')2 contents according to duration of stability study.

Evaluations of all lyophilized products (2SG, SGc, 4SM, SMc and SGM) after stability storage at acceralated, ambient and cold conditions were done. The conformation of Green pit viper antivenin F(ab')2 after stability study show difference from initial (day0) product and dependent on formulation. 2SG and SGc had lowest diffence in CD values than other formulas. Purity was analyzed using SDS-PAGE analysis. Every formula show main band at MW about

100 KD which were similar to standard F(ab')2. The results were analyzed for F(ab')2 contents by densitometer. F(ab')2 contents of all products were more than 75 % and met the require criteria for satisfactory products. The protein contents of stability studied sample had difference value which depended on formulation. When compared with initial products, the products after stability test remained similar protein contents. The all results about protein shown protein of active ingredient were remained and stabled belong 3 months of stability studied.

The thermal analyses of stability studied samples were compared with initial products. Each formula has a differnce DSC patterns and depend on excipient in formulation. When comparison same formula, the initial and the stability studied shown the same DSC pattern. The crystalinity of stability studied products were analyzed and comparison with initial products. All of stability studied products shown the same XRPD pattern with initial products. So the lyophilized products were stable for 3 months upon duration of stability studies. Finally, moisture content of stability studies was analysed and found that stability studied products had lower moisture content than initial procutd. But almost samples had moisture content below 3% moisture content.

The stability studied of five formula lyophilized products for 3 months found that the all formulas were remained physicochemical, chemical properties and upon stable belong stabilty studied time.

CHAPTER V

CONCLUSION

This study confirms that the secondary structure of F(ab')2 Green Pit Viper antivenin is mainly β-sheet. The freezing processes and stabilizers affect the secondary structure of F(ab')2 Green Pit Viper antivenin. Rapid3 freezing pattern produced sample that shows the lowest difference in CD value compared to native F(ab')2. The freezing pattern of Rapid3 is freezing the sample to -40°C with average cooling rate of 1.86°C/min and annealed at -40°C for 30 minutes.

Lyophilized products of F(ab')2 Green Pit Viper antivenin with 2% w/v of either glucose, sorbitol or fructose resulted in collapsed cake structure due to their low glass transition temperatures. Lyophilized products of F(ab')2 Green Pit Viper antivenin with 2% w/v of glycine produced mainly β-glycine structure. Lyophlized products of F(ab')2 Green Pit Viper antivenin with 2% w/v of mannitol resulting in δ-mannitol structure.

The crystalline excipients are physically appropriate for lyophilization of Green Pit Viper antivenin. Mixture of F(ab')2 Green Pit Viper antivenin with glycine (SG) or mannitol (SM) produced rigid structured cakes under desired duration time of lyophilization. The optimal lyophilization process is freezing from room temperature to -40 °C with average cooling rate of 1.86 °C/min and annealed at -40 °C for 30 minutes. Ramp time used to increase the temperature from -40°C to -25°C is 1 hour. Primary drying (pressure of 300 mTorr) at -25°C occured 6 hours and at 0 °C for another 12 hours. Heating up to 30 °C takes 1 hour, and allowed for secondary drying at 30°C for 2 hours under the pressure of 100 mTorr. The total time utilized is approximately 24 hours. This finding may be used to reduce the original lyophilization time reported by QSMI from 50 hours per cycle.

Formulation development of Green Pit Viper antivenin found that mixture of F(ab')2 with 2% w/v glycine is the best formulation which resulted in satisfying cake structure, solid state properties, thermal properties, protein contents and potency (Mouse Neutralizing Test). Stability studies of glycine formulation conforms closely to the initial product at day0 and native F(ab')2.

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APPENDICES

APPENDIX A

Standard curve OLD Lot no. 09002TA

Table 1A CD value at 217 nm of F(ab')2 solution (Conc bulk lot no. 09002TA)

Sample	Concentration (mg/ml)	CD ellipticity value at 217 nm (mdeg)
ConcA	2.64	-29.62748
ConcB	1.32	-14.74654
ConcC	0.7	-7.83889
ConcD	0.33	-3.80964
ConcE	0.22	-2.43593

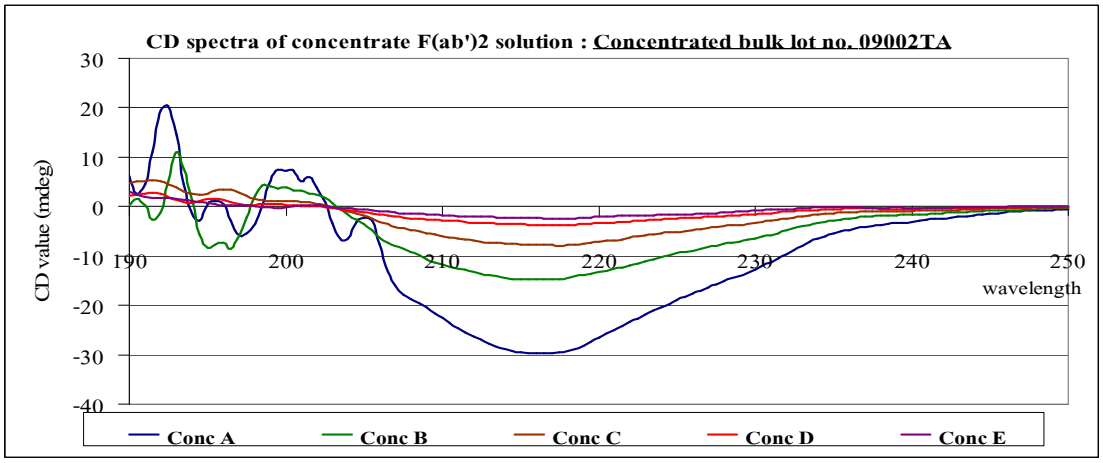

Figure 1A CD spectra of F(ab')2 solution various concentration from lot.no. 09002TA

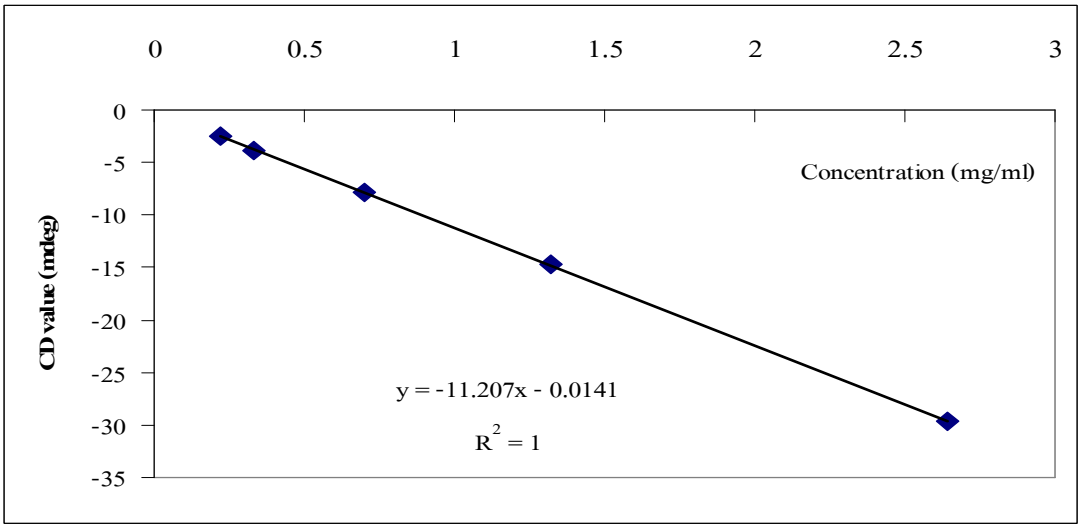

Figure 1B Standard curve of concentrated bulk lot no. 09002TA

Standard curve New Lot no. 10002TA

Table 1B CD ellipticity value at 217 nm of F(ab')2 solution (Conc bulk lot no. 10002TA)

Sample	Concentration (mg/ml)	CD ellipticity value at 217 nm (mdeg)
ConcA	2.76	-21.03209
ConcB	1.38	-10.09418
ConcC	0.7	-5.48507
ConcD	0.3	-2.31198
ConcE	0.15	-1.12077

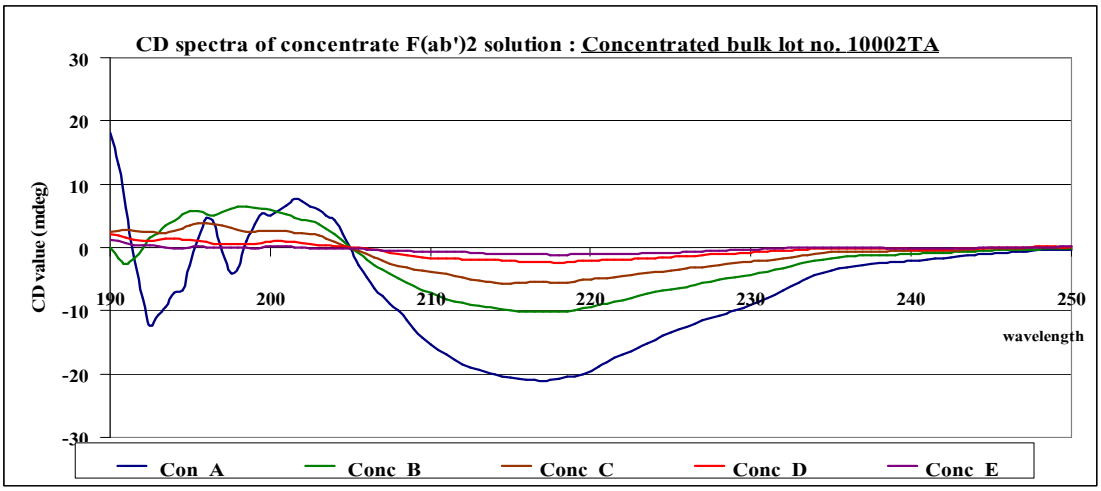

Figure 1C CD spectra of F(ab')2 solution various concentration from lot.no. 10002TA

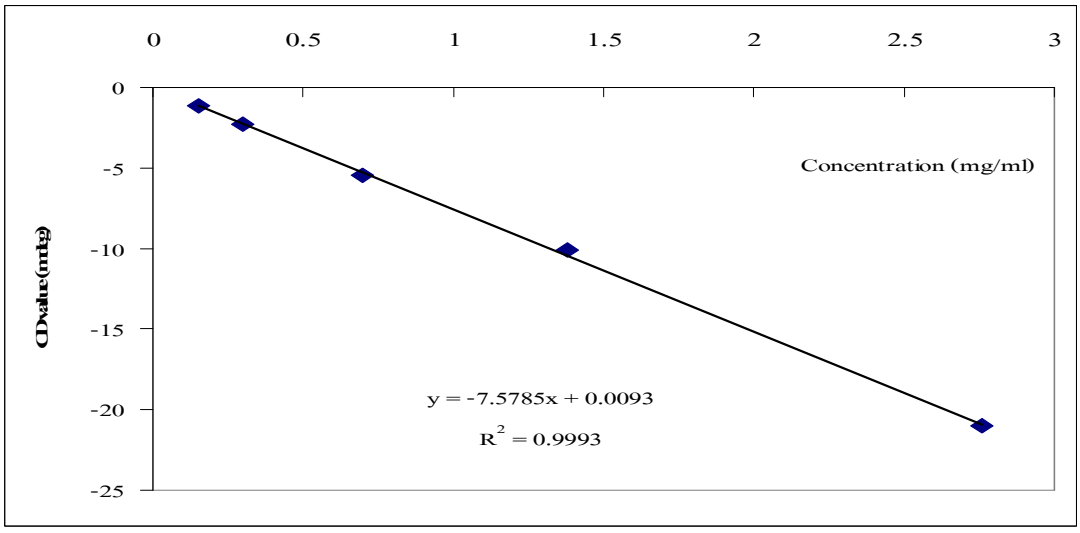

Figure 1D Standard curve of concentrated bulk lot no. 10002TA

APPENDIX B

CD spectra of native F(ab')2

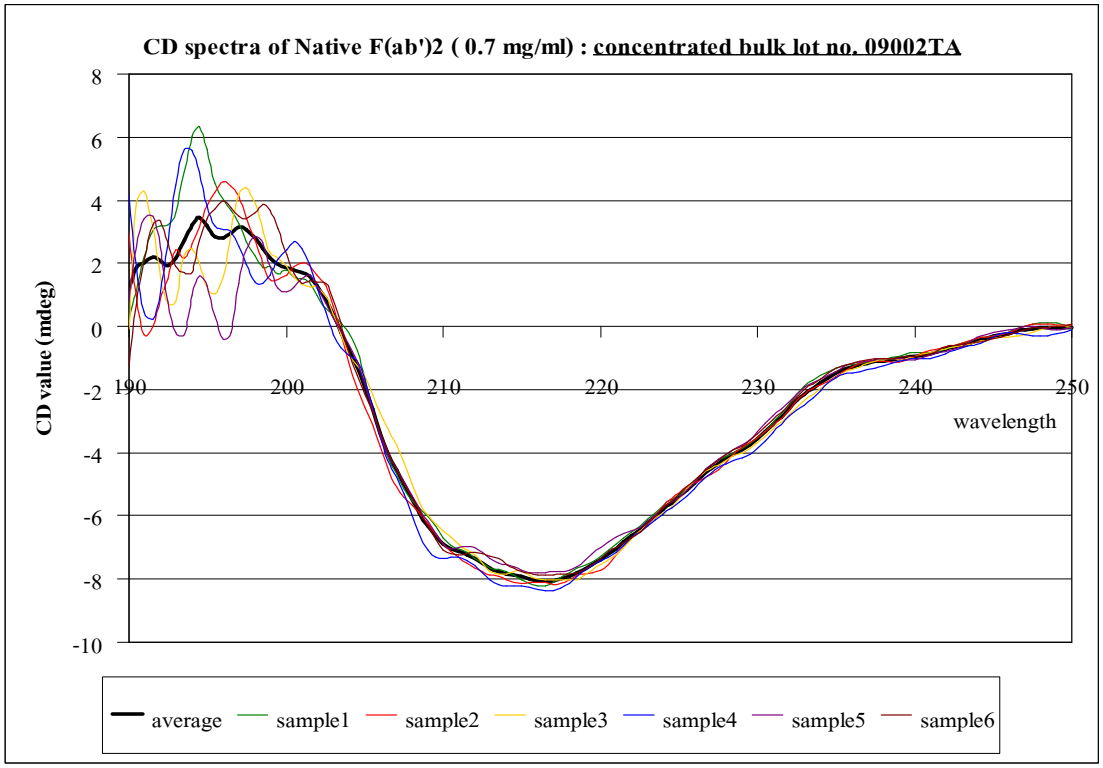

Figure 1E CD spectra of native F(ab')2 : concentrated bulk lot no. 09002TA

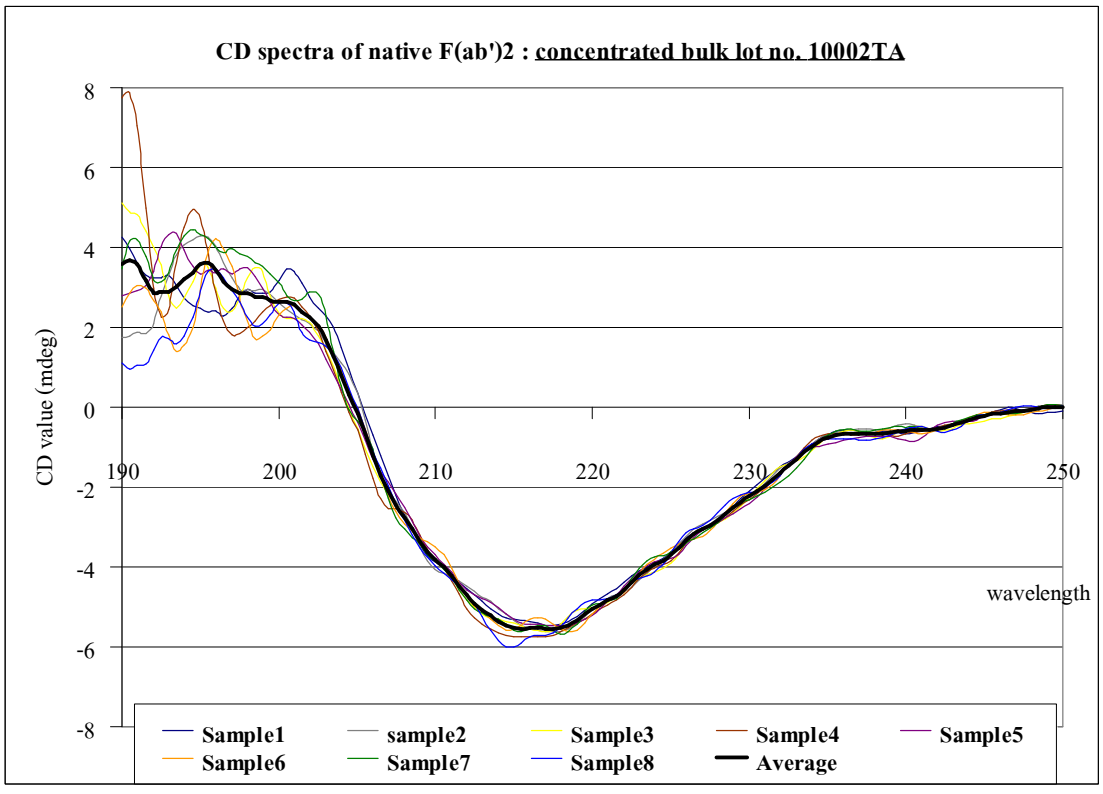

Figure 1F CD spectra of native F(ab')2 : concentrated bulk lot no. 10002TA

APPENDIX C

Table 3A Freezing patterns for study freezing process on secondary structure of F(ab')2

Cycle name / Step parameter	Slow		Rapid1		Rapid3	
	Temperature	Time (minute)	Temperature	Time (minute)	Temperature	Time (minute)
Freezing	25	0	25	0	25	0
	0	40	0	25	0	15
	-10	40	0	30	-40	20
	-20	40	-20	20	-40	20
	-20	30	-20	30	-	-
	-30	40	-40	20	-	-
	-40	40	-40	30	-	-
	-40	30	-	-	-	-
	-40 (extrafreeze)	70	-40 (extrafreeze)	20	-40 (extrafreeze)	35
Total time	330		175		90	

* Freezing process for freezing condition evalution (Lyophilization process development) use the same pattern follow as Table 3A.

Table 3B Primary and Secondary drying for lyophilization process of freezing evaluation

Cycle name / Step parameter	Slow			Rapid1			Rapid3		
	Temperature	Time (min)	Vacuum (mtorr)	Temperature	Time (min)	Vacuum (mtorr)	Temperature	Time (min)	Vacuum (mtorr)
Primary drying	-40	0	1000	-40	0	1000	-40	0	1000
	-30	120	1000	-30	120	1000	-30	120	1000
	-15	120	1000	-15	120	1000	-15	120	1000
	-5	120	1000	-5	120	1000	-5	120	1000
	-5	60	1000	-5	60	1000	-5	60	1000
	0	60	1000	0	60	1000	0	60	1000
	10	120	1000	10	120	1000	10	120	1000
	20	120	1000	20	120	1000	20	120	1000
	20	120	1000	20	120	1000	20	120	1000
	30	120	1000	30	120	1000	30	120	1000
	30	120	1000	30	120	1000	30	120	1000
Secondary Drying	30	120	100	30	120	100	30	120	100

Table 3C Optimization of lyophilization for primary and secondary drying evaluation.

Parameter Of process / Cycle name	Primary Dry								Secondary Drying		
	Step1		Step2		Step3		Step4				
	Temp	Time(hr)	Temp	Time(hr)	Temp	Time(hr)	Temp	Time(hr)	Temp	Time(hr)	Vacuum (mTorr)
PrimaryDry1	-25	1	-25	10	0	1	0	8	30	2	100
PrimaryDry2	-25	1	-25	6	0	1	0	12	30	2	100
PrimaryDry3	-25	1	-25	10	10	1	10	8	30	2	100
PrimaryDry4	-25	1	-25	6	10	1	10	12	30	2	100
PrimaryDry5	-30	1	-30	6	0	1	0	12	30	2	100
PrimaryDry6	-25	1	-25	3	0	1	0	15	30	2	100
PrimaryDry8	-25	1	-25	3	10	1	10	15	30	2	100
PrimaryDry9	-30	1	-30	3	0	1	0	15	30	2	100
PrimaryDry10	-30	1	-30	10	0	1	0	8	30	2	100
Secondary2	-25	1	-25	3	10	1	10	15	30	9	100
	Vacuum pressure : 300 mTorr										

APENDIX D

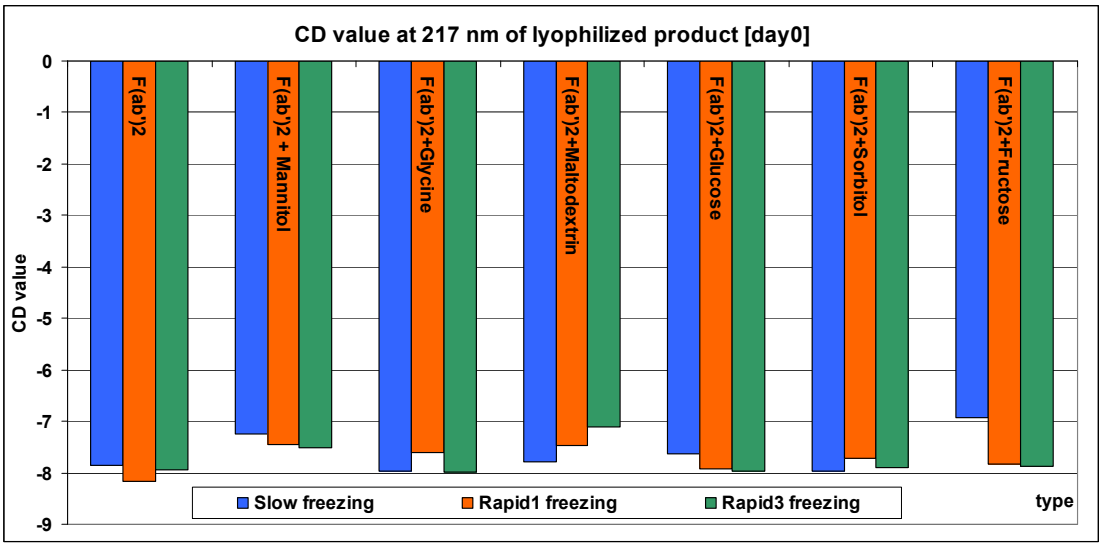

Figure 4A CD value at 217 nm of lyophilized product mixture of F(ab')2 with 2% stabilizer after lyophilized with Slow, Rapid1, Rapid3 freezing lyophilization process.

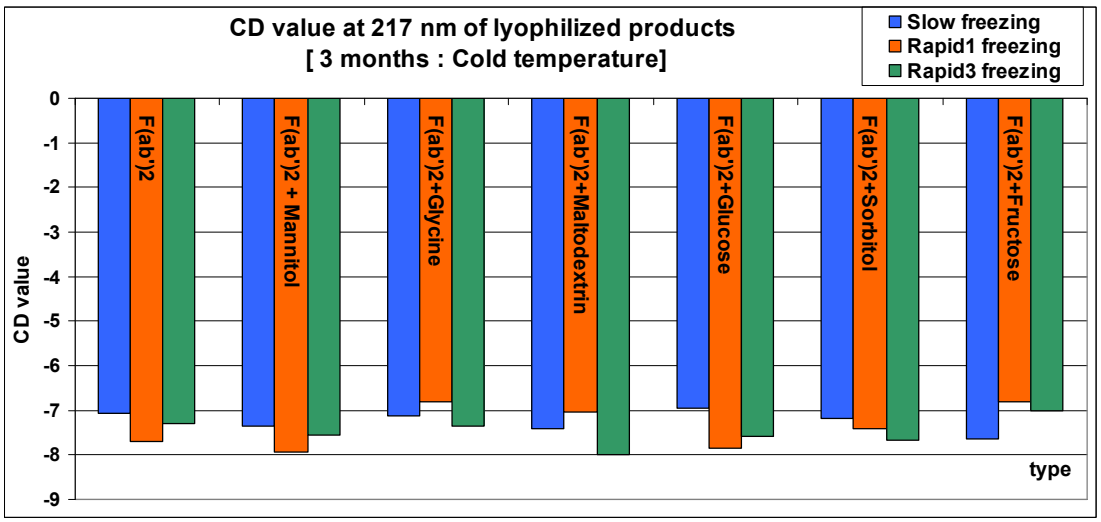

Figure 4B CD ellipticity values at 217 nm of mixture of F(ab')2 with stabilizer 2% lyophilized product after lyophilized with lyophilization process (Slow, Rapid1, Rapid3 freezing) (stored 3 months at 2-8 °C)

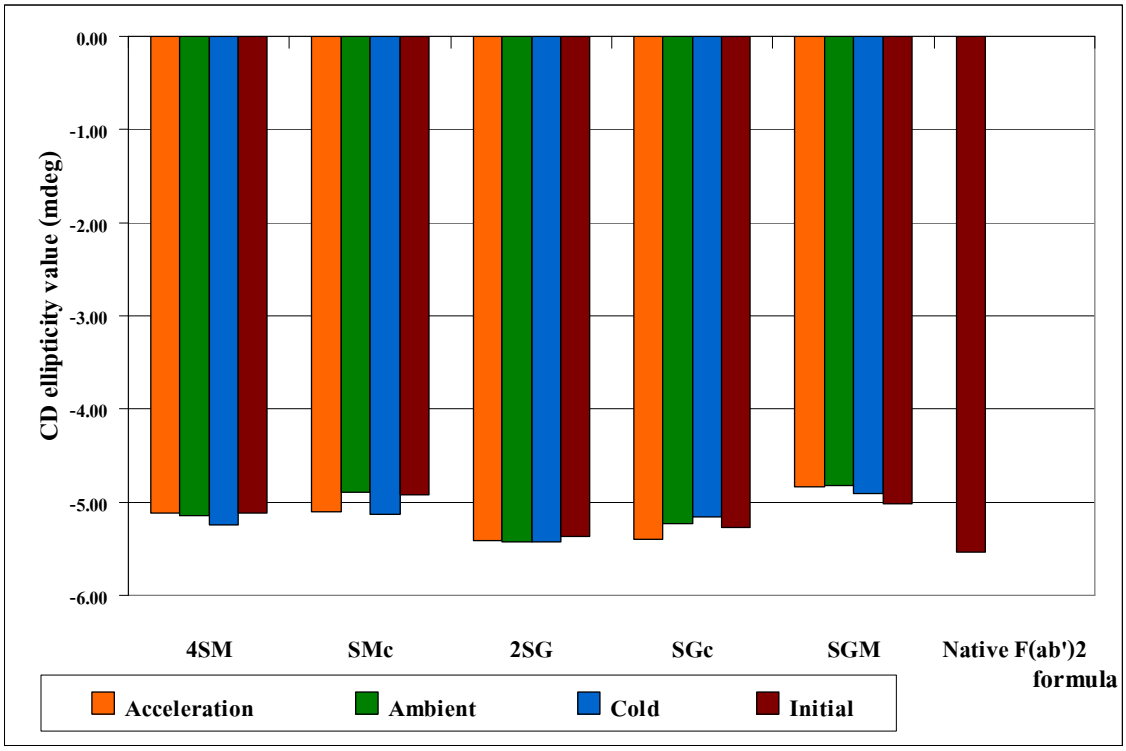

Figure 4C CD ellipticity values of initial lyophilized products and lyophilized products stored under various stability condition for 3 months.

APENDIX E

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 11-33-002	**Approval No. 11-33-002**
Protocol Title The potency determination of green pit viper antivenin product	
Principal Investigator Sumana Khomvilai	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval March 11, 2011	**Date of Expiration** March 11, 2012
Applicant Faculty/Institution Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330	
Signature of Chairperson	**Signature of Authorized Official**
Name and Title THONGCHAI SOOKSAWATE, Ph.D. Chairman	**Name and Title** PARKPOOM TENGAMNUAY, Ph.D. Associate Dean (Research and Academic Service)
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

QUEEN SAOVABHA MEMORIAL INSTITUTE
THAI RED CROSS SOCIETY
(WHO Collaborating Centre for Research on Rabies Pathogenesis and Prevention)

Queen Saovabha Memorial Institute Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Protocol No. 2/2010	
Protocol Title Development of formulation and lyophilization process of green pit viper antivenin	
Principle Investigator Thammanoon Duangsano	
Certification of Institutional Animal Care and Use Committee (QSMI-ACUC) This project has been reviewed and approved by the committee in accordance with Queen Saovabha Memorial Institute regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by National Research Council of Thailand.	
Date of Approval October 20, 2010	**Date of Expiration** October 20, 2012
Applicant Institution Queen Saovabha Memorial Institute, Thai Red Cross Society	
Signature of Chairperson Narongsak Chaiyabutr **Name and Title** Prof. Dr. Narongsak Chaiyabutr Chairman	**Signature of Authorized Official** Visith Sitprija **Name and Title** Prof. Dr. Visith Sitprija Director of Queen Saovabha Memorial Institute
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow institute regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews*	

Division of Science, The Thai Red Cross Society
1871 Rama IV Road
Bangkok 10330, Thailand
Tel : (662) 252-6117
Fax : (662) 254-0212

VITA

Mr. Thammanoon Duangsano was born on March 1, 1979 in Nakhonpathom, Thailand. He obtained his Bechelor degree in Pharmacy (B.Pharm.) from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand in 2000. He obtained his Bechelor degree in Business administration (B.BA.) and Public health (B.PH.) from Sukhothai Thammathirat Open University. He works at Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok from 2004 until present.