"OPTIMIZATION OF DIALYSIS PROCESS FOR LIPOSOMAL FORMULATION USING DESIGN OF EXPERIMENT"

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MASTER OF PHARMACY

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PHARMACEUTICAL TECHNOLOGY &

BIOPHARMACEUTICS

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CERTIFICATE

This is to certify that the dissertation work entitled "Optimization of dialysis process for the liposomal formulation using Design of Experiment" submitted by Mr. Shaikh Mohammed Kasim with Regn. No. (13MPH112) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Technology and Bio pharmaceutics" is a bonafide research work carried out by the candidate at the Controlled Release Injection Department, Wockhardt Research Center, Aurangabad under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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TO WHOM SO EVER IT MAY CONCERN

This is to certify that **Mr. Shaikh Mohammed Kasim,** student of **M. Pharm, from Department of Pharmaceutics, Institute of Pharmacy, Nirma University, Ahmedabad.** has successfully completed his project training from **October 13, 2014** to **April 12, 2015** for the period of **Six Months** at **Wockhardt Research Centre, Aurangabad.**

He has done his project on **"Optimization of Dialysis Process for Liposomal Formulation Using Design of Experiment"** & has shown a keen interest in learning various research procedures.

We have found him to be sincere, honest and hardworking during his training period in this company. To the best of our knowledge he bears a good moral character.

We wish him all success in his life.

For Wockhardt Research Centre

Sucheta Rane
Human Resources

DECLARATION

I hereby declare that the dissertation entitled "Optimization of dialysis process for the liposomal formulation using Design of Experiment", is based on the original work carried out by me under the guidance of Mr. Mukesh Kumar, General Manager, Controlled Release Injection, Wockhardt research Centre, AurangabadandDr. Jigar N. Shah, Assistant professor, Institute of pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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……..Mohammed Kasim

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List of Abbreviations

Cb	Component concentration in the bulk solution [g L-1]
Cf	Component concentration in the filtrate stream [g L-1]
Cross flow	The flow of fluid through the feed channels of the membrane modules created by a pump
CF	Concentration factor
CR	Conversion ratio
Cw	Component concentration at the membrane surface [g L-1]
DF	Diafiltration
DV	Diavolume
CFF	Cross flow filtration
CIP	Cleaning-in-place
ΔP	Pressure differential between retentate and feed
kD	KiloDalton (=1000 Dalton)
LMH	Liter per square meter of membrane surface per hour
NMWC	Nominal molecular weight cut-off
HPTFF	High performance tangential flow filtration
J_f	Filtrate flux [L m-2 h-1]
J_m	Mass flux [g m-2 h-1]
TFF	Tangential flow filtration
TMP	Transmembrane pressure
UF	Ultrafiltration
VCF	Volume concentration factor
VF	Virus filtration
WFI	Water for injection

Optimization of dialysis process for liposomal formulation using Design of Experiment

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Abstract

Major goal of this work is to remove the solvent impurity from the formulation and external buffer exchange by the dialysis process. Dialysis process was done by additional buffer solution to maintain the osmolarity of the formulation. The Purpose of the study was to evaluate the different diafiltration process parameter for the efficient product encapsulation and removing solvent from the stable nanoparticle dispersion. Impact of various process operations on subsequent Nano particulate separation and hence, maximum product recovery was analyzed. Two statistical designs were used in this study as part of an investigation into the possibility and the advantages of applying QbD concepts to diafiltration process. First design (Plackett–Burman) was used to screen high-risk variables obtained from risk analysis and assess their impact on diafiltration process and after that applying the central composite design (CCD) (with more predictive capability) to fully explain the relationship between the variables. Results showed that the trans-membrane pressure and the concentration factor of the feed was major variable for the desire filtration process. Lipidic Nano particle (LPN) separation was done by the tangential flow filtration method. A tangential flow filtration (TFF) system was estimated to purify PEGylated lipid nanoparticles. After sufficient removal of the impurities from the formulation, drug loading was done to better encapsulation of the drug in to the nanoparticle. Minimum concentration of the external buffer concentration for better encapsulation efficiency was also determined at the different level of the diafiltration process. A micro scale cross flow filtration device was designed to concentrate the desired product in the retentate. Overall, the results illustrate the power of micro scale techniques to identify and enable the understanding of key process performance attributes in a Diafiltration process sequence.

Key words: dialysis, tangential flow filtration (TFF), design of experiment**,** Plackett–Burman, Central composite design (CCD), Design Space

1. AIM OF INVESTIGATION

Tangential flow filtration (TFF) is a unit operation that is generally used to separate solutes differing by more than 10 fold in their size; the application is widely used in the area of biotechnology, mainly in purifying cell, virus and bacterial proteins. With the growth of nanotechnology and its application in the biopharmaceutical field, there is a wide application to process Nano sized pharmaceutical colloids. A successful use of Diafiltration and TFF has been established in nanoparticle purification from excess residual solvent and external buffer solution. The use of TFF is not only limited to polymeric nanoparticles, but has been successfully extended to separation of various sized gold nanoparticles from small impurities and in the field of liposomes as well as cationic solid lipid nanoparticles to remove solvents to minimize toxicity.

In my project I have well optimized the TFF system to remove ethanol and buffer solution from PEG based nanoparticles. In the present study, this technique is further explored to purify PEGylated liposome based nanoparticles with quantitative monitoring of the most widely used solvent ethanol and ammonium sulphate buffer.

The overall aim of this thesis was to optimize the various process factor (variable) of the tangential flow filtration for the buffer exchange, impurity removal (solvent removal) & to create the ionic gradient across the liposomal bilayer using Design of experiment (DoE) approach.

In order to achieve the overall project aim a series of objectives were defined as described below.

1. Establish a small-scale filtration platform for the separation of the solvent from the product. Evaluation of various process parameter of the filtration operation. Based on the preliminary trail of the filtration process, determine the optimum variable range of the different parameter which affect the filtration process. Establishment of a small scale cross flow operation, which will require the design of a novel filtration process.

2. The second objective of my thesis was to identify the critical process parameter of the diafiltration process that influence the final product quality and total process time. Optimized the process parameter and identify the range limit of my factor.

3. The third objective was to expand the range of operations that can be studied at the small scale, and involved the design of a novel, well-matched, cross flow filtration (CFF) operating process for the predicted response. Optimized the different factor for the best predicted responses of the diafiltration process. (Placket-Burman and Central composite design of the process parameter)

4. The fourth objective was to optimize the external buffer ions concentration which interfere with the drug loading process, or incomplete drug loading, or less encapsulation efficiency of the formulation. The purpose of this study was to determine any possible benefits through additional Diafiltration step might have on the TFF process. Diafiltration should increase diffusion of impurities through ultrafiltration membranes by continually or intermittently adding buffer to maintain the retentive volume and maintain the osmolarity of the formulation. In this study, different combination of diafiltration and concentration mode was analyzed on the basis of buffer volume consumption, time of filtration impurity removal and filtrate flux rate.

Plan of work:

- Preparation of different buffer concentration of liposome. (300mM- 100Mm salt containing)
- Evaluation of TFF process parameter
    a. Transmembrane Pressure
    b. Cross-flow velocity
    c. Feed concentration
    d. Concentration mode
    e. Diafiltration mode
- Risk Analysis based on the cause and effect diagram (Ishikawa diagram)
- Apply the Plackett-Burman design for the screening of the critical factor
- Apply the Central composite design for the main factor
- Plot the design space of the process and optimize the design space
- Characterization of the concentration and diafiltration mode of the TFF process
- Determine the impact of the filtration process on the physical parameter of the formulation (In process)
    a. Particle Size and PDI
    b. Zeta potential
    c. pH and Conductivity
    d. Viscosity and Osmolarity
    e. Lipid content
- Drug loading at the different stage of the diafiltration
- Determine the effect of the external ion concentration to the free drug
- Removal of the Unentrapped drug from the external buffer of the liposomal formulation using TFF process
    a. Diafiltration step
    b. Concentration step
- Optimization of the different evaluation parameter of the liposome after process
    a. Drug content
    b. Encapsulation efficiency
    c. Particle size & Zeta potential

2. INTRODUCTION

2.1 Background of the project

Liposomes are tiny vesicles composed of natural and synthetic phospholipids and lipids. The size of liposomes can vary from 25 nm to several microns and can be multilaminar (MLV) or uni-or single lamellar (ULV, SUV or SLV) depending on the type of lipids and the method of manufacture. In the 1960's, Bangham[1, 2] discovered that vigorous dispersion of purified phospholipids in water resulted in the formation of microscopic closed membrane spheres. These microscopic membranes form single or multiple lipid bi-layers around an aqueous core and were referred to as liposomes. The application of liposomes to therapeutics was identified by polar/charged small molecules were found to be retained within the liposomes (Sessa and Weissmann, 1968[3]).

Liposomes, defined as microscopic spherical-shaped vesicles, consist of an internal aqueous compartment entrapped by one or multiple concentric lipidic bilayers. Liposomes membrane is composed of natural and/or synthetic lipids which are relatively biocompatible, biodegradable and non-immunogenic material. Because of their unique bilayer-structure properties, liposomes are used as carriers for both lipophilic and water-soluble molecules. Hydrophilic substances are encapsulated in the interior aqueous compartments. Lipophilic drugs are mainly entrapped within lipid bilayers.

Liposomes have attractive biological properties, including the biocompatibility and biodegradability. They show promise as active vectors due to their capacity to enhance the encapsulant performance by increasing drug solubility, and stability; delivering encapsulated drugs to specific target sites, and providing sustained drug release.[4] Their sub-cellular size allows relatively higher intracellular uptake than other particulate systems; improving in vivo drug bioavailability.

Other advantages of liposomes include high encapsulation efficiency inspite of drug solubility, low toxicity due to phospholipids content, drug protection against degradation factors like pH and light and the reduction of tissue irritation.

Liposomes have been extensively studied as drug carriers in the pharmaceutical and medical fields[4-5]. Research has expanded considerably over the last 30 years, increasing

applications area from drug and gene delivery to diagnostics, cosmetics, long-lasting immune-contraception to food and chemical industry.[6] Ten liposomal and lipid-based formulations have been accepted by regulatory authorities and many liposomal drugs are in preclinical development or in clinical trials.[7]

Liposomes, and other particles, of appropriate size (typically <200 nm) and composition can provide longer circulation times as well as provide a passive targeting mechanism for tumor vasculature via the **Enhanced Permeability and Retention** effect (Seymour, 1992[8], Seymour and Baban, 1998 [9] ; Dvorak, 1998). Liposomes have the potential to improve efficacy, safety, and compliance of drugs by delivering more of the drug to the disease site, by providing controlled release, and by reducing dose amounts/frequency. Although liposomes represent an interesting drug delivery approach, the practical application of producing reasonable quantities of medically suitable liposomes is challenging.

2.2 Structure of liposome:

The phospholipid restructuring in aqueous solution is mainly determined by the hydrophobic effect which classifies amphiphilic molecules (phospholipids) so as to minimize entropically unfavorable interactions between hydrophobic acyl-chains and surrounding aqueous medium.[10]This effect is further settled by various intermolecular forces such as electrostatic interactions, hydrogen bonding, as well as Vanderwaals and dispersion forces.[11]

Liposomes were defined as an artificial microscopic vesicle consisting of a central aqueous compartment surrounded by one or more concentric phospholipid layers (lamellas) (Fig. 1). Furthermore, hydrophilic (in the aqueous cavity), hydrophobic (within lipidic membrane) and amphiphilic substances are able to be incorporated within these vesicles developing large potential applications.

Numerous researchers have worked with these structures since Bangham's discovery, making of liposomes the most popular nanocarrier system.[14]

2.3. Classification of liposome

Liposomes can be classified on the basis of composition and mechanism of intracellular delivery into five types.[12]

(i) Conventional liposomes;
(ii) pH-sensitive liposomes;
(iii) Cationic liposomes;
(iv) Immunoliposomes and
(v) long-circulating liposomes.

Otherwise, vesicle size is a critical parameter in determining circulation half-life of liposomes, and both size and number of bilayers influence the extent of drug encapsulation within liposomes. Thus, liposomes were typically classified on the basis of their size and number of bilayers into **(Fig. 2):**

(i) Small unilamellar vesicles (SUV): 20–100 nm;
(ii) Large unilamellar vesicles (LUV): > 100 nm;
(iii) Giant unilamellar vesicles (GUV): > 1000 nm;
(iv) Oligolamellar vesicle (OLV): 100–500 nm and
(v) Multilamellar vesicles (MLV): > 500 nm

New developed types of liposome, designated as double liposome (DL)[15] and multivesicular vesicles (MVV),[16] were recently reported. These liposomes, which could be prepared by novel technique, are thought to improve drug protection against several enzymes.[17]

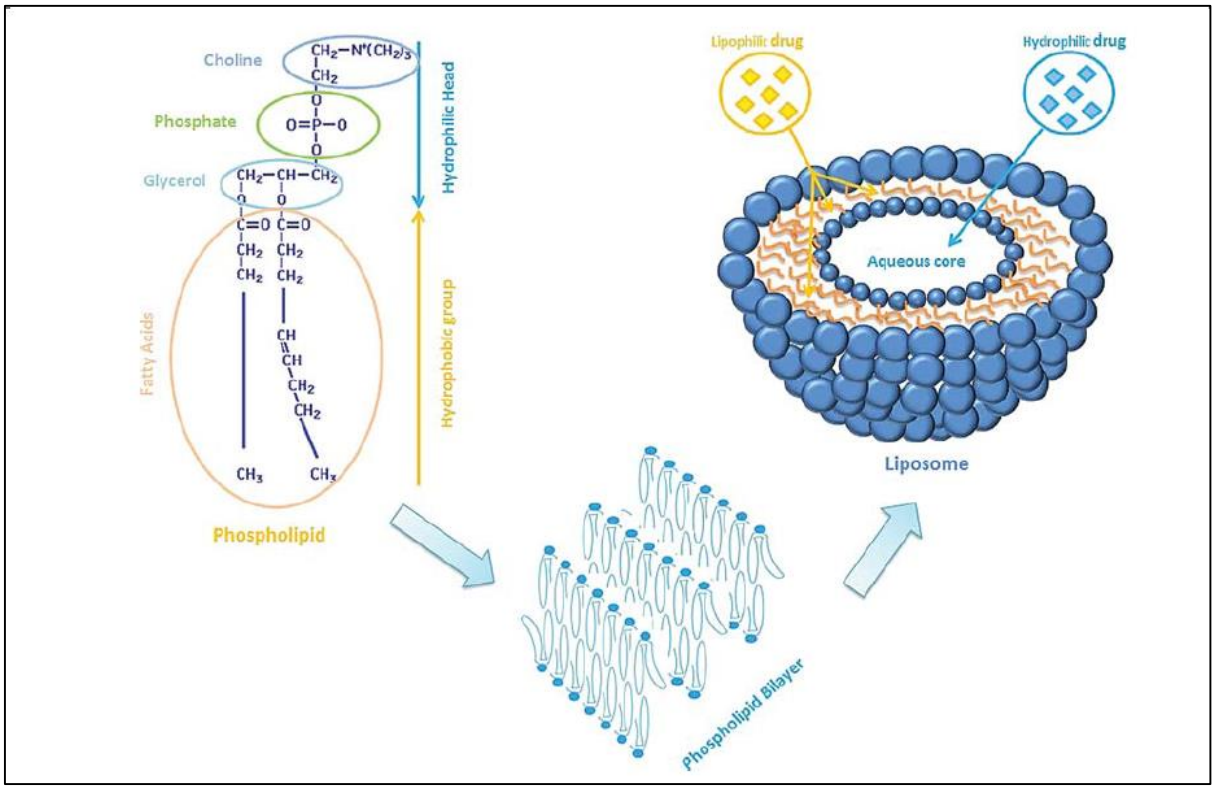


Figure. 1. ***Structure of Liposome: (Hydrophilic and lipophilic portion of the liposome)***[1]

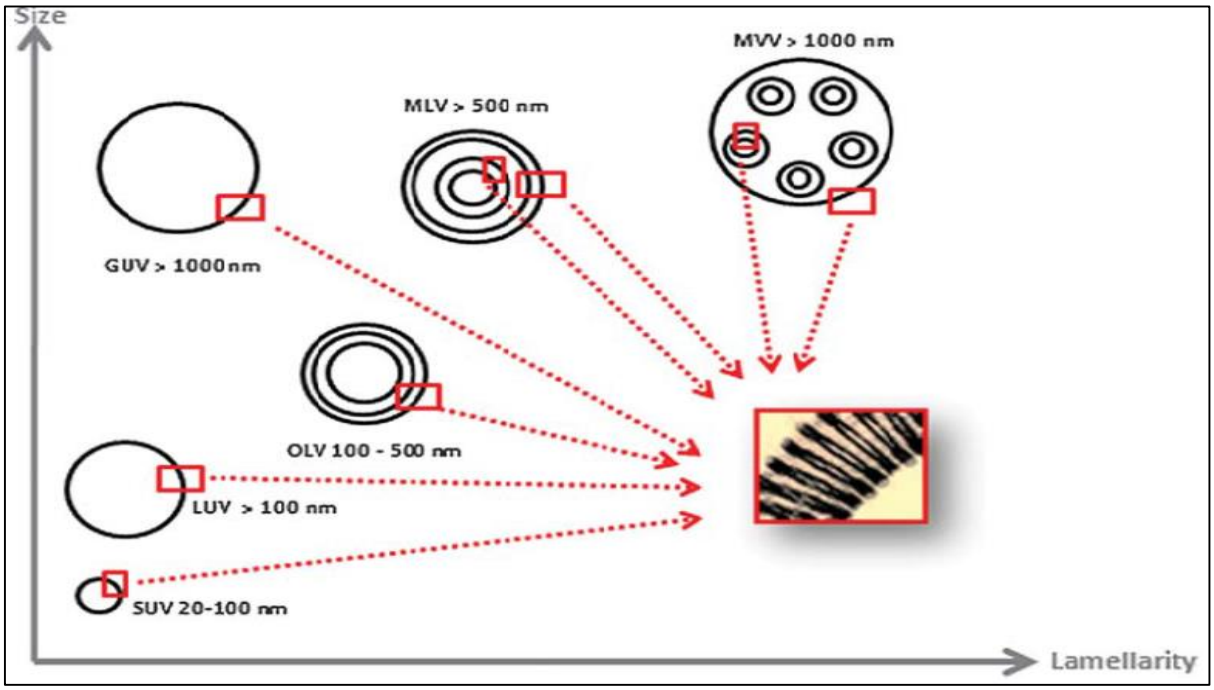


Figure. 2: ***Size and lamellarity of the liposome***[1]

[1]Preparation, Characterization and Applications of Liposomes: State of the Art, Journal of Colloid Science and Biotechnology Vol. 1, 147–168, 2012.

2.4. General Ingredients

Lipid particle: The suspension or solution, lipid particles or liposomes may comprise neutral, anionic and/or preferably cationic lipids. Cationic lipids are preferably comprised in an amount of at least about 30 mol %, more preferably of at least about 40 mol %, most preferably at least about 50 mol % of total liposome forming lipids. Neutral or anionic lipids may be from sterols or lipids such as cholesterol, phospholipids, lysolipids, sphingolipids or pegylated lipids with a neutral or negative net charge.

Neutral and anionic lipids : phosphatidylserine, phosphatidylglycerol, fatty acids, Sterols containing the carboxylic acid group eg.cholesterol, 1-2-diacyl-sn-glycero-3 phospho ethanolamine including 1,2-dioleylphosphoethanolamine (DOPE),1,2 disterylphosphatidyl choline (DSPC), 1,2-dipalmitylphosphatidylcholine (DPPC), 1,2-dimyristylphosphatydyl choline (DMPC), phisphatidylcholine preferably egg PC, soy PC, and sphingomylin.

Cationic lipids: N-[1-(2, 3-dioleoylloxy) propyl]-N, N, N-trimethyl ammonium (TAP) salts, preferably the chloride or methylsulfate. Preferred representative of the TAP lipids are DOTAP (dioleoyl-), DMTAP (dimyristoyl-), DPTAP (dipalmitoyl-), or DSTAP (disteroyl). Two different acyl chain can be linked to the glycerol backbone and form the different catioinic lipids such as N-[1-(2, 3-dioloyloxy) propyl]-N, N-dimethylamine (DOTAP), etc.

Hydrophilic excipients: Hydrophilic polymers suitable for derivatization with a vesicle forming lipids include polyvinyl pyrrolidone, polyvinyl methyl ether, poly methyl oxazoline, poly ethyloxazoline, poly hydroxypropyloxazoline, hydroxymethyl cellulose, hydroxyethyl cellulose, polyethylene glycol, polyaspartame. The polymers may be employed as homopolymers or as block or random copolymers.

A preferred hydrophilic polymer chain is polyethylene glycol(PEG), preferably as a PEG chain having a molecular weight between 500-10000 daltons, more preferably between 500-5000 daltons, most preferably between 1000-2000 daltons. Methoxy or ethoxy- capped analogues of PEG are also preferred hydrophilic polymers, 120-2000 daltons.

Another hydrophilic excipients is a saccharide, a mono, di-, oligo-, or polysaccharides, a sugar alcohol, an amino acid, a peptide, a protein, a water- soluble polymer or combination of their.

A saccharide, or carbohydrate like glucose, fructose, lactose, sucrose, trehalose, maltose, cellobiose, galactose, etc. Other water soluble cellulose derivatives such as methylcellulose, hydroxy propyl cellulose, hydroxy ethyl cellulose, and hypermallose. Particularly preferred saccarides are glucose and trehalose. Other hydrophilic excipients like amino acids, peptides or proteins eg.glycine or other natural amino acids used.

Antioxidants: Stabilizers and antioxidants prevent the oxidation of an active compound nadthelipids which are sensitive to oxidation. Lipids soluble antioxidant such as alpha-, beta-, and gamma-tocopherol, lycopene, alpha- and beta- carotene. Particularly preferred are alpha-tocopherol and ethylenediaminetetraacetic acid.

Organic solvents: Lipid particle are suspended in the aqueous medium. Besides water, other aqueous medium which are at least partially miscible with water, preferably organic solvents, more preferably alcohols (e.g. C1-4 alcohols such as methanol, ethanol, propanol, butanol and combinations thereof or ketones (e.g.C1-4 ketones such as acetone, methyl-ethyl ketone, DMF, DMSO and thereof also used. For preferred method, the aqueous medium is water and ethanol. The ratio between water and the further liquid constituent is preferably between about 99.9:0.1 and about 10:90 (v/v), most preferably between about 80:20 and about 60:40 (v/v).

Advantageous of ethanol alone or in combination was widely used in the formulation.

- Method results in very small and homogenous liposomes
- A decreased viscosity and increase the fluidity, enabling high flow rates of the lipid suspension
- Leads to reduction in drying temperature during the evaporation process.

2.5 Liposomes preparation Technique

Many loading techniques (e.g. passive loading) resulted in poor entrapment efficiencies, limited retention of drugs, polydispersity, and sub-optimal size. Several techniques for loading therapeutic molecules into liposomes have been established in literature as well as practice (Bangham, 1965[1]; Olson, 1979[13]; Hope[14], 1985[15] , 1986 Mayer, 1986[16]; Cullis, Madden, 1990[17] Hope and Wong[18];Wheeler, 1994[19]; Batzri, 1973[20]; Haung, 1969[30], and Fenske, 1998[21]). The most commonly practiced techniques for preparing drug loaded SLV liposomes **include high shear homogenization/extrusion, transmembrane pH gradients, reverse phase evaporation, dialysis, sonication and ionophore processes**.

2.5.1 Hydration of a Thin Lipid Film: Bangham Method[2]

A mixture of phospholipid and cholesterol were dispersed in organic solvent. After that the organic solvent was removed by the evaporation (using a Rotary Evaporator at reduced pressure). Finally, the dry lipidic film deposited on the flask wall was hydrated by adding an aqueous buffer solution under agitation at temperature above the lipid transition temperature (Tg).

Advantages:

- Easy to handle
- Method is widespread

Disadvantages:

- Dispersed-phospholipids in aqueous buffer produce a population of multilamellar liposomes (MLVs) heterogeneous both in size and shape (1–5µm diameter).
- Liposome size reduction techniques, such as sonication for SUVs formation or extrusion through polycarbonate filters forming LUVs were useful to produce smaller and more uniformly sized population of vesicles.

2.5.2 Extrusion/Homogenization Based Processes

In homogenization/extrusion based processes for production of SLV's (Bangham, 1965[22]Olson, 1979[23]; Hope, 1985[24]; Hope, 198[23] and references), lipid films containing drug are typically prepared by mixing lipids, drug and organic solvents (e.g. methanol,

ethanol, chloroform) followed by drying (lyophilization, vacuum, evaporation, etc). Multiple freeze/thawing cycles are often used to increase trapped volume as well as to promote equilibrium solute distributions.

The dried lipid films are hydrated in aqueous media to form MLV's and passed through various sized membranes (e.g. 0.4, 0.2, 0.1 micron polycarbonate) or homogenized over several cycles to produce nanosized SLV's. The hydration step at temperatures above the T_m of lipids to aid in formation of liposomes. The lipid film production can also be employed as a precursor step in other loading techniques (e.g. pH transmembrane, ionophore, and sonication). The extrusion/homogenization process can be used for both neutral lipophilic drugs or water soluble drugs and is a well-established technique used in commercial products (e.g. Myocet, AmBisome).

2.5.3 Solvent (Ether or Ethanol) Injection Technique.

The ethanol injection method was first described in 1973.Another organic solvent technique is an **injection method** (Batzri, 1973[20]). In the injection method, liposomes are produced by continuous injection of the lipid containing organic phase (e.g. ethanol) into the aqueous phase. The solvent injection methods include the dissolution of the lipid into an organic phase (ethanol or ether), followed by the injection of the lipid solution into aqueous media, forming liposomes.

This injection process typically produces MLV's. Production of SLV's from these organic systems can suffer from problems associated with solubility of lipid mixtures in the organic phase as well as the potential need for chromatography or dialysis to remove the organic solvents. (Hope, 1986 and references). The main application of the ethanol injection method resides in the observation that a narrow distribution of small liposomes (under 100 nm) can be obtained by simply injecting an ethanolic lipid solution in water, in one step, without extrusion or sonication.

The ether injection method differs from the ethanol injection method since the ether is immiscible with the aqueous phase, which is also heated so that the solvent is removed from the liposomal product. The method involves injection of ether-lipid solutions into warmed aqueous phases above the boiling point of the ether. The ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms primarily unilamellar liposomes.

Advantage of the ether injection method compared to the ethanol injection method is the removal of the solvent from the product, enabling the process to be run for extended periods forming a concentrated liposomal product with high entrapment efficiencies.

2.5.4 Transmembrane pH Gradient

Weak base drugs could be loaded into existing, preformed SLV liposomes (often prepared by extrusion/homogenization techniques as described above) via a transmembrane pH gradient (Mayer, 1986[16]; Madden, 1990[17], Cullis, 1997[16]; Hope and Wong, 1995[25]). The process is based on membrane permeability of weakly basic drugs (pKa=8.6) incubated at neutral pH with SLV's containing an acidic interior core. The neutral drug will diffuse via concentration gradient into the liposome interior where it is protonated and trapped in the liposome as the charged form is not permeable to the membrane. The liposomal loading continues until the drug is depleted or the buffering capacity of the liposome interior is exhausted. These buffer systems have included **citrate and ammonium sulfate**.

2.5.5 Detergent Dialysis

In this method, liposomes (40–180 nm) are formed when lipids are solubilized with detergent, yielding defined mixed micelles. As the detergent is subsequently removed by controlled dialysis, phospholipids form homogeneous unilamellar vesicles with usefully large encapsulated volume.

In detergent dialysis, dry lipids or preformed vesicles are solubilized in the appropriate detergent containing buffer to form mixed micelles. As the detergent is removed by dialysis, the micelles coalesce and the phospholipids produce the sealed bi-layer structure. This technique has several drawbacks including long processing times for dialysis as well as removal of contaminating detergents. (Hope, 1986[25]and references).

2.5.6 Reverse-Phase Evaporation (REV) Technique

In reverse phase/organic systems, the lipids are hydrated directly from organic solvent systems. This process can produce both MLV's and SLV's depending on lipids and processes used. **US4235871** (Papahadjopoulos, Skoka) and **US4485054** (Mezei, Nugent) provide examples of this process where a water in oil emulsion is formed with the lipids in the organic solvent and the active substances in the aqueous buffer solution. The organic

solvent is removed under vacuum which results in a gel-like mixture. This mixture is converted to liposomes/lipid vesicles by agitation or dispersion in an aqueous media.

A lipidic film is prepared by evaporating organic solvent under reduced pressure. The system is purged with nitrogen and the lipids are re-dissolved in a second organic phase which is usually constituted by diethyl ether and/or isopropyl ether. Large unilamellar and oligolamellar vesicles are formed when an aqueous buffer is introduced into this mixture. The organic solvent is subsequently removed and the system is maintained under continuous nitrogen. These vesicles have aqueous volume to lipid ratios that are 30 times higher than sonicated preparations and 4 times higher than multilamellar vesicles. Most importantly, a substantial fraction of the aqueous phase (up to 62% at low salt concentrations) is entrapped within the vesicles, encapsulating even large macromolecular assemblies with high efficiency.

2.5.7 Sonication

Sonication of MLV's has been used to prepare SLV's (Haung, 1969[26]; Hope, 1986[14]and references). This procedure produces limit size vesicles (25-50 nm). Sonication of MLV's has several drawbacks such as limited vesicle size, liposome instability, and low trapped volumes.

2.5.8 Ionophore

Ionophore loading (Fenske, 1998[25]; Wheeler, 1994[25]) involves the use of ion driven, secondary pH gradients or ionic metal/drug complexes (e.g. doxorubicin-Mn^{+2}) to load liposomes. In the ion driven systems, a primary ion gradient is generated when SLVs manufactured by extrusion in K_2SO_4, $MnSO_4$, or $MgSO_4$ solutions are passed down a column equilibrated in a sucrose-containing buffer. After the primary ion gradient is established, the drug (e.g. vincristine, mitoxantrone, ciprofloxacin, and doxorubicin) is added followed by the ionophore (nigericin for K, A23187 and EDTA for Mn and Mg).

The ionophores couple the outward flow of the metal ions and the inward flow of protons. The ionophore mediated transport results in an acidification of the liposome interior. This creates a pH gradient which in turn provides a loading mechanism for the drug. Encapsulation efficiencies of 80-90% with these systems (Fenske, 1998). The nigericin systems were reported to have poor in-vivo circulation and drug retention while the

A23187/EDTA systems report similar in-vivo circulation/drug retention as citrate and ammonium loaded systems. In metal ion/drug complex systems, metal ion drug conjugates (e.g. doxorubicin-Mn^{+2}) are able to load the liposomes without the presence of a pH gradient.

2.5.9 New Large-Scale Liposome Technique

A number of techniques such as Heating Method, Spray drying, Freeze Drying, Super Critical Reverse Phase Evaporation (SCRPE), and several modified ethanol injection techniques for the of liposome preparation which are increasingly attractive.

I. Heating Method

This method involves the hydration of liposome components in an aqueous medium followed by the heating of these components, in the presence of glycerol (3% v/v), up to 120^0C. Glycerol is a water-soluble and physiologically acceptable chemical with the ability to increase the stability of lipid vesicles and does not need to be removed from the final liposomal product. Temperature and mechanical stirring provide adequate energy for the formation of stable liposomes.

By the TLC that no degradation of the used lipids occurred at the above mentioned temperatures (Reza Mozafari et al). The particle size can be controlled by the phospholipid nature and charge, the speed of the stirring and the shape of the reaction vessel. Otherwise, employment of heat stops the need to carry out any further sterilisation procedure reducing the time and cost of liposome production

II. Spray-Drying

Spray-drying is a very simple and industrially applicable method, the direct spray-drying of a mixture of lipid and drug was applied in the preparation of liposomes. The spray-drying process is a fast single-step procedure applied in the nanoparticles formulation. Liposomes were prepared by suspending lecithin and mannitol in chloroform. The mixture was sonicated for 8 min (bath sonicator) and after subjected to spray-drying on a different spray dryer.

The main factor influencing the liposomal size was the volume of aqueous medium used for hydration of the spray-dried product.[27] Mannitol plays an important role in increasing the surface area of the lipid mixture, enabling successful hydration of the spray-dried product.

III. Freeze Drying

This new method was described for the preparation of sterile and pyrogen-free submicron narrow sized liposomes.[28] It is based on the formation of a homogenous dispersion of lipids in water-soluble carrier materials. Liposome-forming lipids and water-soluble carrier materials such as sucrose were dissolved in tert-butyl alcohol/water cosolvent systems in appropriate ratios to form a clear isotropic monophase solution. Then the monophase solution was sterilized by filtration and filled into freeze-drying vials.

IV. Super Critical Reverse Phase Evaporation (SCRPE).

The SCRPE is a one-step new method that hasbeen developed for liposomes preparation using supercritical carbon dioxide.[29-30] This method allowed aqueous dispersions of liposomes to be obtained through emulsion formation by introducing a given amount of water into a homogeneous mixture of supercritical CO_2 /LR dipalmitoyl phosphatidyl choline / ethanol under sufficient stirring and subsequent pressure reduction. The trapping efficiency of these liposomes indicated more than 5 times higher values for the water-soluble solute compared to multilamellar vesicles prepared by the Bangham method. The trapping efficiency for an oil soluble substance, the cholesterol, was about 63%. The SCRPE is an excellent technique that permits one-step preparation of large unilamellar liposomes exhibiting a high trapping efficiency for both water-soluble and oil-soluble compounds.[31-32]

V. Modified Ethanol Injection Method

Novel approaches based on the principle of the ethanol injection technique such as the microfluidic channel method[33-34], the crossflow-injection technique[35], and the membrane contactor method[36] were recently reported for liposome production.

1. The Crossflow Injection Technique

It is a novel scalable liposome preparation technique for pharmaceutical application. A cross flow injection module made of two tubes welded together forming a cross. [35] At the connecting point, the modules were adapted with an injection hole. The influencing parameters such as the lipid concentration, the injection pressure, the injection hole diameter, the buffer flow rate, and system performance were investigated. (**Wagner et al)** A minimum of buffer flow rate is required to affect batch homogeneity and strongly influencing parameters are lipid concentration in combination with increasing injection pressures. After exceeding the upper pressure limit of the linear range, where injection velocities remain constant, the vesicle batches are narrowly distributed, also when injecting higher lipid concentrations. Reproducibility and scalability data show similar results with respect to vesicle size and size distribution and demonstrate the stability and robustness of the novel continuous liposome preparation technique.

2. Microfluidization

A microfluidic hydrodynamic focusing (MHF) platform, generated liposomes by injecting the lipid phase and the water phase into a microchannel Microfluidic flow is generally laminar due to the small channel dimensions and relatively low flow rates. Well-defined mixing is then obtained by interfacial diffusion when multiple flow streams are injected in a microchannel. The size of the liposomes was mainly controlled by changing the flow rate.[40] (**Jahn et al**)

3. Membrane Contactor

The ethanol injection technique while using a membrane contactor for large scale liposomes production is recently used. In this method, a lipid phase (ethanol, phospholipid and cholesterol) was pressed through the membrane with a specified pore size. Nitrogen gas at pressure below 5 bar was sufficient for passing the organic phase through the membrane. At the same time, the aqueous phase flew tangentially to the membrane surface and swept away the formed liposomes within the membrane device. The new process advantages are the design simplicity, the control of the liposome size by tuning the process parameters and the scaling-up abilities.[36](**Jaafar- Maalej et al)**

Table 2.1: ***Described the brief detailed about the major pharmaceutical companies mostly used main drug loading technique.***

Sr No	Drug Loading Technique	Pharmaceutical Company
1	**Extrusion/Homogenization**	Elan, Celsion, GeneExcel, Gilead, Merrimack, QLT, Pharmexa, Regulon, Terumo, Taiwan Liposome Company(TLC), and Zilip-Pharma
2	**Transmembrane pH Gradient**	Alza, Gilead Tekmira, Pharmexa, Taiwan Liposome Company and Zilip-Pharma.
3	**Reverse Phase Evaporation/ Injection methods**	AgenusNeoPharm, Polymun, Taiwan Liposome Company, and YM Biosciences.
4	**Detergent Dialysis**	Regulon
5	**Sonication**	ALZA/SEQUUS,AGI Dermatics, GeneExcel, Oakwood, Xenetic

VI. Other Preparation/Loading Techniques

The selection of lipid components in the final composition depends on the nature of the drug, most importantly, its lipophilicity, size and charge. The circulation time as well as drug retention are dependent on lipid composition. While neutral lipids (e.g. phosphatidylcholine and cholesterol) are preferred for many applications, the use of cationic lipids is necessary in the delivery of anionic molecules such as DNA and oligonucleotides. Since the type of lipids used in liposomal formulations are similar to the lipid components of the membranes, liposomal compositions are considered safe as drug delivery vehicles from all routes. However, cationic lipids appear to carry some adverse effects on systemic application (Kircheis *et al.*, 1999 and D. Goula *et al.*, 1999). A unique loading procedure from **Aphios** utilizes sub/supercritical processes to load liposomes. The SLIT technology from **Transave** utilizes temperature cycling. The liposomes from **Abnoba** are produced during a pressing process of the mistletoes plant.

The level of toxic effects was found to be dependent on the particle size (most pronounced for particles of 300–400 nm in diameter). Inclusion of cholesterol, or cholesterol derivatives, is important in regulating the release of liposome entrapped therapeutic compounds into the bloodstream. The in vitro (in the presence of human serum) and in vivo release of encapsulated drugs from liposomes is strongly dependent on cholesterol concentration. In general, the **release of drugs decreases as the cholesterol levels increase in the liposomes.**

Liposomes can encapsulate both the water-soluble and water-insoluble drug molecules. When encapsulated, water insoluble molecules are solubilized within the lipid bilayer and soluble ones are encapsulated within internal water phase of the liposome. Hydrophobic neutral drugs or drugs with intermediate solubility appear to be rapidly released in the presence of plasma proteins and cell membranes. Hydrophobic weak bases, such as doxorubicin and vincristine, have better retention characteristics.

The final product can be either a ready to use liposomal suspension or a lyophilized powder ready for reconstitution before use. Aseptic processing and sterile filtration are typically used for sterilization. During manufacture of liposomal preparations, size/distribution, surface charge, aggregation properties must be carefully studied. During development, one must show the sameness of GLP and later batches. Lipids used in liposomes must be characterized at the same level of detail expected for drug substance. Protein binding to the liposome must be recognized and the in vitro release test must be done.

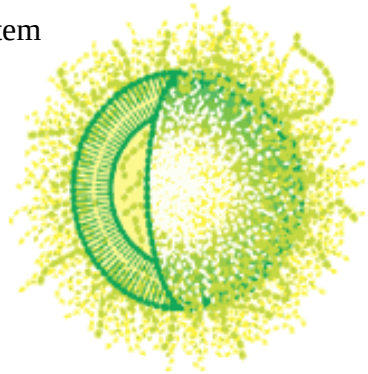
2.6 Injectable liposomes for cancer treatment

Liposomes are a platform technology used in the delivery of water insoluble, water soluble (charged and neutral), peptides, proteins and negatively charged DNA. The most common route is injectable delivery. Other routes include pulmonary, topical, and nasal. **Cancer targeting** is the most widely used application of liposomal formulations. Liposomes provide longer circulation times as well as passive targeting based on particle size/EPR as mentioned above. Further, the lipid components of the liposome formulations can be chemically attached to targeting molecules in active targeting applications. The mechanism of action of the liposomal drugs without active targeting component is thought to be due to sustained release of drug from the liposomes and diffusion of the released drug throughout the tumor interstitial fluid, with subsequent uptake of the released drug by tumor cells.

Upon administration in the body, unmodified liposomes will be recognized by the immune/RES system as foreign bodies and destroyed before significant amounts reach the intended disease site, limiting their effectiveness. Such lipid-based dispersions can also be problematic as products to develop and manufacture because of issues relating to physical and chemical stability, drug release rate, etc.

2.6.1 STEALTH® Liposomes

STEALTH® Liposomes avoid recognition by the immune system because of polyethylene glycol (PEG) coating. Hydrophilic PEG coating and relatively small size of liposomes (about 100 nm) limit binding of plasma opsonins to liposomes and their subsequent uptake by the RES organs. (**Gabizon et al.1990, Huang et al.1992**).



Results in increased circulation time and residence of liposome encapsulated drug within the plasma space, minimizing wide distribution in normal tissues and maximizing uptake by tumors through leaky endothelial junctions. (**US5213804**)

2.6.2 Liposomal Drug Formulation in Cancer Treatment

Cancer is a general term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumours and neoplasms. One major feature of cancer is the rapid formation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs, the latter process is referred to as metastasizing. Metastases are the major cause of death from cancer.

Epidemiology of cancer: Cancers figure among the primary reasons of disease and death worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012.[ii] The number of new cases is probably rise by about 70% over the next 2 decades. Among men, the 5 most common sites of cancer analyzed in 2012 were lung, prostate, colorectum, stomach, and liver cancer. Among women the 5 most common sites analyzed were breast, colorectum, lung, cervix, and stomach cancer. More than 60% of world's total new annual cases arise in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths.[ii]

Cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next 2 decades.[ii]

The most common causes of cancer death are cancers of:

- Lung (1.59 million deaths)
- liver (745 000 deaths)
- stomach (723 000 deaths)
- colorectal (694 000 deaths)
- breast (521 000 deaths)
- Esophageal cancer (400 000 deaths) [ii].

Lipophilic and amphiphilic drugs can be incorporated into the liposomal bilayers whereas hydrophilic drugs can be incorporated into the inner aqueous compartment lead to a controlled release effect **(Fig. 1).** Advantages of the liposome is non-toxic and degradable in the body because of their naturally occurring lipids as main content lead to a controlled

release effect. Thus, the systemic situation does not recognize the free anticancer drug. It recognizes only the liposomes and the drug pharmacokinetics is now replaced by the pharmacokinetic behavior of the Liposomal vesicles. Besides the ability of liposomes to protect drugs from their degradation in the blood stream and that liposomes themselves are of low intrinsic toxicity, non-immunogenic and biodegradable, the most important feature of liposomes is their ability to accumulate in tumors due to the enhanced permeability and retention effect [EPR].[8-37]

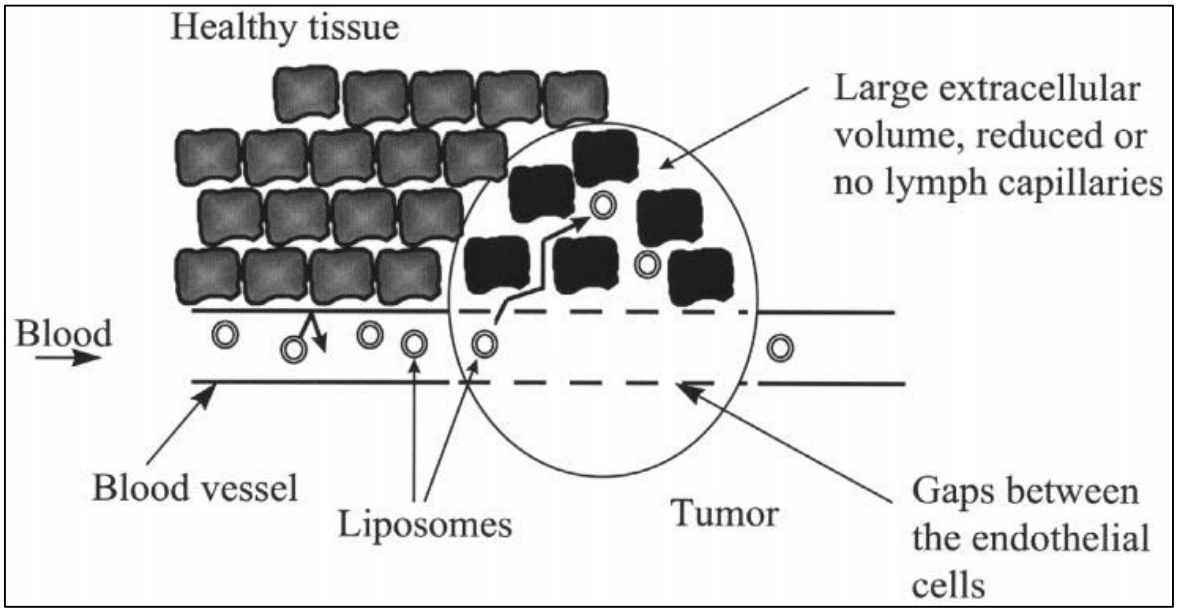


Figure. 3 ***: Accumulation of liposomes in tumor tissues due to the enhanced permeability and retention (EPR) effect.***

The EPR-effect is mostly based on differences between the vasculature in tumors and healthy organs or tissues. Blood vessels in tumors are more leaky due to their accelerated growth to enable rapid tumor growth.[8] Tumor cells are very often not as densely packed as cells in healthy tissues. The lymphatic system, important for removing substances and also nanoparticles like liposomes from the tissues or organs is very often only marginally expressed. Thus, nanoparticles such as biological macromolecules or synthetic polymers bigger than 30–40 kDa[10] and also liposomes up to a diameter of 400–600 nm are able to diffuse out of the leaky tumor blood vessels and accumulate in the tumor tissues, but not in healthy organs and tissues[9] (passive targeting).

2.7 Excipients profile

2.7.1 Hydrogenated phosphatidylcholine from soybean (HSPC)

1. Synonyms: HSPC

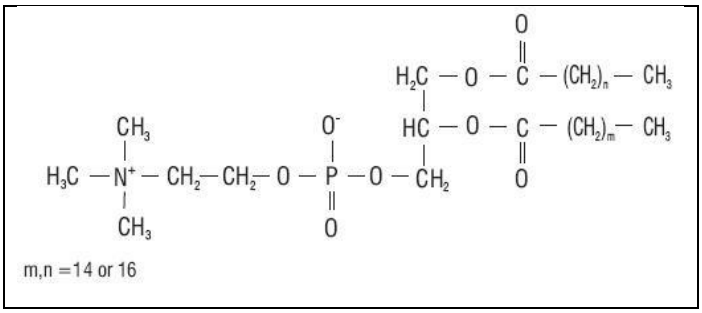
2. Chemical Name: Hydrogenated soybean phosphatidylcholine

3. CAS Registry Number: 97281-48-6

4. Empirical Formula: - $C_{44}H_{88}NO_8P$

5. Molecular Weight: -Average MW: 783.774

6. Structural Formula:



8. Purity: >99%

9. Stability: 1 year

10. Storage: -20˚c

2.7.2 Cholesterol

Nonproprietary Names

BP: Cholesterol, JP: Cholesterol, PhEur: Cholesterol, USP-NF: Cholesterol

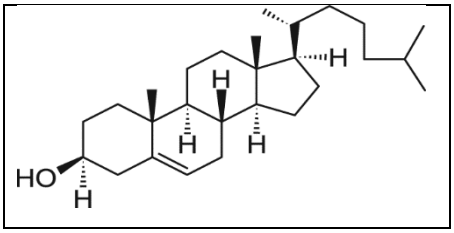
1. Chemical Name: cholest-5-en-3ß-ol

2. CAS Registry Number: 57-88-5

3. Empirical Formula: - $C_{27}H_{46}O$

4. Molecular Weight: 386.654

5. Structural Formula:



6. Purity: >98%

7. Stability: 6 months

8. Storage: -20˚c

Functional Category: Emollient; emulsifying agent.

Applications in Pharmaceutical Formulation or Technology

In cosmetics and topical pharmaceutical formulations at concentrations of 0.3–5.0% w/w as an emulsifying agent. It imparts water-absorbing power to an ointment and has emollient activity. It is the major sterol of the higher animals, and it is found in all body tissues, especially in the brain and spinal cord. It is also the main constituent of gallstones.[38]

Description

It occurs as white or faintly yellow, almost odorless, pearly leaflets, needles, powder, or granules. On prolonged exposure to light and air, cholesterol acquires a yellow to tan color.

Typical Properties

Boiling point3608C (some decomposition)

Density 1.052 g/cm3 for anhydrous form.

Dielectric constant D20 = 5.41

Melting point 147–1508C

Specific rotation

$[\alpha]^{20}D$= - 39.58 (2% w/v solution in chloroform);

$[\alpha]^{20}D$ = - 31.58 (2% w/v solution in ether).

2.7.3 DSPE-mPEG-2000

1. Synonyms: DSPE-MPEG-2000 (Na^+ salt)

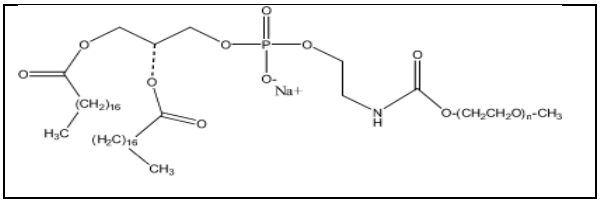
2. Chemical Name: 1, 2-Distearoyl-phosphatidylethanolamine-methy polyethylene glycol conjugate-2000 (Na^+ salt)

3. CAS Registry Number: 147867-65-0

4. Empirical Formula: - $C_{133}H_{267}N_2O_{55}P$

5. Molecular Weight: 2805.497

6. Structural Formula:



8. Purity: 98+%

9. Stability: 1 year

10. Storage: -20˚c

11. Solubility: chloroform

12. Physical Appearance: solid

2.7.4 Ethanol

1. Nonproprietary Names

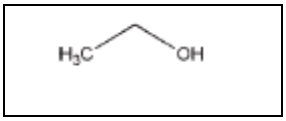
BP: Ethanol (96%), JP: Ethanol, PhEur: Ethanol (96 per cent) ,USP: Alcohol

2. Synonyms: Ethanolum (96 per centum); ethyl alcohol; ethyl hydroxide; grain alcohol; methyl carbinol

3. Chemical Name and CAS Registry Number: Ethanol [64-17-5]

4. Empirical Formula and Molecular Weight: C_2H_6O - 46.07

5. Structural Formula



6. Functional Category: Antimicrobial preservative; disinfectant; skin penetrant; solvent.

7. Applications in Pharmaceutical Formulation or Technology

Ethanol is primarily used as a solvent, it is also employed as a disinfectant, and in solutions as an antimicrobial preservative.[53]Topical ethanol solutions are used in the development of transdermal drug delivery systems as penetration enhancers and co surfactant.[54]

8. Description: It is a clear, colorless, mobile, and volatile liquid with as light, characteristic odor and burning taste.

9. Typical Properties

Antimicrobial activity Ethanol is bactericidal in aqueous mixture sat concentrations between 60% and 95% v/v; the optimum concentration is generally considered to be 70%

v/v. Antimicrobial activity is enhanced in the presence of edetic acid or edentate salts.[53] Ethanol is inactivated in the presence of nonionic surfactants and is ineffective against bacterial spores.[38]

10. Boiling point: 78.158C

Flammability Readily flammable, burning with a blue, smokeless flame.

11. Flash point: 148C (closed cup)

12. Solubility: Miscible with chloroform, ether, glycerin, and water (with rise of temperature and contraction of volume).

13. Specific gravity: 0.8119–0.8139 at 208C

14. Stability and Storage Conditions

Aqueous ethanol solutions may be sterilized by autoclaving or by Filtration and should be stored in airtight containers, in a cool place.

2.7.5 Water for Injection (WFI)

1. Nonproprietary Names

BP: Purified water, JP: Purified water, PhEur: Aqua purificata, USP: Purified water

2. Synonyms: Aqua; hydrogen oxide.

3. Chemical Name: Water

4. CAS Registry Number: [7732-18-5]

5. Empirical Formula: H_2O

6. Molecular Weight: 18.02

7. Structural Formula: H_2O

8. Functional Category: Solvent.

9. Applications in Pharmaceutical Formulation or Technology

Water is the most widely used excipient in pharmaceutical production operations. Specific grades of water are used for particular applications in concentrations up to 100%; Purified water and water for injection are also used for cleaning operations during production of pharmaceutical products.

10. Description: Water is a clear, colorless, odorless, and tasteless liquid.

11. Typical Properties

Boiling point: 100°C

Critical pressure: 22.1MPa (218.3 atm)

Critical temperature: 374.2°C

Dielectric constant: D25 = 78.54

Dipole moment: 1.76 in benzene at 25°C; 1.86 indioxane at 25°C.

Ionization constant: 1.008 - 10-14 at 25°C.

Latent heat of fusion: 6 kJ/mol (1.436 kcal/mol)

Latent heat of vaporization: 40.7 kJ/mol (9.717 kcal/mol)

Melting point: 0°C

Refractive index: nD20 = 1.3330

Solubility: miscible with most polar solvents.

Specific gravity: 0.9971 at 25°C.

Specific heat (liquid):4.184 J/g/°C (1.00 cal/g/°C) at 14°C.

Surface tension: 71.97 mN/m (71.97 dynes/cm) at 25°C.

Vapor pressure: 3.17 kPa (23.76 mmHg) at 25°C.

Viscosity (dynamic): 0.89 mPa s (0.89 cP) at 25°C.

12. Stability and Storage Conditions

Water is chemically stable in all physical states (ice, liquid, and vapor). Water for specific purposes should be stored in appropriate containers.

2.8.6 Ammonium Sulfate

1. Synonyms: Diammonium sulfate, Mascagnite, diazanium sulfate, Ammonium sulphate, Sulfuric acid diammonium salt

2. Chemical Name: diazanium;sulfate

3. Empirical Formula: $H_8N_2O_4S$

4. Molecular Weight: 132.13952

5. Description: White or brown orthorhombic crystals

2.8.7 Sucrose

1. Nonproprietary Names

BP: Sucrose, JP: Sucrose, PhEur: Sucrose, USP-NF: Sucrose

2. Synonyms: saccharose, sugar, Table sugar, White sugar, Saccharum, Cane sugar, D-Sucrose, Amerfand, Amerfond

3. Chemical Name: (2R,3R,4S,5S,6R)-2-[(2S,3S,4S,5R)-3,4-dihydroxy-2,5 bis(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol

5. Empirical Formula: $C_{12}H_{22}O_{11}$

6. Molecular Weight: 342.29648

7. Description: Monoclinic white crystals

8. Functional Category: Confectionery base; coating agent; granulation aid; suspending agent; sweetening agent; tablet binder; tablet and capsule diluent; tablet filler; therapeutic agent; viscosity-increasing agent.

9. Applications in Pharmaceutical Formulation or Technology

It is widely used in oral pharmaceutical formulations. Sucrose syrup, containing 50–67% w/w sucrose, is used in tableting as a binding agent for wet granulation. In the powdered form, sucrose serves as a dry binder (2–20% w/w) or as a bulking agent and sweetener in chewable tablets and lozenges. Tablets that contain large amounts of sucrose may harden to give poor disintegration. Sucrose syrups are used as tablet-coating agents at concentrations between 50% and 67% w/w. With higher concentrations, partial inversion of sucrose occurs, which makes sugar coating difficult. Sucrose syrups are also widely used as vehicles in oral liquid dosage forms to enhance palatability or to increase viscosity. Sucrose has been used as a diluent in freeze-dried protein products and foods and confectionery, and therapeutically in sugar pastes that are used to promote wound healing.

10. Description: Sucrose is a sugar obtained from sugar cane (Saccharum officinarum Linne´ (Fam. Gramineae)), sugar beet (Beta vulgaris Linne´ (Fam. Chenopodiaceae)), and other sources. It contains no added substances. Sucrose occurs as colorless crystals, as crystalline masses or blocks, or as a white crystalline powder; it is odorless and has a sweet taste.[38]

11. Typical Properties

Density (bulk)

0.93 g/cm3 (crystalline sucrose); 0.60 g/cm3 (powdered sucrose).

Density (tapped)

1.03 g/cm3 (crystalline sucrose); 0.82 g/cm3 (powdered sucrose).

Density (true): 1.6 g/cm3

Dissociation constant pKa = 12.62

Flow ability: Crystalline sucrose is free flowing, whereas powdered sucrose is a cohesive solid.

Melting point: 160–1868C (with decomposition)

Moisture content finely divided sucrose is hygroscopic and absorbs up to 1% water.

Osmolarity: A 9.25% w/v aqueous solution is isoosmotic with serum. Particle size distribution Powdered sucrose is a white, irregular sized granular powder. The crystalline material consists of colorless crystalline, roughly cubic granules.

Refractive index nD25 = 1.34783 (10% w/v aqueous solution)

*Table 2. 2.**Marketed liposomal products:***

Sr. No	Molecule	Product	Company	Disease	Status
1	**Doxorubicin HCl**	**Innovator**	Johnson and Johnson (USA)	Kaposi sarcoma and AIDS related cancer. Ovarian cancer	Market since 1995 (USA) and 1996 (Europe)
2	Daunorubicin	DaunoXome	NeXstar Pharmaceuticals Diatos in 2006	Specific types of leukemia (acute myeloid leukemia and acute lymphocytic Leukemia).	Market since 1996 (USA and Europe)
3	**Amphotericin B**	**Ambisome**	NeXstar Pharmaceuticals acquired by Gilead Sciences in 1999.	Systemic fungal Infections	Market since 1990 (Europe) and 1997 (USA)
4	Inactivated hepatitis A virus	EPAXAL	Crucell Company who merged with the Swiss Serum and Vaccine Institute in 2006	Hepatitis A	Swiss market since 1994.
5	Lidocaine	LMX4 LMX5	Ferndale Laboratories, USA	Anaesthesia for skin Itching, burning or pain.	On the US market since 1998

2.8 Membrane filtration for biopharmaceutical processes

Membrane filtration, a pressure-driven separation process, is a well-known unit operation and has long been used to recover and concentrate biotechnological products. There are many novel separations are currently being studied, membrane filtration remains a key unit operation in bioprocessing. In the case of large scale production of biopharmaceutical product like monoclonal antibodies, vaccines, protein and peptides and some of the liposomal formulation membrane filtration is the conventional unit operations processes of choice due to reduced risks in scale-up, technology transfer and process robustness.[39]

Membrane filtration processes separate components according to size in which the primary role of the membrane is to act as a selective barrier for the different components of the feed stream. As opposed to conventional filtration processes, which are usually applied to the separation of fine and course particles greater than 10 microns, membrane filtration is a classification of filtration processes involving particles less than 10 microns using membrane filters that are usually less than 10 microns.

2.9.1 System design and mode of operation

The design of a membrane filtration process requires certain key process elements:
(1) The size of the material that has to be retained on or be allowed to permeate through the membrane;
(2) The type of filter required in terms of pore size, whether micro porous filter or ultra-filter; and
(3) The mode of operation of the separation process.

Membrane filtration processes were mostly performed using ultrafiltration membranes, increased use of microfiltration membrane processing has increasing since the late 1990s.[40] **Table 2.3** shows some of the different membranes currently available for microfiltration and ultrafiltration of suspensions and solutions. When choosing membrane filters, the type of material of the membrane should be compatible to the feed stream which is going to be processed and that it could provide the desired separation. It is essential that the membranes do not bind to the product as this will decrease recovery. Some of the membrane materials

in **Table 2.3** are naturally hydrophobic (PVDF and PTFE). Therefore, modified versions of these membranes should be used. Most manufacturers will have hydrophilic PVDF membrane, which is the suitable type of membrane for cell harvesting and clarification.

Table 2. 3 Common membrane filters for biopharmaceuticals processing

Material	**Microfiltration**	**Ultrafiltration**
Polyvinylidene fluoride (PVDF)	X	
Polyethersulfone (PES)	X	X
Polytetrafluoroethene (PTFE)	X	
Regenerated cellulose (RC)		X
Polysulfone (PS)	X	X

For their practical application, membranes are packaged according to the design of the equipment housing. Typical membrane filtration configurations in industrial bio-separation are as flat-sheet membranes in cartridges (similar to the plate-and-frame type for particulate filtration) or flat-sheets in spiral wound; and as bundles of hollow fiber membrane.

In recent years, membrane separations in bioprocessing have mostly focused on the use of flat-sheet and hollow fiber membranes for cell harvest or cell debris removal. Flatsheet membranes in cassette format is the most common format for the process. Therefore it is very important to select membrane formats which have available filtration systems at different scales of development, from laboratory to pilot and then to manufacturing scale.[41]

The classical mode of operation of membrane filtration processes is via normal flow (NFF) or dead-end filtration (DEF). This has followed from conventional filtration processes which were also operated in this way[42]. But in case of dead end filtration, give the low permeate flux. Different strategies for increasing flux were developed.

The fundamentals of membrane separation, cross flow microfiltration has found more application in industrial bioseparation because of improved permeate fluxes[43]. **Figure 4** illustrates these two modes of operation. DEF is a process where the feed flows perpendicular to the membrane surface. As a result, retained components build up on the

feed/retentate side and lead to membrane fouling and concentration polarisation. The permeate flux during dead-end filtration decreases over time.[44]

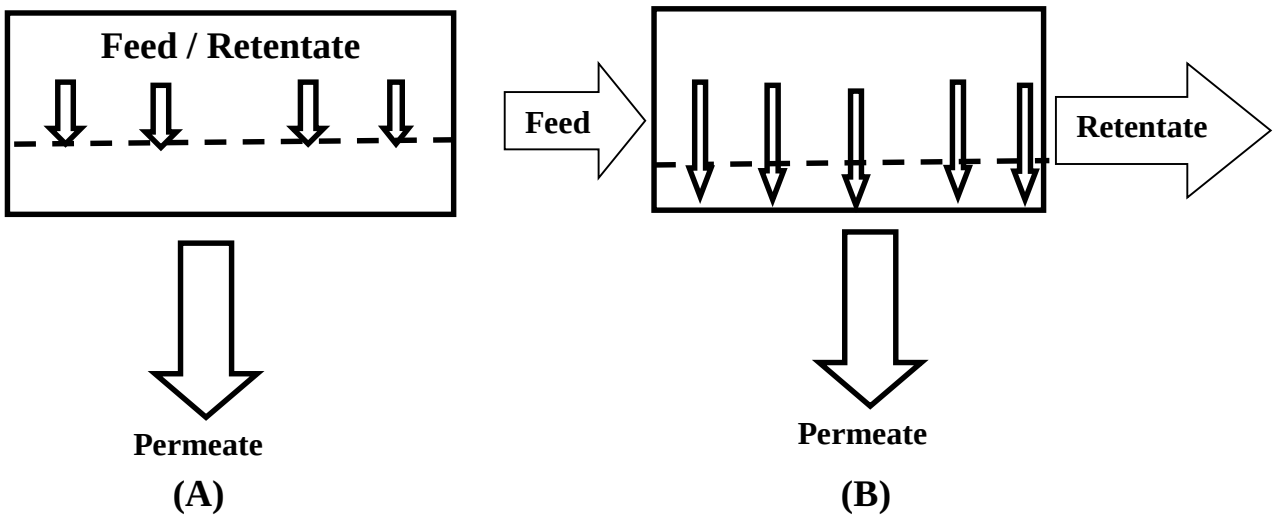


Figure. 4 ***: Modes of membrane filtration operation: (A) Dead-end filtration; and (B) cross flow filtration.***

Tangential (TFF) or cross flow filtration (CFF) have the feed stream flowing parallel to the membrane. The feed flow then allows the retained components to be brushed off along the membrane surface and out of the CFF device.

2.9 Theories and models of membrane filtration

A key performance indicator of membrane filtration processes is the permeate flux, *J*, which is the measure of volume of liquid, *V*, which has permeated through the membrane of a certain area, *Am*, for a certain period of time (*t)*. Based on Below Equation (1) , once the flux is known, the processing time for certain volume of material can be determined for a given membrane area, or if given a fixed processing time, the required membrane area can be determined. Thus, flux modelling has been an important undertaking in understanding membrane filtration processes[45].

$J = V/A*t$ *(1)*

2.9.1 Hagen-Poiseuille flow through a membrane pore: ideal flux

In the most ideal and simplest situation, flow through a membrane can be treated as a fluid flow situation analogous with viscous flow through pipes as in fluid transport problems. **Figure 5** is a schematic representation of this situation.

If the Hagen-Poiseuille flow equation is applied to flow of fluid through a membrane pore, Equation 2 is derived.[45]

$$J = \epsilon * d^2_{ch*} \Delta P_{TM} / 32 * l * \mu \qquad (2)$$

Where: *J* is the permeate flux; is the membrane porosity; d_{ch} is the channel diameter or membrane pore size; Δ*PTM* is the transmembrane pressure; *l* is the membrane thickness; and μis the viscosity of the fluid.

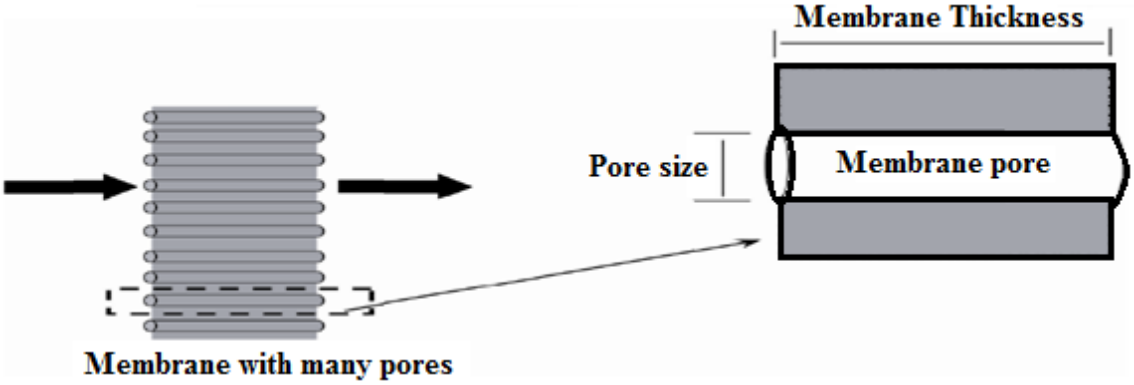


Figure. 5 ***: Flow through membrane pores to permeate flux by the Hagen-Poiseuille equation.***

The Hagen-Poiseuille equation assumes that the fluid flows through a constant circular cross-section with the diameter that is very small compared to the length. Also, in this situation, the flow is viscous, incompressible and laminar. The fluid follows Newtonian law and that there is no slip of fluid particles at the boundary.

2.9.2 Flux modelling based on flow resistances

The determination of flux through a series of resistances is commonly encountered in heat transfer in the form of Fourier's law of conduction.[46] Modelling flux by the resistance model takes the general form of:

Flux =Driving force/Total resistance

Different resistances are considered for flows through a membrane filter.

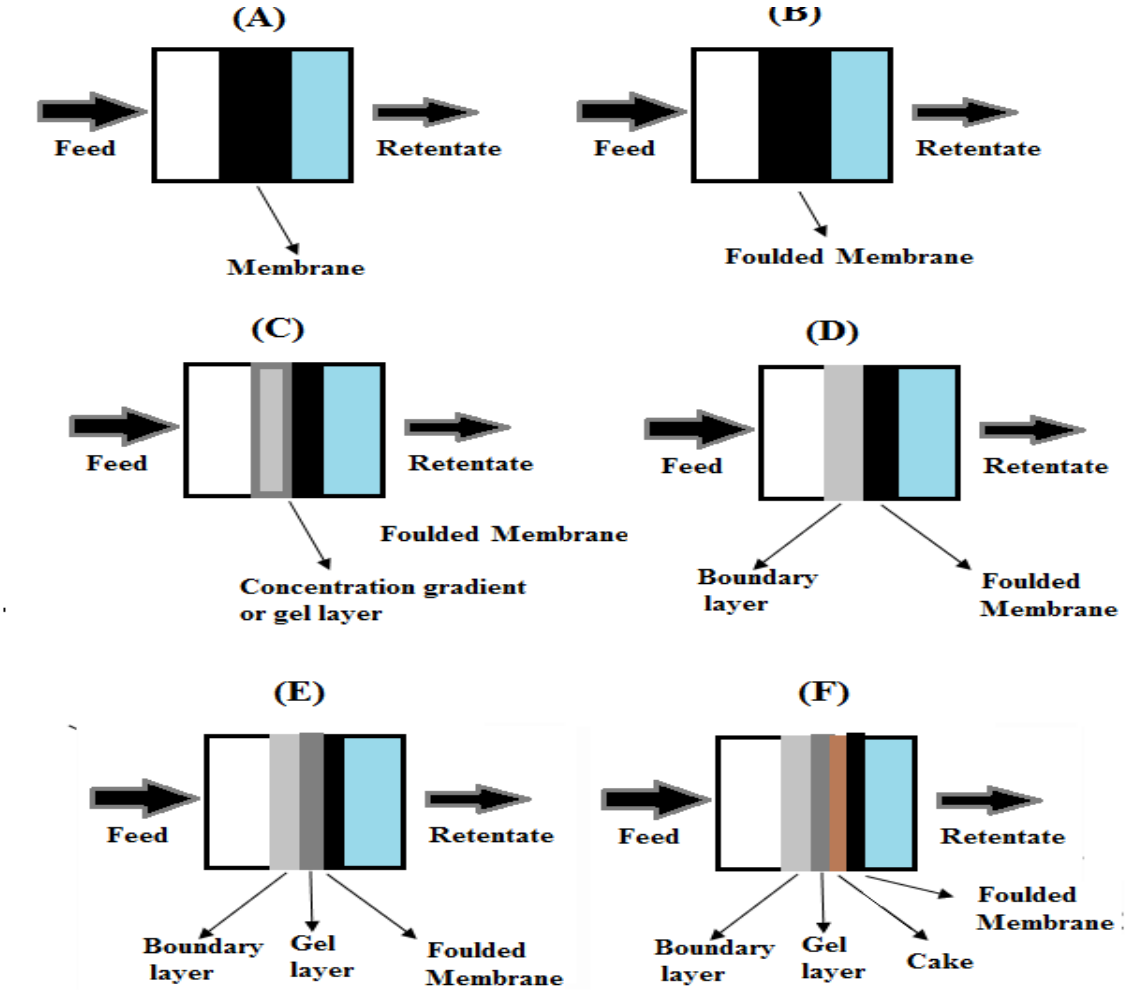


*Figure. 6**: Different Resistances in series for membrane filtration process.***

Figure 6 illustrates these which include: (A) the clean membrane; (B) the fouled membrane; (C) the fouled membrane with a gel layer; (D); the fouled membrane with a boundary layer; (E) the fouled membrane with a boundary layer and gel layer; and (F) the fouled membrane with a boundary , gel and cake layer.

From **Figure 6**, it can be seen that the different levels of resistances should be determined if flux is to be known using the general equation in Equation 3. In the following paragraphs, the determination of these resistances is discussed.

$$J = \Delta P_{TM} / R_T * \mu \qquad (3)$$

Where: *RT* is the total resistance which could be:

(A) The clean membrane

$$RT = Rm \qquad (3.1)$$

(B) The fouled membrane

$$RT = Rm + Rf \qquad (3.2)$$

(C) The fouled membrane with a gel layer

$$RT = Rm + Rg \qquad (3.3)$$

(D) The fouled membrane with a boundary layer

$$RT = Rm + Rbl \qquad (3.4)$$

(E) The fouled membrane with a boundary layer and gel layer

$$RT = Rm + Rbl + Rg \qquad (3.5)$$

(F) The fouled membrane with a boundary, gel and cake layer

$$RT = Rm + Rbl + Rg + Rc \qquad (3.6)$$

1. **Membrane resistance**

Predicting a pure solvent flux, e.g. for water, is straightforward if the membrane resistance is known. *Rm* can be determined from experimental flux measurements and calculated by using Equation 1.4 and Equation 1.5. *Rm* can also be determined by employing the Kozeny-Carman equation in Equation 4.[47]

$$\mathbf{Rm = K (1-\varepsilon_m)^2 * S_m * \Delta x_m / \varepsilon^3_m} \qquad (4)$$

In Equation 4, *K* is the **Kozeny -Carman constant** which depends on membrane morphology, ε_m is the membrane surface porosity, Δxm is the membrane thickness and *Sm* is the pore internal surface area per unit volume. The membrane resistance can be estimated using the Kozeny-Carman equation under the assumption that each membrane consists of closely packed spheres. According to this equation, a small difference in porosity may have a significant effect on the calculated membrane resistance.

2. Fouled membrane resistance

The fouled membrane resistance (*Rm*+ *Rf*) is determined by performing the water flux experiment, as described above, before and after passing through to the membrane the feed suspension of interest. The first water flux experiment is to determine the clean membrane resistance, *Rm*, and the second to determine the total resistance *RT*. From Equation 3 and Equation 3.2, *Rf* can be determined.[48]

3. Boundary layer, concentration polarization layer and gel layer

The retention of certain components on one side of the membrane results in the phenomenon called **concentration polarization**. At a certain distance from the membrane, the concentration of particles or solutes starts to deviate from the bulk concentration. This concentration increases, with the highest near the surface of the membrane. For certain feeds, the concentration near the membrane becomes very high such that a layer of particles become viscous and gel-like, hence the term gel layer.[49] This is schematically shown in **Figure 6** C. The resistance of the gel layer is calculated from Equation 3 and Equation 3.3.

The boundary layer on the other hand is formed at a distance away from the membrane where the concentration changes from the bulk concentration towards the highest (gel concentration) concentration near the membrane. This is schematically shown in **Figure 6** D. The resistance of the gel layer is calculated from Equation 3 and Equation 3.4. **Figure 6** E also illustrates a situation of concentration polarization wherein the resistance is contributed by the resistances due to both the gel and the solutes within the boundary layer. This is also shown schematically in **Figure 6.**A. A disadvantage of this approach is the difficulty of distinguishing between these different layers.[49]

4. Cake resistance

Equation 5 is based on the classical cake filtration theory that was supported by the influential work of Grace (1953a). This was developed based on the Poiseuille law of viscous flow.[50] It has become one of the most important equations in classical cake filtration which only assumes that aside from the membrane resistance, the other contribution to resistance to flow is the cake which formed due to particles being deposited on the membrane surface. For membrane filtration, there has been no clear distinction between a "cake" layers, "gel" layer, "boundary" or "concentration polarisation" layer. It has been commonly applied to dead-end microfiltration analysis.[51]

$$\frac{dV}{dt} = \frac{Am * \Delta PTM}{(\alpha * \rho o * V/A + Rm) * \mu} \qquad (5)$$

Simple integration of Equation 5yields the widely known parabolic law of constant pressure filtration in Equation 6. From Equation 6, the average specific cake resistance, α, can be determined if ρ*o* is known or can be estimated. α is a measure of how easily a feed can be filtered.[50] With Equation 7, and Equation 3, the permeate flux can be predicted if it could be assumed that the resistance is due to cake formation.

$$A_m * t / V = \mu / \Delta P_{TM} (\rho o * \alpha / 2A_m * V + Rm) \qquad (6)$$

$$R_T = R_C = \alpha * mC \qquad (7)$$

5. Theories of concentration polarization

It is based on the mass transfer film theory model (Belfort et al., 1994).Based on this model, the transport of solutes or particles towards the membrane is by convective transport mechanism due to permeate flow through the membrane while transport away from the membrane may be by diffusion, inertia or shear forces. At steady state, these two opposing transport mechanisms reach a balance thus resulting in a steady flux.[52]

2.10 Tangential Flow Filtration (TFF)

It also known as cross flow filtration, where the feed stream passes parallel to the membrane face as one portion passes through the membrane (permeate) while the remainder (retentate) is recirculated back to the feed reservoir. The flow of sample solution across the membrane surface sweeps away aggregating molecules that form a membrane-clogging gel (gel polarization), allowing molecules smaller than the membrane pores to move toward and through the membrane.[53]

The primary applications for TFF are concentration, diafiltration (desalting and buffer exchange), and fractionation of large from small biomolecules.

Advantages of TFF:

- Easy to set up and use
- Fast , Efficient and Economical
- Perform two steps with one system
- Can be scaled-up or scaled-down

2.10.1 Tangential Flow Filtration Overview

Membrane filtration is a separation technique widely used in the life science laboratory. Depending on membrane porosity, it can be classified as a **microfiltration or ultrafiltration** process.

1. Microfiltration membranes, with pore sizes typically between 0.1 µm and 10 µm, are generally used for clarification, sterilization, and removal of micro particulates or for cell harvesting.

2. Ultrafiltration membranes, with much smaller pore sizes between 0.001 and 0.1 µm, are used for concentrating and desalting dissolved molecules (proteins, peptides, nucleic acids, carbohydrates, and other biomolecules), exchanging buffers, and gross fractionation. Ultrafiltration membranes are typically classified by molecular weight cut-off (MWCO) rather than pore size.[53]

1) Direct Flow Filtration (DFF), also known as ”**dead-end”** filtration, applies the feed stream perpendicular to the membrane face and attempts to pass 100% of the fluid through the membrane, and

2) Tangential Flow Filtration (TFF), also known as **cross-flow filtration**, where the feed stream passes parallel to the membrane face as one portion passes through the membrane (permeate) while the remainder (retentate) is recirculated back to the feed reservoir.

Two processes is there

1. Concentration

Concentration is a simple process that involves eliminating fluid from a solution while retaining the solute molecules. The concentration of the solute increases in direct proportion to the decrease in solution volume, i.e. having the volume effectively doubles the concentration.

For concentrate a sample prefer a UF membrane with a MWCO that is substantially lower than the molecular weight of the molecules to be retained. This is important in order to assure complete retention and high recovery of the target molecule. A good general rule is to select a membrane with a MWCO that is 3 to 6 times lower than the molecular weight of the molecules to be retained. For example, if flow rate (or processing time) is a major attention, selection of a membrane with a MWCO toward the lower end of this range (3x) will yield higher flow rates. If recovery is the primary concern, selection of a tighter membrane (6x) will yield maximum recovery (with a slower flow rate).

The membrane is installed (or a disposable TFF capsule selected), and the TFF system is initialized (typically flushed with water and tested for water filtrate flow rate and integrity). Sample is added, a cross flow is established, feed and retentate pressures are set, then filtrate is collected. When the desired concentration is reached, the process is stopped, and sample recovery or Diafiltration may begin.

2. Diafiltration:

Diafiltration is the fractionation process that washes smaller molecules through a membrane and leaves larger molecules in the retentate without ultimately changing concentration. It can be used to remove salts or exchange buffers. It can remove ethanol or other small solvents or additives.

There are several ways to perform diafiltration. In **continuous diafiltration,** the diafiltration solution (water or buffer) is added to the sample feed reservoir at the same rate as filtrate is generated. In this way the volume in the sample reservoir remains constant, but the small molecules (e.g. salts) that can freely permeate through the membrane are washed away. Using salt removal as an example, each additional diafiltration volume (DV) reduces the salt concentration further. (A diafiltration volume is the volume of sample before the diafiltration solution is added.) Using 5 diafiltration volumes will reduce the ionic strength by ~99% with continuous diafiltration.

In **discontinuous diafiltration,** the solution is first diluted and then concentrated back to the starting volume. This process is then repeated until the required concentration of small molecules (e.g. salts) remaining in the reservoir is reached. Each additional diafiltration volume (DV) reduces the salt concentration further. A diafiltration volume is the volume of sample before the diluting solution is added. Using 5 diafiltration volumes will reduce the ionic strength by ~96% with discontinuous diafiltration.

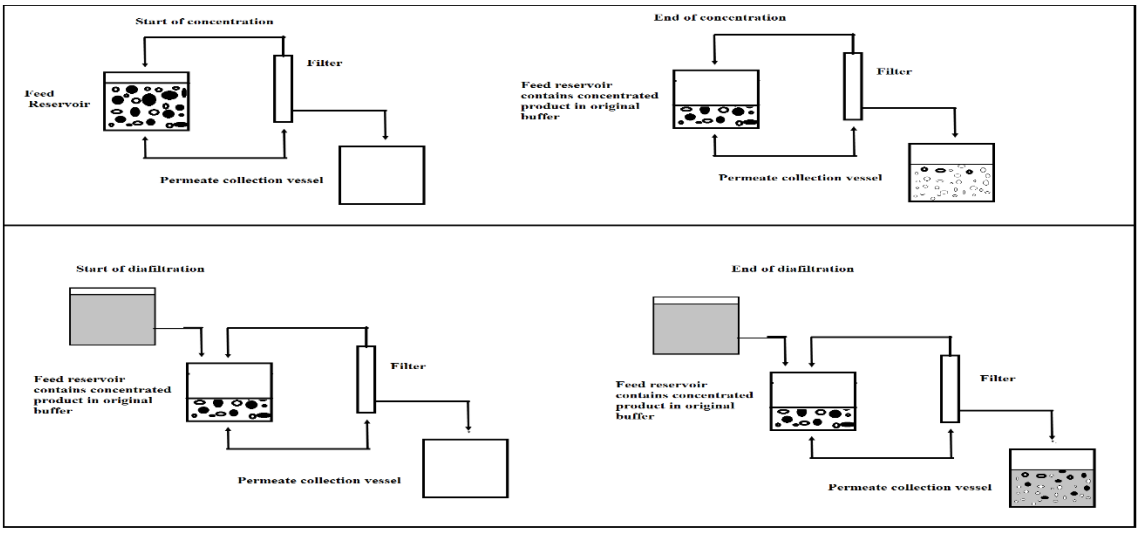


Figure. 7: Concentration and diafiltration mode of the process

Tangential Flow Device Assembly

Tangential flow filtration systems typically require a TFF device (capsule, cassette and holder, hollow fiber module, etc.) with a pump (peristaltic or equivalent), tubing, valves or clamps, one or more pressure gauges, and a sample reservoir (Figure). Pressure gauges are typically installed at the feed, retentate, and filtrate ports in development and process TFF systems.

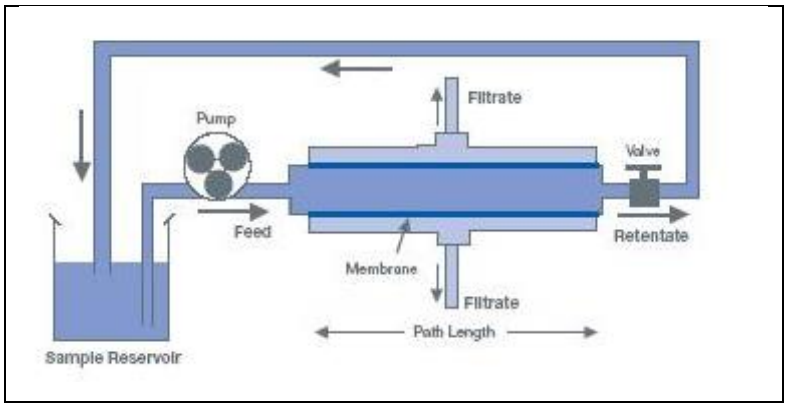


Figure. 8: **Flow Path through a Simple TFF Device**

Operation of a TFF system consists of the following steps:

1. Rinse the TFF device before use to remove the storage agent.
2. Establish the normalized water permeability (NWP) of the membrane to establish a baseline for the device performance. (This step is not necessary but strongly recommended if the device will be cleaned and reused.)
3. Condition system with the sample buffer. (Conditioning helps remove air from the system, adjust system temperature and prevent possible precipitation or denaturation of biomolecules resulting from contact with flushing solution.)
4. Process the sample (concentration and/or diafiltration, or fractionation).
5. Clean; determine cleaning efficiency.
6. Store TFF device

General Steps for the proper TFF System

Step 1: Define the purpose of the TFF process

A membrane can be chosen that passes the product while retaining higher molecular weight components in the sample.

Step 2: Choose the membrane molecular weight cutoff

The molecular weight cutoff (MWCO) of a membrane is defined by its ability to retain a given percent of a molecule in solution (typically 90% retention). To retain a product, select a membrane with a MWCO that is 3 to 6 times lower than the molecular weight of the target protein. For fractionation, select a membrane MWCO that is lower than the molecular weight of the molecule to be retained but higher than the molecular weight of the molecule you are trying to pass.[42-53]

Step 3: Choose the flow channel configuration

There are three different configurations available. Not all configurations are available with different TFF devices.

A. **Screen channel configuratio**n is used with a clean, filtered (0.2 µm) solution (no particles or aggregates that can get trapped in the screen). A woven separator in the channel creates gentle turbulence along the membrane surface, minimizing membrane fouling.

B. **Suspended screen channel configuratio**n has a more open structure in the retentate channel that provides better performance when highly viscous fluids (for example, serum) or particle-laden solutions are being used. It can also be used to concentrate cells or clarify cell or fermentation broths.

C. **Open channel configuration** is used in the same applications as the suspended screen channel. This configuration has no screen in the feed channel. Instead, it uses spacers to define the channel height. Devices may be available in several channel heights. Typically, a channel height between 0.5 and 1.0 mm is used for cell harvest applications. This structure minimizes cell disruption and maximizes recovery of intact cells after concentration.

2.11 TFF operating parameter design (QbD principle of the TFF)

There are four major operating parameters that can be controlled on a TFF system:

1. Transmembrane pressure
2. Cross-flow velocity which is converted to cross-flow flux (CFF) by the membrane area
3. Feed concentration
4. Temperature

Other parameter like viscosity, pH, and surface area chemistry also included.

Transmembrane pressure: It is the primary driving force for convective transport through the membrane pores and results in solvent flow from the feed to the permeate stream.

It is calculated by the following equation:

$$TMP = \frac{(Pfeed - Ppermeate) + (Pretentate - Ppermeate)}{2}$$

$$= \frac{TMPfeed + TMPretentate}{2}$$

Cross-flow flux is calculated as

$$CFF = \frac{QR}{A}$$

Where P_{feed} = Retentate side inlet pressure, Permeate = permeate side pressure, $P_{retentate}$= retentate side outlet pressure, Q_R= retentate flow rate, A= membrane area.

A simplified mass transfer model based on the hydraulic resistance of the membrane and the gel layer. If for the process where constant viscosity can be assumed, the governing equation for permeate flux becomes

$$Jfiltrate = k * TMP = [1/Rg + Rm] * TMP$$

$J_{Filtrate}$ = K × TMP

= [1/ Rg+Rm] × TMP

Where, k = mass transfer coefficient, TMP = Transmembrane pressure; Rg = gel layer resistance, and R_m = membrane resistance.

Mainly three distinct regions where the transmembrane pressure and the cross-flow rate influence permeate flux to varing degrees. At lower transmembrane pressures, permeate flux is influenced by both membrane and gel layer resistance and is proportional to the pressure. In this region, permeate flux is membrane controlled and the flux is pressure dependent.[54]

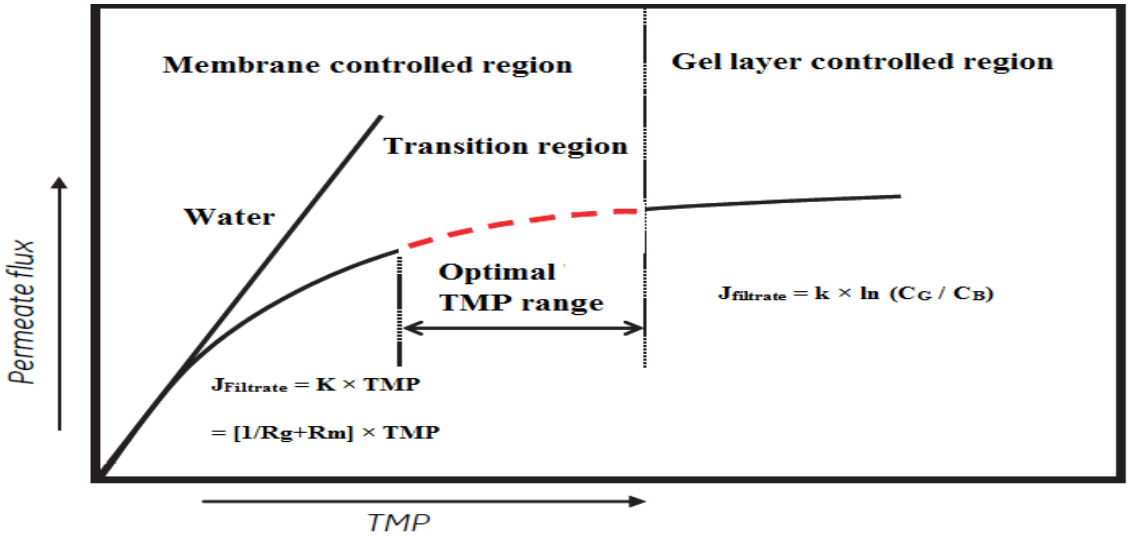


*Figure. 9: **Different region of the TFF process***

At high transmembrane pressures, a significant gel layer of the rejected solutes accumulates on the membrane and restricts flow. At this point, mass transfer is no longer controlled by the operating parameters of flow and transmembrane pressure. In this region, permeate flux is said to be gel layer controlled and the flux is independent of the pressure. In this region, there is a balance between the rate at which solute is deposited at the membrane due to convection by the solvent flow through the membrane and diffusion of the solute from the membrane to the bulk fluid. Permeate flux is governed the mass transfer coefficient and the relative solute concentration in the gel layer and bulk solution (feed concentration), which is described by the film theory model that predicts that flux decreases exponentially with feed concentration.[54]

$$\mathbf{J_{filtrate} = k \times \ln (C_G / C_B)}$$

Where C_G = gel layer concentration and C_B = bulk stream concentration.

The optimal operating point is in the transition region between the membrane controlled region and the gel layer controlled region. In this region, the beneficial effect of increasing trnsmembrane pressure on permeate flux become nonlinear, but flux is still controlled by TMP. Both transmemrane pressure and cross flow flux are important. Cross-flow provides turbulence and shear that improve the mass transfer of solutes from the membrane surface back to the bulk liquid.

The agitation and mixing provide by the cross-flow turbulence effectively sweeps accumulated solute from the membrane and reduce the gel layer thickness by controlling concentration polarization. Increasing cross-flow initially has a linear effect on reducing the gel layer thickness and moves the gel layer controlled region to higher transmembrane pressure as shown in **Fig .**In the transition region, cross-flow affects filtrate flux by extending the pressure/ membrane controlled region and delaying the onset of the gel layer controlled region to a higher TMP. Cross-flow turbulence can be used characterize the TFF system and can be expressed in terms of cross-flow rate, shear rate, or Reynolds number.[54]

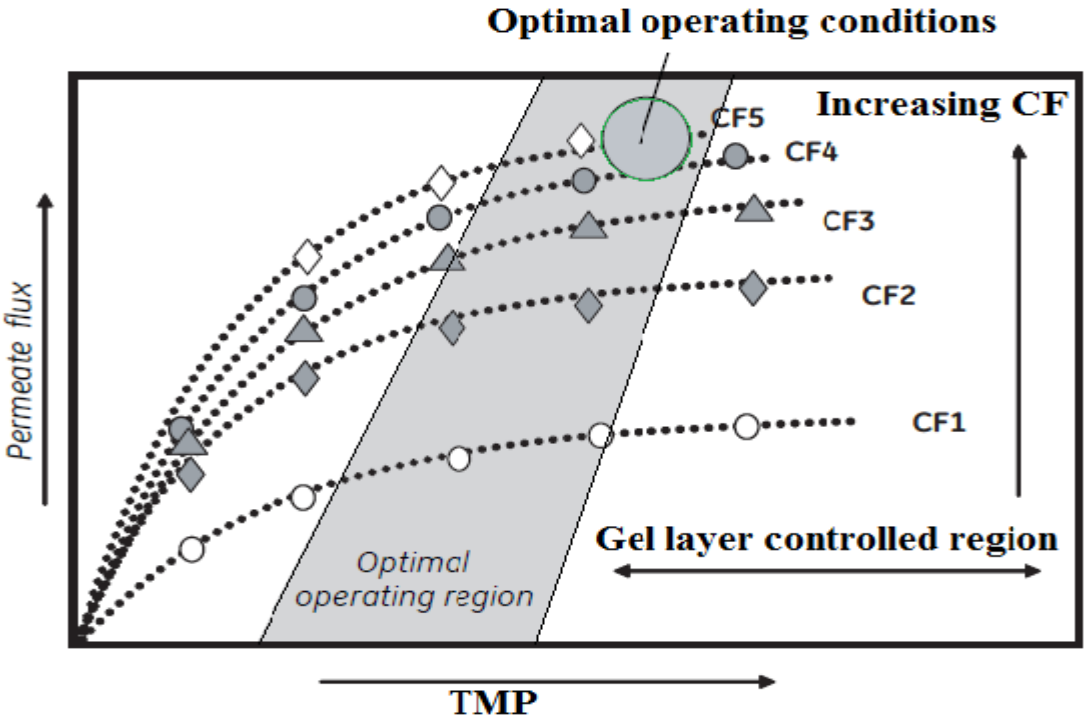


Figure. 10 ***: Effect of cross-flow rate on permeate flux***

For the acceptable product retention, the optimal operating point is in the transition region just prior to the gel layer controlled region. The transition point provides the optimal

balance between flux performance and external pressure. The plot in fig can be used to determine the transition region and the optimal TMP that maximizes permeate flux. Higher cross-flow also results in greater product shear and heat generation and requires large pumps and piping. A suitable cross flow flux is selected to achieve the desired permeate flux for the process.

TMP affect the gel layer, it is in effect a dynamic membrane. As TMP increases, the gel layer restricts solute transmission increasing the rejection coefficient.

Temperature influences fluid viscosity and density and solute diffusivity. As a result, increasing the operating temperature increases permeate flux. It is important to assess the effects of temperature on the product and specify the highest possible temperature within product and membrane stability constraints.

TFF Diafiltration operating mode design

Diafiltration is most commonly employed to transport contaminants from the retentate to permeate, thereby removing from the product. It can also be used fractionate the product from larger or higher molecular weight species. Quality design of the Diafiltration operating mode is critical to minimizing operating time, buffer volume, and product recovery for shear sensitive product. Continuous diafiltration is more efficient than discontinuous Diafiltration. Continuous Diafiltration involves feeding Diafiltration buffer to the retentate, while maintaining a constant retentate volume.

For the number of continuous Diafiltration volumes (diavolumes) required to reduce contaminants to acceptable levels, the rejection coefficient for the contaminants should be determined at the selected operating parameter. The rejection coefficients of the product depend on the gel layer that is impacted by the TMP and cross-flow flux. The number of diavolumes (with an over design factor of two diavolumes) canbe calculated from

$$N = \frac{\ln(1 - \frac{YF}{100\ \%})}{(R-1)} + 2$$

Where, N =number of diavolumes, YF = % solute in permeate, and R= solute rejection coefficient.

Figure 10 illustrate the transport of solute from the retentate to permeate as a function of the solute rejection. This represents ideal, theoretical, mass transport. The optimal concentration at which to commence diaflitration is derived from the equation for permeate flux in the gel layer controlled region. From this equation, the optimal Diafiltration concentration has been derived from the film model.[54]

$C_D = C_G / 2.7$

Where C_D = optimal retentate concentration for Diafiltration,

C_G= gel layer concentration

C_G is generally in the range of 20-40% (200-400g/L).

Operating at a feed concentration as close as possible to C_D will minimize the total Diafiltration volume and processing time. TFF system are often operated by concentrating to the final desired concentration and performing Diafiltration at this concentration. Generally, the most time efficient Diafiltration operating mode is to concentrate the feed close to C_D, perform the required number of diafiltrations, and then concentrate or dilute to the final desired concentration.[54]

2.12 Design of Experiment

A statistics-based approach to design of experiments to achieve a predictive knowledge of a complex, multi variable process with the fewest acceptable trials.

A few exploratory experiments or from past experience or Based on some underlying theory or hypothesis. This process is called as design of experiment

1. Achieving maximum realistic information with the minimum number of well-designed experiments.

2. An experimental program recognizes the major "factors" that affect the outcome of the experiment.

3. The factors may be identified by looking at all the quantities that may affect the outcome of the experiment.

Benefits of Design of Experiments

- Experimental design involves manipulating the independent variable to observe the effect on the dependent variable. This makes it possible to determine a cause and effect relationship.
- Controlling the independent variable the experimenter attempts to eliminate unwanted extraneous variables.
- Control over extraneous variables is usually greater than in other research methods.
- Because of strict conditions and control the experimenter can set up the experiment again and repeat or 'check' their results. Replication is very important as when similar results are obtained this gives greater confidence in the results.

Use of Design of experiment

Design of experiments is used to determine the causes of variation in the response, the find conditions under which the optimal (maximum or minimum) response is achieved, to compare responses at different levels of controlled variables & to develop a Model for predicting response. The particle size and the size distribution of the final liposome product which largely depend on the processing steps where usually homogenization steps are employed. Upon selection of the key variables the liposomal formulation will be optimized using experimental design where for each of the selected variable will be tested.[55]

Key steps for Design of experiments

Obtaining good results from a Design of experiments involves those six steps.

- Set objective
- Select process variables
- Select an experimental design
- Execute the design
- Check that the data are consistent with the experimental assumptions.
- Analyze and interpret the results.

Major Approaches to DOE

1) **Factorial Design**
2) **Taguchi Method**
3) **Response Surface Design**

Two types of design

1. **First-Order Design**
   I. The 2k Factorial Design
   II. The Plackett–Burman Design
   III. The Simplex Design
2. **Second-Order design**
   I. The 3k Factorial Design
   II. The Central Composite Design (CCD)
   III. The Box–Behnken Design

1) Factorial Design:

I. **Full factorial design**
II. **Fractional Factorial Designs**
III. **One half factorial design**

1. First-Order Design

The most common first-order designs are 2*k* factorial (*k* is the number of control variables)

I. The 2k Factorial Design

In a 2*k* factorial design, each control variable is measured at two levels, which can be coded to take the values, −1, 1, that related to the low and high levels, respectively, of each variable. This design consists of all possible combinations of such levels of the *k* factors.

The corresponding 2^3 design matrix is of order 8 × 3 of the form

$$D = \begin{bmatrix} -1 & -1 & -1 \\ 1 & -1 & -1 \\ -1 & 1 & -1 \\ -1 & -1 & 1 \\ 1 & 1 & -1 \\ 1 & -1 & 1 \\ -1 & 1 & 1 \\ 1 & 1 & 1 \end{bmatrix}$$

I. The Plackett–Burman Design

The Plackett–Burman design allows two levels for each of the k control variables, just like a 2k design, but requires a much smaller number of experimental runs, especially if k is large. It is therefore more economical than the 2k design. Its number, n, of design points is equal to k + 1, which is the similar as the number of factors in model. In this respect, the design is said to be saturated because its number of design points is equal to the number of parameters to be estimated in the model. Also, this design is available only when n is a multiple of 4. Therefore, it can be used when the number, k, of control variables is equal to 3, 7, 11, 15,... To construct a Plackett–Burman design in k variables, a row is first selected whose elements are equal to −1 or 1 such that the number of 1s is k +12 and the number of −1s is k − 12 . The next k − 1 rows are generated from the first row by shifting it cyclically one place to the right k − 1 times. Then, a row of negative ones is added at the bottom of the design. For example, for k = 7, the design matrix, D, has eight points whose coordinates are x1, x2, . . ., x7 and is of the form

Design arrangements for *k* = 3, 7, 11, . . . , 99 factors can be found.

2. Second-Order design

I. The 3k Factorial Design

II. The Central Composite Design (CCD)

III. The Box–Behnken Design

I. The 3k Factorial Design

The 3*k* factorial design consists of all the combinations of the levels of the *k* control variables which have three levels each. If the levels are equally spaced, then they can be coded so that they correspond to −1, 0, 1. The number of experimental runs for this design is 3*k*, which can be very for a large *k*. Fractions of a 3*k* design can be considered to reduce the cost of running such an experiment.[56]

II. The Central Composite Design (CCD)

This is perhaps the most popular of all second-order designs. This design consists of the following three portions:

1. A complete (or a fraction of) 2*k* factorial design whose factors' levels are coded as −1, 1. This is called the factorial portion.

2. An axial portion consisting of 2*k* points arranged so that two points are chosen on the axis of each control variable at a distance of α from the design center (chosen as the point at the origin of the coordinates system).

3. $n0$ center points. Thus, the total number of design points in a CCD is $n = 2k + 2k + n0$. For example, a CCD for $k = 2$, $\alpha = \sqrt{2} = 1.41$, $n0 = 2$ has the form

CCD is obtained by enlarging a first-order design, namely, the 2*k* factorial with additional experimental runs, specifically, the 2*k* axial points and the $n0$ center point replications. Thus, this design is developed in a style with the consecutive nature of a response surface study in starting with a first-order design, to fit a first-degree model, followed by the addition of design points to fit the larger second-degree model. The first-order design serves in a preliminary phase to get initial information about the response system and to assess the importance of the factors in a given experiment. The additional experimental runs are chosen for the purpose of getting more information that can lead to the determination of optimum operating conditions on the control variables using the second-degree model. The values of α (or the axial parameter) and $n0$, the number of center-point replications, are chosen so that the CCD can acquire certain desirable properties. For example, choosing $\alpha = F1/4$, where *F* denotes the number of points in the factorial portion, causes the CCD to

be rotatable. The value of *n*0 can then be chosen so that the CCD can achieve either the orthogonality property or the uniform precision property. The orthogonality of a second-order design is attainable only after expressing model in terms of orthogonal polynomials.[57]

III. The Box–Behnken Design

This design was developed by Box and Behnken. It provides three levels for each factor and consists of a particular subset of the factorial combinations from the 3^kfactorial design. The use of the Box–Behnken design is popular in industrial research because it is an economical design and requires only three levels for each factor where the settings are −1, 0, 1. Some Box–Behnken designs are rotatable, but, in general, this design is not always rotatable. Box and Behnken number of design arrangements for k = 3, 4, 5, 6, 7, 9, 10, 11, 12, and 16 factors.[41]

Interpretation

Analysis of Variance Table -P-Value

The p-values (P) in the analysis of variance table to determine which of the effects in the model are statistically significant. Typically, the interaction effects in the model are analysed. A significant interaction will influence the interpretation of the main effects.

For the p-value:

- Identify the p-value for the effect for the evaluation.
- Compare this p-value to **α-level**. A commonly used **α -level is 0.05**.
- If the p-value is less than or equal to **α**, conclude that the effect is significant.
- If the p-value is greater than **α**, conclude that the effect is not significant.

In theory, any VIF value greater than 1 can conclude the variance of the coefficients so much that statistical significance is a less useful way to identify candidate models. In practice, values greater than 5–10 can produce unstable coefficients that are difficult to interpret and, thereby, prompt corrective measures.

Model Summary: R-Sq and R-Sq (Adj) Values

S, R, adjusted R, and predicted R are measures of how well the model fits the data. These values can help you select the model with the best fit.

S is measured in the units of the response variable and represents the standard distance that data values fall from the regression line. The lower S for the better the equation predicts the response.

R (R-Sq) describes the amount of variation in the observed response values that is explained by the predictor(s). R always increases with additional predictors. For example, the best five-predictor model will always have a higher R than the best four-predictor model. Therefore, R is most useful when comparing models of the same size.

Adjusted R is a modified R that has been adjusted for the number of terms in the model. If you include unnecessary terms, R can be artificially high. Unlike R, adjusted R may get smaller when you add terms to the model. Use adjusted R to compare models with different numbers of predictors.

R^2(pred) is a measure of how well the model predicts the response for new observations. Large differences between predicted R^2 and the other two R^2 statistics can indicate that the model is overfit. An overfit model does not predict new observations nearly as well as the model fits the existing data. Predicted R^2 is more useful than adjusted R^2 for comparing models because it is calculated with observations not included in the model calculation.

The analysis of variance table shows the following results:

Main effects: A Linear Model: $Y = b_o + b_1X$ (first order)

Squared Terms: A Quadratic Model: $Y = b_o + b_1X + b_{11}X^2$ (second order)

Interaction effects: A Cubic model: $Y = b_o + b_1X + b_{11}X^2 + b_{111}X^3$ (third order)

Regression Equation: The regression equation is an algebraic representation of the relationship between the response and predictor variables.[55]

The regression equation takes the form of:

Response = constant + coefficient * predictor + ... + coefficient * predictor

or $y = b_o + b_1X_1 + b_2X_2 + ... + b_kX_k$

Where: Response (Y) is the value of the response.

Constant (b_o) is the value of the response variable when the predictor variable(s) is zero. The constant is also called the intercept because it determines where the regression line intercepts (meets) the Y-axis.

Predictor(s) (X) is the value of the predictor variable(s). The predictor can be a polynomial term.

Coefficients (b_1, b_2, ... ,b_k) represent the estimated change in mean response for each unit change in the predictor value. In other words, it is the change in Y that occurs when X increases by one unit.

Normal Effects Plot

A normal effects plot to compare the relative magnitude and the statistical significance of both main and interaction effects. Minitab draws a line to indicate where the points would be expected to fall if all effects were zero. Points that do not fall near the line usually signal significant effects. Such effects are larger and generally further from the fitted line than unimportant effects. By default, Minitab uses an a-level of 0.05 and labels any effect that is significant.

Half Normal Effects Plot

Half normal effects plot to compare the magnitude and the statistical significance of both main and interaction effects. The fitted line indicates the expected points to fall if all effects were zero. Points that do not fall near the line usually signal significant effects. Such effects are larger and generally further from the fitted line than unimportant effects. Significant effects are labeled and fall away from the line on the right side. By default, Minitab uses an a-level of 0.05 and labels any effect that is significant.[58]

3. LITERATURE REVIEW

1. Technology Specific Review

Tenzel et al. (1991) reviewed the preparation of liposome in an ethanol water mixture and the solvent exchange by the different technique like reverse osmosis, evaporation and sterile filtration. The average size of the liposomes can be controlled by changing the ionic strength and lipid composition of the mixture.

Yuiurnas et al. (1990) reviewed an apparatus and method for manufacturing of the lipid vesicles by the shear mixing of the composition. The shear mixing in a substantially cylindrical mixing chamber having at least one tangential input for rapid production of the vesicles.

Edgerly-Plug (2003) reviewed a process for producing liposomes having a desired mean particle size; wherein the desired mean particle size obtained by the varying the initial organic solvent concentration.

Bhamidipati et al. (2006) reviewed the manufacturing process for the liposomal formulation. In this method, a lipid fraction added to the water-miscible organic solvent. This solution added to the aqueous solution and after size reduction and removal of the water miscible organic solvent from the preparation using the Diafiltration method.

Wiggernhorn et al. (2010) reviewed the general method of preparing liposomes by different technique like extrusion, spray drying or spray freezing technique for better size optimization. It described the general idea about the component of the extrusion process and the component of the liposome formulation.

M. Najlah et al. **(2014)** a niosome nanodispersion was manufactured using high-pressure homogenization following the hydration of proniosomes. This study has shown that both sonication and high-pressure homogenization were capable of generating noisome Nano dispersions from micro-sized vesicles manufactured using a proniosome technology. Compared with sonication, high pressure homogenization produced vesicles with a similar drug entrapment efficiency, superior homogenization output rate, and absence of sample contamination and overheating

2. **Dosage form specific review**

Barenholz et al. (**1993)** reviewed the efficient active loading of the weak amphiphatic drugs into liposomes using the transmembrane pH gradient. The liposomes loaded with the amphiphatic drug are stable and safe.

3. **Filtration specific review**

R. van Reis et al. (1999) focused on exploring the effects of membrane charge, in combination with buffer pH, on protein separation using HPTFF (High-performance tangential flow filtration). Optimization of buffer pH and ionic strength have a significant impact on the sieving behaviour of proteins in membrane systems.

Lenk et al. (1999) reviewed the Tangential flow filtration (TFF) process for the size separation of the liposome and lipid particles. TFF apparatus having different pore sizes may be used to sequential separation of the particles having a defined size range.

Slater et al. (2002) reviewed the composition of liposomes having an outer surface and an inner surface defining an aqueous liposome compartment, and other additives which are generally used in the liposome formulation. General idea about the drug loading principle and unentrapped drug removed from the external medium by the simple tangential flow filtration method.

Bonner et al. (2011) reviewed a separation of the material based on the design of the single pass tangential flow process without the recirculation loop. The separation module includes the external feed reservoir and retentate reservoir. The single pass TFF process provides high conversion while operating with relatively low pressure sources. It is also used for the separation of blood.

Bosch et al. (2011) reviewed tangential flow filtration devices and methods for elevating heterogeneous mixture of blood constituents for leukocytes by removal of non-leukocyte blood constituents.

Wang at el. (2012) reviewed a method for purifying a protein by processing the sample through a capture chromatography resin to provide a first eluating the protein; and

inactivating viruses in the first eluate. After that processing the inactivated eluate through at least one depth filter to provide a filtered eluate comprising the protein.

Pompiati et al. (2011) reviewed a method for purifying an immunoglobulin in monomeric form in aqueous buffered solution using the anion exchange resin chromatography. Anion exchange chromatography useful to separate the monomeric form of the immunoglobulin, because of the anionic form does not bind to the anion exchange material.

4. QbD specific review

P. Guichardon et al. (2005) deals with the purification of a semi-solid liposome (SSL) preparation which consists in the removal of free hydrogel present in external solution. Two separation methods, a cross-flow ultrafiltration and a centrifugation have been investigated and compared. As filter systems, membrane processes using different membranes consist in a diafiltration step until the free hydrogel were almost completely eliminated followed by a concentration step. To develop an industrial plant, a membrane filter system was preferred to a centrifuge because of its flexibility, compactness and cost.

X. *Xu et al. (2012)* described the different case study related to the liposomal formulation with respect to the different design. Two statistical design applying QbD concepts to liposome based complex parenteral controlled release system. In this article placket-burman and central composite design used to determine the design space for the encapsulation efficiency. On comparing the CCD model generated response surface with additional data points, the accuracy and robustness of the model was confirmed. Using this developed model, the design space for Tenofovir liposomes preparation has been established in a laboratory scale.

X. Xu et al. (2012) disclosed the Quality by design (QbD) principles to gain a comprehensive understanding of the preparation of superoxide dismutase (SOD) containing liposome formulations and risk analysis and D-optimal statistical design were performed. Different design space for the prediction data can be analyzed.

4. MATERIALS AND METHOD

4.1 Innovator product profile summary

Table 4. 1 ***: Innovator Product Profile***

Sr. No.	Title	Description
1	Product Name	--*
2	Innovator	--*
3	Dosage Form	Injection
4	Therapeutic category	Anticancer
5	Route of Administration	Parentally (Intravenous)
6	Brief Pharmacokinetic	**Half Life:** 55 hrs **Apparent V_d :** 700 to 1100 L **Protein Binding:** 70%
7	Description	A brick red aqueous injection
8	Storage Condition	At 2-8˚C

4.2 Innovator evaluation study

Table 4. 2 ***: Innovator Product Analytical Evaluation:***

Sr. No.	Details of Test	Innovator Product
1	Brand Name	--*
2	Label Claim	--*
3	Pack	10 ml
4	**TESTS** Appearance Osmolality Assay of Drug ‘A’	**RESULTS** Red orange 305 mOsmol 98 %

**** Data reported is as per the test report released from Analytical Department of Wockhardt Research Center.**

4.3 List of components

The following table shows the list of components used for the formulation and evaluation study of Liposome.

Table 4. 3 ***: List of Components***

Sr. No	Components	Size	Manufacturer
4	0.22µm PF filter	32mm	Pall Life science
5	Disposable syringes	0.5,2, 5,10,20 ml	Dolphin BT
6	Schott Bottles	250,500, 1000 ml	Schott Duran, Made in Germany
7	Glass ware	All	

4.4 LIST OF EQUIPMENTS

The following table shows the list of equipment used for the formulation and evaluation study of Liposome.

Table 4. 4 ***: List of Equipment***

Sr. No.	Instruments	Manufacturer/ Supplier	Model No
1	Electronic weighing balance	Sartorius, Mettler Toledo	CE7101
2	High Pressure Homogenizer	Avastin	C50
3	Dynamic Light Scattering	Malvern	ZS Nano
5	Quixstand Benchtop Dialysis	GE life science	QSM-03SP/50
5	Orbital Incubator-Shaker	Scigenics Biotec	356L
6	Mechanical Stirrer IKA	IKA lab	C-MAG HS10
7	Dry Heat Sterilizer	Matchine Fabricks	

8	pH-meter	Thermo orion	Orionstar A215
9	Conductivity meter	Thermo Scientific	
11	UVvisible spectrophotometer	Schimadzoo	UV 1800
14	Osmometer Model	Advanced Instrument Inc.	3250
15	Magnetic Stirrer	Tarsons Spinot Digital	5560
16	FTIR	Jasco, Japan	6100 Type A

4.5 LIST OF EXCIPIENTS

The following table shows the list of excipients used for the formulation and evaluation study of Liposome.

Table 4. 5 ***: List of Excipients***

Sr. No.	**Excipients**	**Manufacturer/ Supplier**
1	**Hydrogenated phosphatidyl choline from soybean (HSPC)**	Lipoid GMBH, Made in Germany.
2	**Cholesterol**	Dishman, Made in France
3	**DSPE-mPEG-2000**	Lipoid GMBH, Made in Germany.
4	**Ethanol**	Brompton, Ontario,Canada
5	**Water for injection**	Milli Q
6	**Sucrose**	Merck
7	**Ammonium Sulfate**	Merck
8	**Histidine**	Merck

4.6 METHODOLOGY

4.6.1 Preparation of Standard Solution

Weigh accurately 20 mg in 100 ml volumetric flask and dissolve in 10 ml of methanol. Sonicate for 10 minute and make up the volume up to 100 mL with methanol. (200 ppm) Take 2 ml of the solution in to the 10 ml volumetric flask and make up the volume up to 10ml with methanol. (40ppm)

Determination of UV absorption maxima

Concentration 40 µg/ml was prepared using methanol and then scanned between wavelength 200-800 nm. The wavelength at which maximum absorbance is observed was noted as maximum wavelength (λ_{max}).

Determination of Calibration Curve

Prepare primary stock solution of 2000 ppm in methanol (20 mg of Drug A in 100 ml of methanol). Take 12.5 ml of primary stock solution to the 50 ml volumetric flask and make up the volume up to 50 ml with methanol. (50 ppm) (Secondary stock solution)Take 1, 2, 3, 4, 5, 6, 7, 8, and 9 ml of the secondary stock solution to the different 10 ml volumetric flask and volume make up to the mark with methanol. Take the absorbance of the each solution at 482 nm (5 – 45 ppm).

4.6.2 Calibration Curve of Ammonium Sulphate Conductivity in 10% sucrose solution

Preparation of Primary Stock Solution (500 mM)

Take 66 gm of ammonium sulphate into the 100ml volumetric flask and add 20 ml milli Q water. Sonicate for 10min and volume make up to the mark with milli Q water. (500 mM)

Preparation of secondary stock solution (500 mM)

Take 2 ml, 4 ml, 6ml, 8 ml, 10 ml, and 12 ml primary stock solution in to the 20 ml volumetric flask and volume make up with 10% sucrose solution.

Preparation of 10 % sucrose solution:

Weigh 10gm of sucrose in to the 100 ml volumetric flask and add 20 ml milli Q water. Sonicate for 10 min and volume make up to the mark with milli Q water. (10 % sucrose)

Determination of conductance

Measure the conductance of the all sample with the conductometer at the temp 25^{0} C. Stable conductance reading noted down.

4.7 Pharmaceutical Pre-formulation studies

Pre-formulation testing is designed to assess the influence of physicochemical properties of drug substances and excipients on formulation properties of dosage form, method of manufacture and pharmacokinetic biopharmaceutical properties of the resulting product. A thorough understanding of physicochemical properties may ultimately confirm that no significant barriers are present for the formulation development.

Drug Excipient Compatibility Study:

DSC

DSC Study of pre-formulation Sample was performed using a Diamond DSC (Mettler Star SW 8.10) to determine the drug excipient compatibility study.

Operating Procedure:

Weigh an appropriate quantity of the sample shown in table to be examined and place in the sample crucible. Set the initial temperature 30^0C and final temperature 350^0C, and the heating rate 10^0C/min. Begin the analysis and record the thermo-gram, with the temperature and/or time on the x-axis and the energy change on the y-axis. The temperature at which the phenomenon occurs (the onset temperature) corresponds to the intersection of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve. The peak of the curve indicates the end of the thermal phenomenon.

4.8 Liposome Formulation Procedure

High Pressure Homogenization

Crude liposome is forced under high pressures to pass through a narrow gap. Thus, it creates a fast acceleration undergoing an extreme drop in pressure as the liposome exits the homogenization valve. Which leads to formation of required size and polydispersity. This will be depend on the number cycles perform with applying pressure like 1000 psi, 1500 psi, 2000 psi etc.

Cassette Dialysis:

Primary applications for TFF are concentration, Diafiltration. In concentration this is a simple process that involves removing fluid from a solution while retaining the solute molecules. The concentration of the solute increases in direct proportion to the decrease in

solution volume, i.e. having the volume effectively doubles the concentration. And in Diafiltration stage the fractionation process that washes smaller molecules through a membrane and leaves larger molecules in the retentate without ultimately changing concentration. It can be used to remove salts or exchange buffers. It can remove ethanol or other small solvents or additives. So the process of making the liposome to a more concentric by exchange of buffer i.e ammonium sulfate with sucrose solution at about 50%. This will be determined by checking the conductance in between process

4.9 EVALUATION

1. Particle Size determination

Particle size analysis was conducted using a Malvern ZS90 zeta-sizer. Prepared liposome formulations were filled in the cuvette by the injection and analysed it.All measurements were conducted at 25 ^{0}C and in triplicate. The values were reported as intensity weighted mean ± SD (distribution width).Poly dispersity index (PDI) was also measured along with size.

2. Zeta potential analysis

Zeta potential was measured using a Malvern ZS90 zeta-sizer and a folded capillary cell. The samples should be same which used for particle sizing for zeta-potential measurement. All tests were conducted at 25 ^{0}C and in triplicate and reported as mean ± SD.

3. Assay for Drug A

Add the reagent i.e Ammonium Ferrothiocynate and Chloroform with Drug A Liposome into the glass centrifuge tube. Each tube was subjected to vortex for 1 min. Tubes were kept in centrifuge for 10 min. at 1000 rpm. Lower layer ($CHCl_3$) was removed with the help of glass pipette. Absorbance of $CHCl_3$ Containing concentration of Drug A Liposome was measured at 482 nm.

The pharmacopoeia limit of % Drug assay is 95-105 %

$$\text{Assay (mg/mL)} = \frac{A_T}{A_S} \times \frac{WS}{10} \times \frac{0.15}{10} \times \frac{10}{0.15} \times \frac{P}{100} \times 1$$

Where; A_T= Absorbance of Test

A_S= Absorbance of Standard

WS= Weight of standard

WT=Weight of test

4. Lipid Assay (lipid concentration by Stewart Assay Method)

Step I: - Preparation of Ammonium Ferrothiocynate :- Dissolve 27.3 gm of Ferric Chloride hexahydrate and 30.4 gm of Ammonium Thiocyanate in WFI make up the volume up to 1000 ml with WFI.

Step II: - Add the reagent i.e. Ammonium Ferrothiocynate and Chloroform with lipid solution into the glass centrifuge tube

Step III: - Each tube was subjected to vortex for 1 min.

Step IV: - Tubes were kept in centrifuge for 10 min. at 1000 rpm.

Step V: - Lower layer ($CHCl_3$) was removed with the help of glass pipette.

Step VI: - Absorbance of $CHCl_3$ Containing standard was measured at 485 nm and conc. of lipid was calculated.

The pharmacopoeia limit of % Lipid assay is 90-110 %

$$\text{Assay (mg/mL)} = \frac{A_T}{A_S} \times \frac{WS}{2} \times \frac{0.2}{10} \times \frac{1}{4} \times \frac{4}{WT} \times \frac{P}{100} \times 1$$

Where; A_T= Absorbance of Test

A_S= Absorbance of Standard

WS= Weight of standard; WT=Weight of test

5. **Calculation of the % EE**

Encapsulation Efficiency was determined by the HLB cartridge.

Procedure:

Prepare the standard solution of the drug in methanol. (40 ppm) Label the triplicate cartridge sample for the individual analysis of the sample. Each cartridge having same pre-treatment like pass methanol 1 ml for conditioning, water 1 ml for equilibration and ammonium carbonate 1 ml for conditioning of the cartridge by applying the vacuum pressure (> 5 Hg). Load the dilute sample of the formulation to the 3 HLB cartridge and pass it through the cartridge by applying pressure. Wash the cartridge with sufficient quantity of the water at three time and volume make up to 10 ml with methanol. Collect the filtrate sample at the different stage of the filtration. Vortex the test tube of the entrapped drug for 10 min for breaking the liposome. Repeat the same procedure with methanol to the same cartridge and final volume make up with methanol. Prepare standard solution of the drug and same concentration of the formulation for the analysis.

Entrapped drug

Formula: Entrapped Drug = [Total Drug- Free Drug]. Limit is more than 95 %

Free Drug

Determined by HLB Cartridge, Extraction and UV analysis.

Limit is Less than 10 %

Encapsulation efficiency

The % Encapsulation efficiency was calculated by the formula

% EE = [Total Drug – Free Drug / Total Drug] X100 .Limit is 90-100 %

6. **Osmolality**

Osmolality was measured using an Advanced Instruments Osmometer-3250.250µl of liposomal formulation was required to determine Osmolality. Osmolality was determined which is based on freezing point depression principle. Limit is 270-330 mOsmol.

5. EXPERIMENTAL WORK

5.1 Calibration curve of drug

Table 5. 1 ***Calibration Curve of the drug in Methanol***

Sample ID	Concentration (ug/ml)	Absorbance			Mean	S.D.
		I	II	II		
Blank	0	0	0	0	0	0
Standard	50	1.140	1.15	1.14	1.14	
5 PPM	5	0.103	0.105	0.107	0.105	0.00231
10 PPM	10	0.224	0.225	0.225	0.224	0.00058
15 PPM	15	0.35	0.344	0.345	0.346	0.00321
20 PPM	20	0.468	0.468	0.468	0.468	0
25 PPM	25	0.589	0.59	0.591	0.59	0.001
30 PPM	30	0.706	0.706	0.71	0.7073	0.00231
35 PPM	35	0.877	0.874	0.88	0.877	0.003
40 PPM	40	0.944	0.944	0.944	0.944	0
45 PPM	45	1.06	1.06	1.062	1.060	0.00115
50 PPM	50	1.162	1.161	1.165	1.162	0.00208
Std BKT	50	1.156				

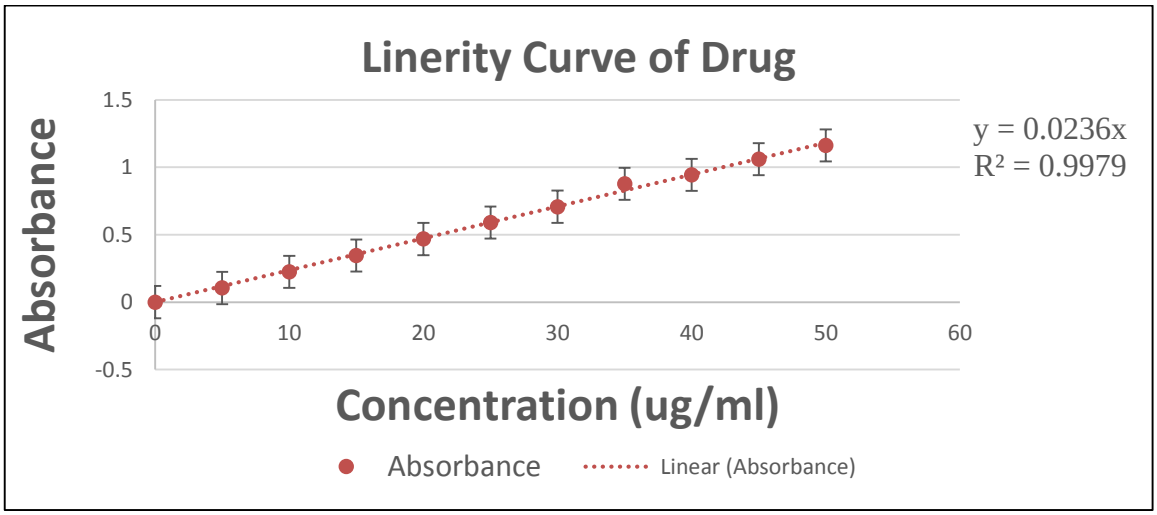


Figure. 11: calibration curve of drug A in methanol

5.2 Conductivity of the buffer soluion (Ammonium Sulphate solution in the 10 % sucrose solution)

Table 5. 2: ***Relationship between molarity and concentration of the*** *$(NH_4)_2SO_4$*

1 M $(NH_4)_2SO_4$ = 132.14 gm/ 1 L
1 M $(NH_4)_2SO_4$ = 132.14 mg/ ml
1 mM $(NH_4)_2SO_4$ = 0.13214 mg/ ml
1 mM $(NH_4)_2SO_4$ = 2.6428 mg/ 20ml

Table 5. 3 : ***Linear conductivity of the $(NH_4)_2SO_4$ in Sucrose solution***

(NH4)2SO4 (mM)	$(NH_4)_2SO_4$ mg /20 ml	Conductivity (mS/cm)			Mean	S.D
		I	II	III		
0 Mm (Sucrose 10%)	0 mg	0.00688	0.005	0.0056	0.0058	0.00096
50 mM	66 mg	9.233	9.4	9.56	9.39	0.163
100 mM	132 mg	16.84	16.85	16.78	16.82	0.037
150 mM	198 mg	24.45	24.51	24.42	24.46	0.045
200 mM	264 mg	31.16	30.23	31.5	30.96	0.657
250 mM	330 mg	40.48	40.58	40.63	40.56	0.076
300 mM	396 mg	50.88	51.23	51.5	51.20	0.310

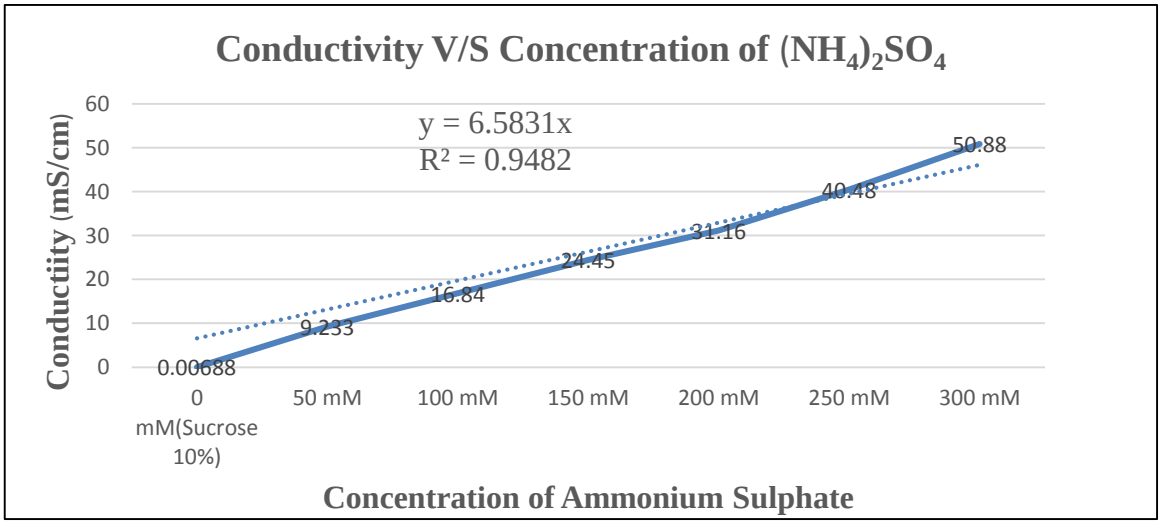


Figure. 12: ***Linear curve of $(NH_4)_2SO_4$ in sucrose solution***

5.3. Preformulation Study:

5.3.1 Differential Scanning Colorimetry (DSC)

DSC Thermogram for Drug A, HSPC, Cholesterol, m-PEG and combination of drug with this excipients were shown in fig respectively.

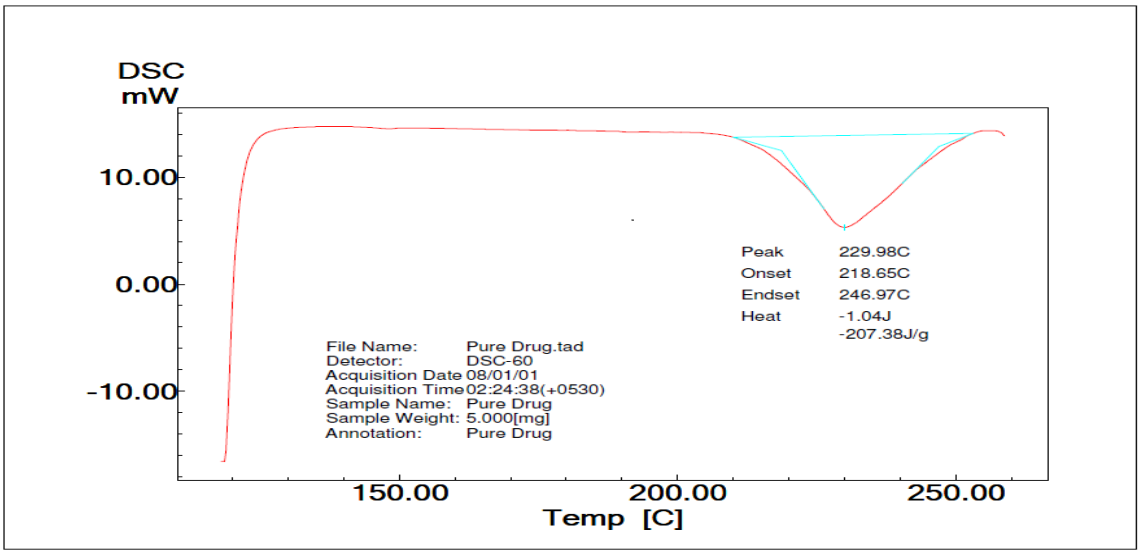


Figure. 13 : ***DSC Thermogram for Drug A***

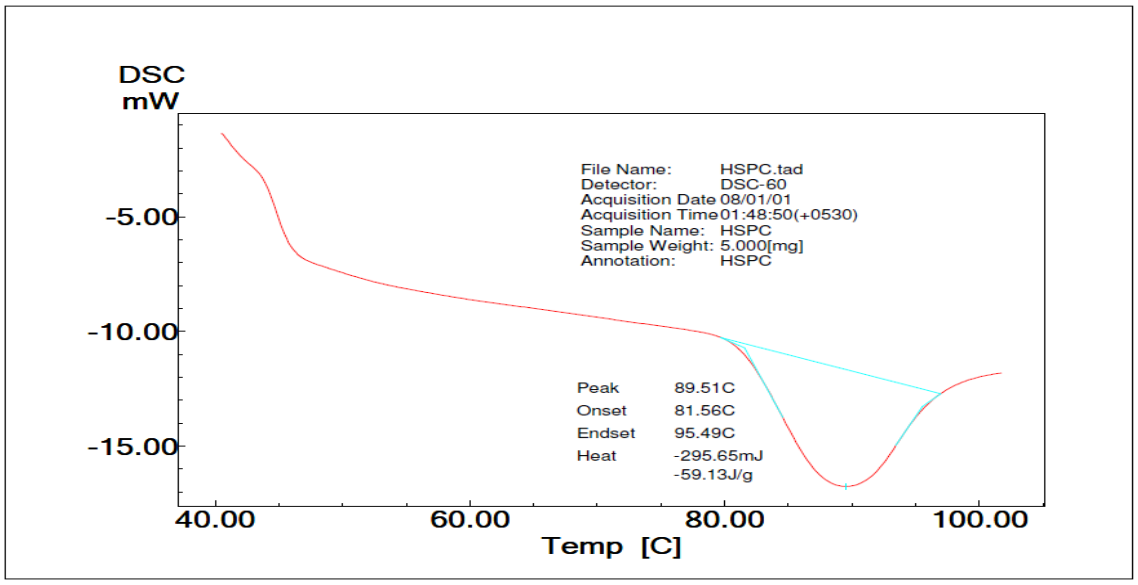


Figure. 14 : ***DSC Thermogram for HSPC***

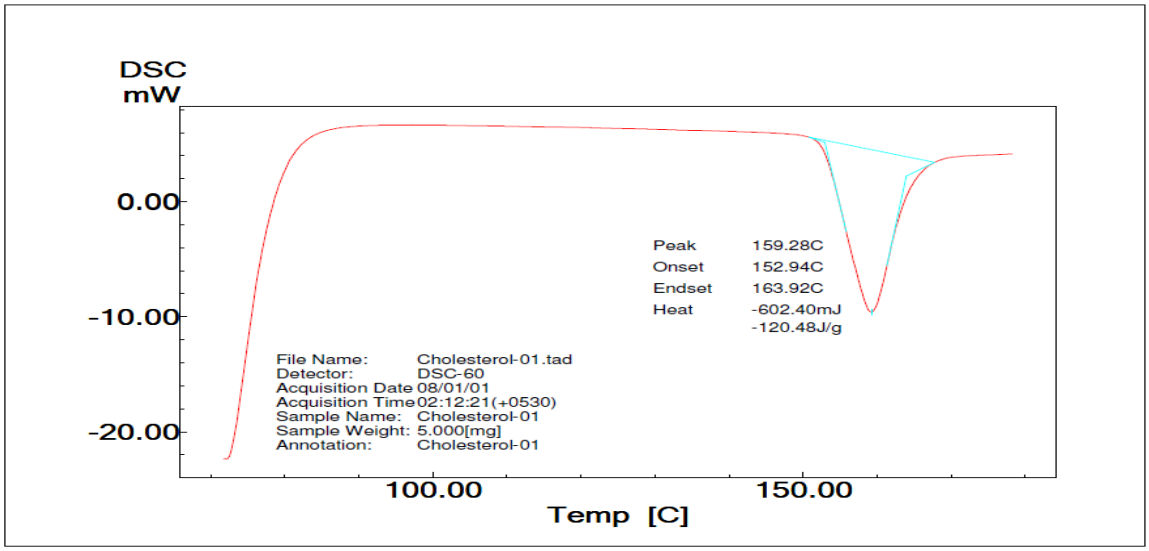


Figure. 15 : ***DSC Thermogram for Cholesterol***

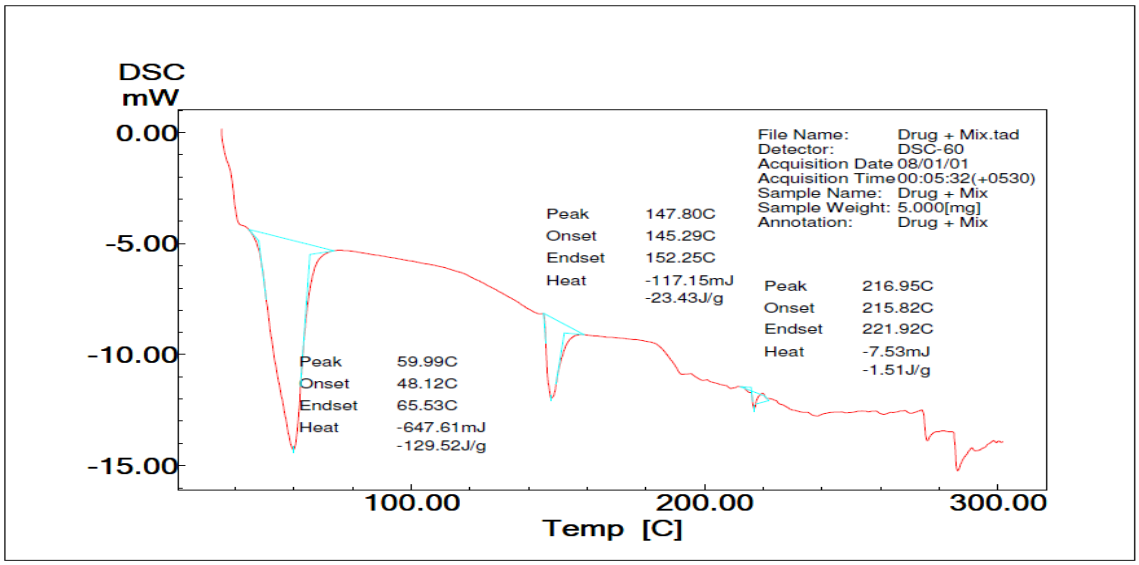


Figure. 16 : ***DSC Thermogram for drug + excipients (lipids)***

Observations:

All the Thermogram for API, HSPC, and cholesterol and cholesterol showed endothermic reaction. Also there is no significant change in DSC thermogram for combination of API and lipid excipient.

5.4 Experimental trials

Table 5. 4: ***Preparation of the placebo liposome:***

ID	Lipid (mM)	Chol (%w/v)	Drug (mg/ml)	Buffer (mM)	Solvent/ Non solvent volume ratio	Conductivity (mS/cm)	Size (nm)	PdI
1	20	0.5	2	100	0.1	10.56	Not measured	
2	20	0.5	2	200	0.1	22.78		
3	20	0.5	2	300	0.1	36.96	341.3	0.508

Table 5. 5 ***Optimized batch formula for placebo liposome:***

ID	Lipid (mM)	Chol (%w/v)	Drug (mg/ml)	Buffer (mM)	Solvent/ Non solvent volume ratio	Conductivity (mS/cm)	Size (nm)	PdI
1	20	0.5	2	300	0.1	36.96	341.3	0.508

(Note: Lipid= HSPC +m-PEG (3:1), Chol =cholesterol, Buffer = Ammonium sulphate solution, Solvent / Nonsolvent = ethanol/ WFI)

Procedure:

All the liposome formulations were prepared using a modified ethanol injection method. Briefly, the desired amount of lipids were weighed into a 250 ml Schot Duran bottle. Heating and dissolving lipid component in ethanol at a temperature of 50^0 C to 75^0 C and injecting in to an aqueous solution of the buffer. In process maintain the temperature at 50^0 C to 75^0 C. Liposome prepare with three different concentration of buffer (300, 200,100 mM) and after proper size achieved, continue to the TFF system. Size reduced liposome were introduced to the TFF system for removal of the solvent molecule (ethanol) and buffer ion with the sucrose solution. Optimize the TFF system with the diafiltration time and volume of buffer to produce the appropriate purification of the external buffer. The inlet flow, inlet pressure, outlet pressure (back pressure), and filtrate flow were monitored during the process. Compare the initial buffer concentration for optimum diafiltration volume.

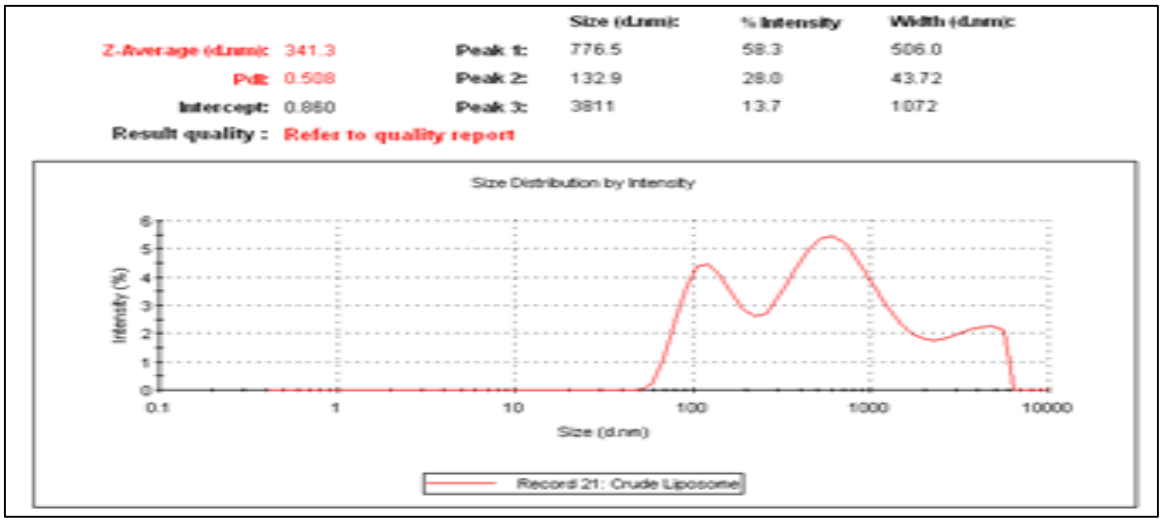


Figure. 17 : ***Particle size report of placebo liposome:***

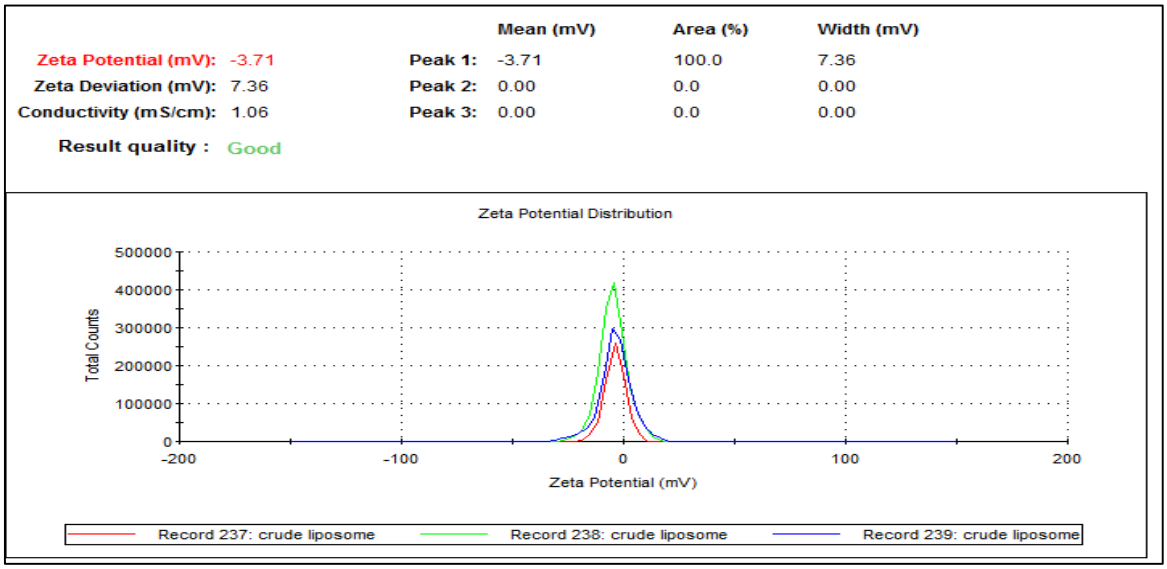


Figure. 18 : ***Zeta potential report of placebo liposome:***

Result:

In all the batches (PL-1 to PL-3) different concentration of the buffer solution was analyzed. Different concentration of the buffer solution was produce the different conductivity of the pro-liposomal suspension.

Conclusion: To study the better ionic difference across the liposomal bilayer, 300 ppm of the buffer solution was selected for the further optimization.

5.5 Optimization of TFF process parameter

5.5.1 Flux versus TMP

Procedure:

Run the TFF system with WFI at the flow rate approximately 50 ml/min. (Watson marlow pump at 200 rpm) Increasing the inlet pressure with the time period and optimize the Filtrate rate against TMP at particular time. (1 hr) Calculate the filtrate flux of the water at constant surface area of 500kd cartridge (0.085 m^2).Repeat the same process with the liposomal formulation maintaining all the process factor constant. Plot the graph of Filtrate flux v/s TMP.

Table 5. 6: Water trail at 200 RPM

Water trail at 200 RPM										
Time (min)	Duration (min)	Filtrat (ml)	Total Filtrat (ml)	Filtrat Rate (ml/min)	Filtrat Rate (l/hr)	Inlet (psi)	Outlet (psi)	TMP (psi)	TMP (bar)	Filtrat Flux (LMH)
0	0	0	0	0	0	0	0	0	0	0
10	10	80	80	8	0.48	1	0	0.5	0.034	5.647
20	10	90	170	17	1.02	2	0	1	0.069	12
30	10	80	250	25	1.5	3	1	2	0.138	17.65
40	10	80	330	33	1.98	4	1	2.5	0.172	23.29
50	10	70	400	40	2.4	5	1	3	0.207	28.24
60	10	100	500	50	3	6	1	3.5	0.241	35.29

Table 5. 7: Lipid trail at 200 RPM

Lipid trail at 200 RPM										
Time (min)	Duration (min)	Filtrat (ml)	Total Filtrat (ml)	Filtrate Rate (ml/min)	Filtrat Rate (l/hr)	Inlet (psi)	Outlet (psi)	TMP (psi)	TMP (bar)	Filtrat Flux (LMH)
0	0	0	0	0	0	0	0	0	0	0
10	10	90	90	9	0.54	3	1	2	0.138	6.353
20	10	60	150	15	0.9	6	1	3.5	0.241	10.59
30	10	50	200	20	1.2	8	2	5	0.345	14.12
40	10	20	220	22	1.32	10	3	6.5	0.448	15.53
50	10	15	235	23.5	1.41	12	4	8	0.551	16.59
60	10	13	248	24.8	1.488	15	5	10	0.689	17.51
70	10	12	260	26	1.56	18	6	12	0.827	18.35
80	10	10	270	27	1.62	20	6	13	0.896	19.06
90	10	5	275	27.5	1.65	20	7	13.5	0.931	19.41

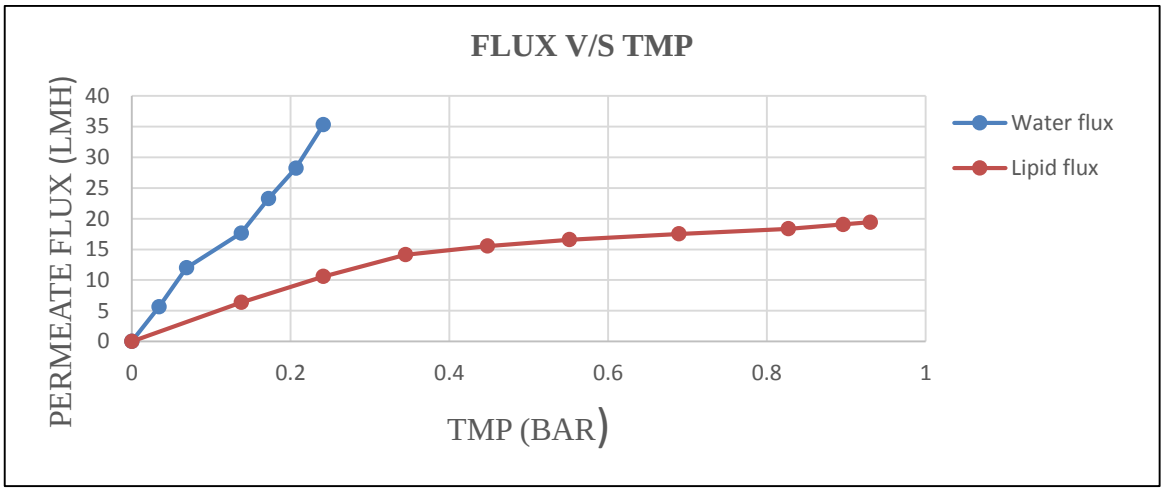


Figure. 19 : Optimal TMP range for water and lipid at the different cross flow rate.

Result:

The water filtrate flux was increased with the TMP at constant level. While in the liposomal filtrate flux was increased with the TMP at starting point after that it was approximately stable and no significantly increased, although increasing the TMP.

Conclusion:

There was no significant gel layer formed on the hollow fiber cartridge in the water trail, so filtrate flux increased with the TMP. But in liposome case, TMP and cross flow rate affect the Flux at various stage. At lower TMP, permeate flux was influenced by both membrane & gel layer resistance and is proportional to the pressure.

5.5.2 TMP and cross flow

Procedure:

Run the TFF system with liposomal solution (1L) at the flow rate approximately 50 ml/min (Watson marlow pump about 200 rpm). Increasing the inlet pressure with the time period and subsequently increase the outlet pressure. Optimize the Filtrate rate against TMP at particular time. (1-2 hr) Calculate the filtrate flux of the sample at constant surface area of 500kd cartridge (0.085 m^2).Repeat the same process with another sample of liposome at the different rpm 150 and 250 respectively, maintaining all the process factor constant. Plot the graph of Filtrate flux v/s TMP.

Observation table:

Table 5. 8 Cross Flow at the 200 RPM

200 RPM										
Time (min)	**Duration (min)**	**Filtrat (ml)**	**Total Filtrat (ml)**	**Filtrate Rate (ml/min)**	**Filtrat Rate (l/hr)**	**Inlet (psi)**	**Outlet (psi)**	**TMP (psi)**	**TMP (bar)**	**Filtrat Flux (LMH)**
0	0	0	0	0	0	0	0	0	0	0
10	10	90	90	9	0.54	3	1	2	0.14	6.352
20	10	60	150	15	0.9	6	1	3.5	0.24	10.58
30	10	50	200	20	1.2	8	2	5	0.34	14.11
40	10	20	220	22	1.32	10	3	6.5	0.45	15.52
50	10	15	235	23.5	1.41	12	4	8	0.55	16.58
60	10	13	248	24.8	1.488	15	5	10	0.69	17.50
70	10	12	260	26	1.56	18	6	12	0.83	18.35
80	10	10	270	27	1.62	20	6	13	0.9	19.05
90	10	5	275	27.5	1.65	20	7	13.5	0.93	19.41
100	10	5	280	28	1.68	20	8	14	0.97	19.76

Table 5. 9 Cross flow at the 250 RPM

250 RPM										
Time (min)	**Duration (min)**	**Filtrat (ml)**	**Total Filtrat (ml)**	**Filtrate Rate (ml/min)**	**Filtrat Rate (l/hr)**	**Inlet (psi)**	**Outlet (psi)**	**TMP (psi)**	**TMP (bar)**	**Filtrate Flux (LMH)**
0	0	0	0	0	0	0	0	0	0	0
10	10	120	120	12	0.72	3	1	2	0.14	8.470
20	10	60	180	18	1.08	6	1	3.5	0.24	12.70
30	10	50	230	23	1.38	8	2	5	0.34	16.23
40	10	20	250	25	1.5	10	3	6.5	0.45	17.64

50	10	20	270	27	1.62	12	4	8	0.55	19.05
60	10	15	285	28.5	1.71	15	5	10	0.69	20.11
70	10	15	300	30	1.8	18	6	12	0.83	21.17

Table 5. 10: Cross Flow at the 150 RPM

150 RPM										
Time (min)	**Duration (min)**	**Filtrat (ml)**	**Total Filtrat (ml)**	**Filtrate Rate (ml/min)**	**Filtrat Rate (l/hr)**	**Inlet (psi)**	**Outlet (psi)**	**TMP (psi)**	**TMP (bar)**	**Filtrate Flux (LMH)**
0	0	0	0	0	0	0	0	0	0	0
10	10	60	60	6	0.36	3	1	2	0.14	4.2353
20	10	40	100	10	0.6	6	1	3.5	0.24	7.0588
30	10	35	135	13.5	0.81	8	2	5	0.34	9.5294
40	10	25	160	16	0.96	10	3	6.5	0.45	11.294
50	10	20	180	18	1.08	12	4	8	0.55	12.706
60	10	15	195	19.5	1.17	15	5	10	0.69	13.765
70	10	9	204	20.4	1.224	18	6	12	0.83	14.4
80	10	11	215	21.5	1.29	20	6	13	0.9	15.176
90	10	7	222	22.2	1.332	20	7	13.5	0.93	15.671
100	10	5	227	22.7	1.362	20	8	14	0.97	16.024
110	10	5	232	23.2	1.392	22	8	15	1.03	16.376
120	10	3	235	23.5	1.41	24	8	16	1.1	16.588

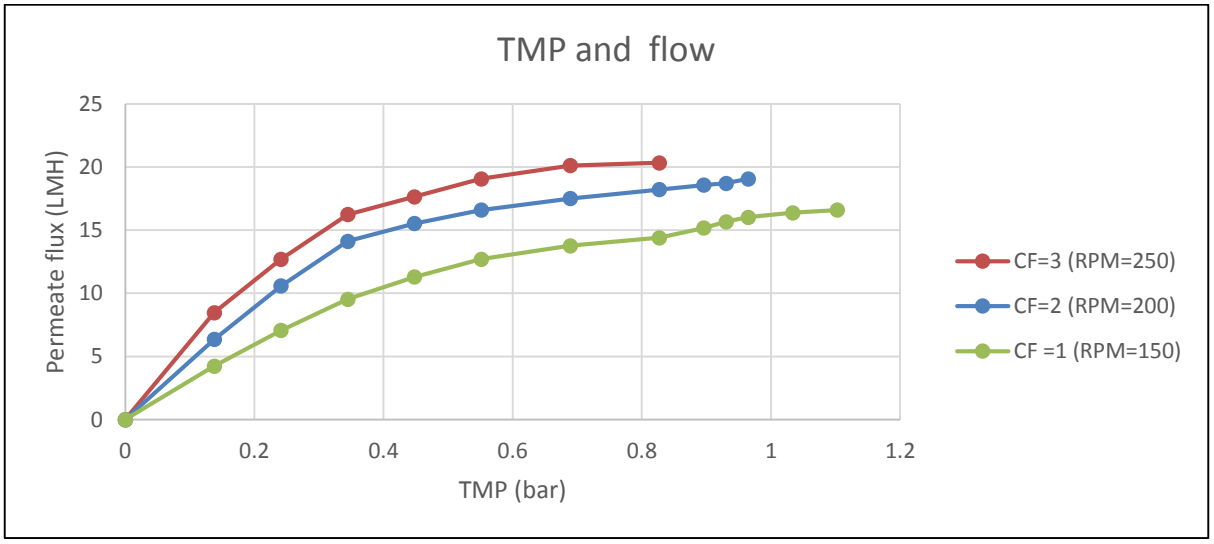


Figure. 20: Relationship between flux and TMP at different cross flow rates.

Result:

Increasing the flow rate, filtrate flux was also increased at particular TMP. But in the gel layer region it was steady state, approximately constant.

Conclusion:

At a particular TMP, increasing the cross flow rate helps to reduce the concentration gradient layer in the cartridge and increase flux. Cross flow rates may be increased until process yield, product quality or process economics are adversely affected through, like shear stress effects. Optimization of a CFF process for the product concentration must include an examination of the interaction of the two most important variables: **cross flow and TMP**.

5.5.3 TMP scouting:

Theory:

TMP scouting (or ***TMP excursions***) is an important part of process optimization. Increasing TMP when ultrafiltering pure water results in a proportional increase in flux. With a process fluid that contains solutes the rate of increase in flux drops as the TMP increases and the concentration gradient restricts passage of liquid through the filter. At high TMP values, formation of a gel layer effectively blocks the filter and no further increase in flux is seen. Higher cross flow rates help to prevent the formation of a gel layer, allowing higher flux rates to be achieved before the flux becomes independent of TMP. TMP scouting involves measuring the interdependence of cross flow rate, TMP and flux in order to determine the optimum conditions for filtration, where flux is high but is still dependent on TMP.

Procedure:

The standard procedure is to perform a TMP scouting experiment in which a series of TMP set points (6) is measured at different cross flow rates (in this case 3) for particular time period (approximately 30 min). From these experiments the effect on flux is evaluated, and optimal cross flow and TMP may be identified.

Run the TFF system with liposomal solution (1L) at the flow rate approximately 50 ml/min (Watson marlow pump about 200 rpm). Increasing the inlet pressure with the time period and subsequently increase the outlet pressure. Optimize the Filtrate rate against TMP at particular time period. (Approximately 30 min at the concentration factor 1X and after 3X). Calculate the filtrate flux of the sample at constant surface area of 500kd cartridge (0.085 m^2).Flux is measured at 6 TMP points and 3 cross flow rates with permeate recycled to the feed reservoir to maintain a steady state. Repeat the same process with another sample of liposome at the different rpm 150 and 250 respectively, maintaining all the process factor constant. Plot the graph of Filtrate flux v/s TMP of all the filtration process.

- Initial concentration = 1X and target concentration = 2.5X

Table 5. 11: ***Low concentration (1x) at 200 rpm cross-flow***

LOW CONCENTRATION (1X) 200 RPM										
Time (min)	**Duration (min)**	**Filtrat (ml)**	**Total Filtrat (ml)**	**Filtrate Rate (ml/min)**	**Filtrate Flow Rate (L/Hr)**	**Pressure Drop(psi)**		**TMP (psi)**	**TMP (bar)**	**Filtrat Flux (LMH)**
						Inlet	**Outlet**			
0	0	0	0	0	0			0	0	0
5	5	90	90	18	1.08	7	1	4	0.276	12.70
10	5	110	200	20	1.2	10	2	6	0.414	14.11
15	5	135	335	22.333	1.34	12	4	8	0.552	15.76
20	5	148	483	24.15	1.449	14	5	9.5	0.655	17.04
25	5	155	638	25.52	1.531	16	6	11	0.758	18.01
30	5	170	808	26.933	1.616	18	8	13	0.896	19.01
									Avg :	16.11

Table 5. 12 : ***Low concentration (1x) at 150 rpm cross-flow***

LOW CONCENTRATION (1X) 150 RPM										
Time (min)	**Duration (min)**	**Filtrat (ml)**	**Total Filtrat (ml)**	**Filtrat Rate (ml/min)**	**Filtrat Flow Rate (L/Hr)**	**Pressure Drop (psi)**		**TMP (psi)**	**TMP (bar)**	**Filtrat Flux (LMH)**
						Inlet	**Outlet**			
0	0	0	0	0	0	0	0	0	0	0
5	5	60	60	12	0.72	7	1	4	0.276	8.470
10	5	85	145	14.5	0.87	10	2	6	0.414	10.23
15	5	102	247	16.467	0.988	12	4	8	0.552	11.62
20	5	120	367	18.35	1.101	14	5	9.5	0.655	12.95
25	5	128	495	19.8	1.188	16	6	11	0.758	13.97
30	5	135	630	21	1.26	18	8	13	0.896	14.82
									Avg :	12.01

Table 5. 13: ***Low concentration (1x) at 250 rpm cross-flow***

LOW CONCENTRATION (1X) 250 RPM										
Time (min)	**Duration (min)**	**Filtrat (ml)**	**Total Filtrat (ml)**	**Filtrate Rate (ml/min)**	**Filtrat Flow Rate (L/Hr)**	**Pressure Drop (psi)**		**TMP (psi)**	**TMP (bar)**	**Filtrat Flux (LMH)**
						Inlet	**Outlet**			
0	0	0	0	0	0	0	0	0	0	0
5	5	120	120	24	1.44	7	1	4	0.276	16.94
10	5	145	265	26.5	1.59	10	2	6	0.414	18.70
15	5	162	427	28.467	1.708	12	4	8	0.552	20.09
20	5	185	612	30.6	1.836	14	5	9.5	0.655	21.6
25	5	205	817	32.68	1.961	16	6	11	0.758	23.06
30	5	220	1037	34.567	2.074	18	8	13	0.896	24.4
									Avg:	20.80

Table 5. 14 : **High concentration (2.5x) at 200 rpm cross flow**

HIGH CONCENTRATION (2.5X) 200 RPM										
Time (min)	Duration (min)	Filtrat (ml)	Total Filtrat (ml)	Filtrate Rate (ml/min)	Filtrat Flow Rate (L/Hr)	Pressure Drop (psi)		TMP (psi)	TMP (bar)	Filtrat Flux (LMH)
						Inlet	Outlet			
0	0	0	0	0	0	0	0	0	0	0
5	5	60	60	12	0.72	7	1	4	0.276	8.470
10	5	85	145	14.5	0.87	10	2	6	0.414	10.23
15	5	110	255	17	1.02	12	4	8	0.552	12
20	5	125	380	19	1.14	14	5	9.5	0.655	13.41
25	5	140	520	20.8	1.248	16	6	11	0.758	14.68
30	5	150	670	22.333	1.34	18	8	13	0.896	15.76
									Avg :	12.43

Table 5. 15: **High concentration (2.5x) at 150 rpm cross flow**

HIGH CONCENTRATION (2.5X) 150 RPM										
Time (min)	Duration (min)	Filtrat (ml)	Total Filtrat (ml)	Filtrate Rate (ml/min)	Filtrat Flow Rate (L/Hr)	Pressure Drop (psi)		TMP (psi)	TMP (bar)	FiltratFlux (LMH)
						Inlet	Outlet			
0	0	0	0	0	0	0	0	0	0	0
5	5	40	40	8	0.48	7	1	4	0.276	5.647
10	5	55	95	9.5	0.57	10	2	6	0.414	6.705
15	5	70	165	11	0.66	12	4	8	0.552	7.764
20	5	85	250	12.5	0.75	14	5	9.5	0.655	8.823
25	5	110	360	14.4	0.864	16	6	11	0.758	10.16
30	5	120	480	16	0.96	18	8	13	0.896	11.29
									Avg :	8.400

Table 5. 16: **High concentration (2.5x) at 250 rpm cross flow**

HIGH CONCENTRATION (2.5X) 250 RPM										
Time (min)	Duration (min)	Filtrat (ml)	Total Filtrat (ml)	Filtrate Rate (ml/min)	Filtrat Flow Rate (L/Hr)	Pressure Drop(psi)		TMP (psi)	TMP (bar)	Filtrat Flux (LMH)
						Inlet	Outlet			
0	0	0	0	0	0			0	0	0
5	5	50	50	10	0.6	7	1	4	0.276	7.058
10	5	115	165	16.5	0.99	10	2	6	0.414	11.64
15	5	125	290	19.333	1.16	12	4	8	0.552	13.65
20	5	142	432	21.6	1.296	14	5	9.5	0.655	15.25
25	5	155	587	23.48	1.409	16	6	11	0.758	16.57
30	5	170	757	25.233	1.514	18	8	13	0.896	17.81
									Avg :	13.66

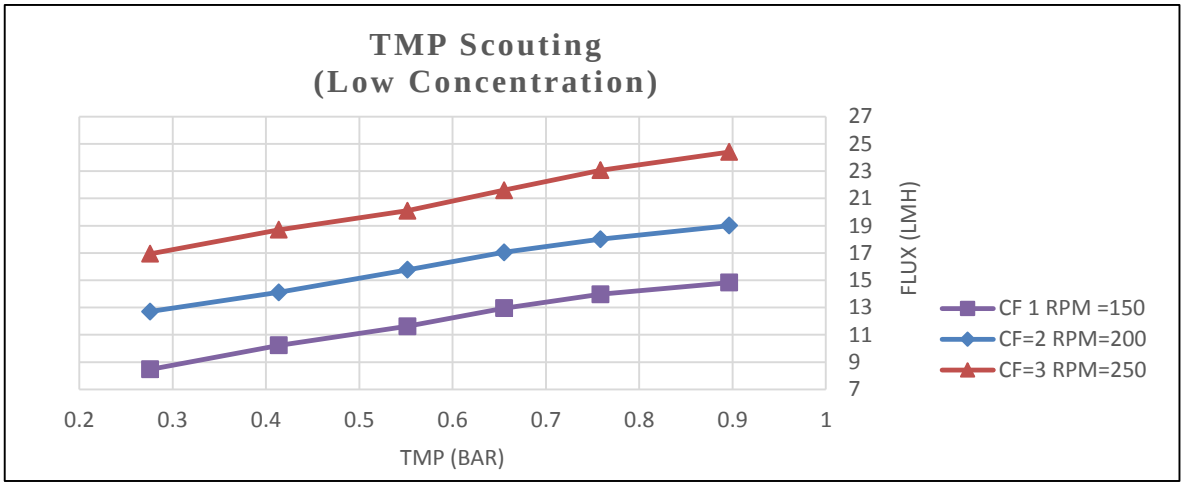


Figure. 10 : TMP scouting results at different cross flow rate at the low concentration

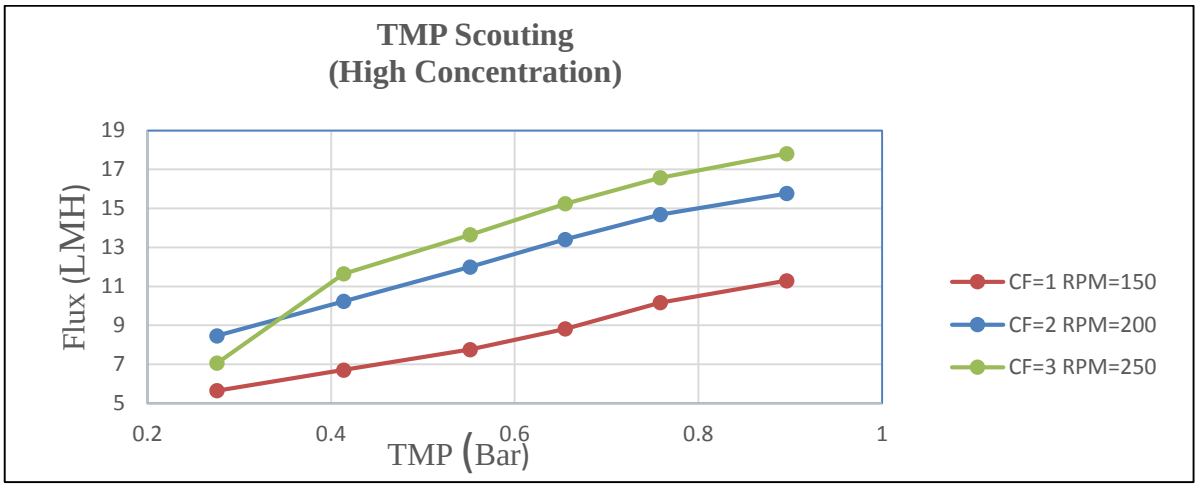


Figure. 11: TMP scouting results at different cross flow rate at the low concentration

Result:

At the low concentration (1X), flux increases with TMP at all cross flow rates (40, 50, 60 ml/min). 0.4 to 0.8 bar (6-12 psi) TMP significant filtrate flux obtain (10 – 20 LMH)**.** At the high concentration (2.5X), drastically increase the flux at the high TMP(>0.8 bar).So, to maintain the constant flux rate throughout process optimal design space set at the linear portion of the flux v/s TMP graph. Approximately 0.4 to 0.7 bar (6 -11psi) TMP produce flux 10 -15 LMH.

Conclusion:

It is possible to design a process control scheme that maintains a high flux value (12-20 LMH) for a reasonable process time (4-5 hr) for 1 liter batch and stable process conditions.

5.5.4 Process time (Optimization of Concentration mode)

Total Process time of the filtration is an important part of process optimization also. In a concentration process, the optimized cross flow and TMP conditions established above can be used to identify the diafiltration point (the point which provides the fastest buffer exchange), and optimal buffer consumption.

Procedure:

Run the TFF system with liposomal solution (1L) at the flow rate approximately 50 ml/min (Watson marlow pump about 200 rpm).Increasing the inlet pressure with the time period and subsequently increase the outlet pressure to maintain the TMP 5-6 psi. Optimize the Filtrate rate against TMP at particular time period. (Approximately 1-2 hr from the concentration factor 1X to 2X, 3X and after 4X).Calculate the filtrate flux of the sample at constant surface area of 500 kd cartridge (0.085 m^2).Plot the graph of Filtrate flux v/s concentration factor, concentration factor * flux v/s concentration factor of all the filtration process.

- Initial concentration =1X; Target concentration = 2X, 2.5X, 3X, 3.5X, 4X.

Observation table:

*Table 5. 17 : **Optimization of Flow rate in concentration mode***

Mode	Time (min)	Duration (min)	Filtrat (ml)	Total Filtrate (ml)	Filtrate Rate (ml/min)	Filtrate Flow Rate (L/Hr)	Filtrate Flux (LMH)	Average Flux (LMH)	Retentate (ml)
1X	0	0	0	0	0	0	0	0	1000
2X	6	6	100	100	16.66	1	11.764	12.228	900
	11	5	100	200	18.18	1.090	12.834		800
	16	5	100	300	18.75	1.125	13.235		700
	23	7	100	400	17.39	1.0434	12.276		600
	32	9	100	500	15.625	0.9375	11.029		500
2.5X	35	3	25	525	15	0.9	10.588	9.6028	475
	39	4	25	550	14.102	0.8461	9.9547		450
	44	5	25	575	13.068	0.7840	9.2245		425
	49	5	25	600	12.244	0.734	8.6434		400
3X	58	9	25	625	10.775	0.6465	7.6065	6.9358	375
	67	9	25	650	9.7015	0.5821	6.8481		350
	75	8	25	675	9	0.54	6.3529		325
3.5 X	90	15	25	700	7.7778	0.4667	5.4902	5.1821	300
	105	15	25	725	6.9048	0.4143	4.874		275

4X	130	25	25	750	5.7692	0.3462	4.0724	4.0724	250

Table 5. 18 ***Optimization of Transmembrane Pressure (TMP)***

Mode	Time (min)	VRR	VRF	CF	Pressure Drop (psi)		TMP (psi)	TMP (bar)	Conductivity (mS/cm)	Conc Factor * Flux
					Inlet	Outlet				
1X	0	1	0	1	0	0	0	0	33.4	0
2X	6	0.9	0.1	1.11	7.5	2	4.75	0.32	32.5	13.07
	11	0.8	0.2	1.25						16.04
	16	0.7	0.3	1.43						18.90
	23	0.6	0.4	1.67						20.46
	32	0.5	0.5	2						22.05
2.5X	35	0.475	0.525	2.10	8	2	5	0.34	31.8	22.29
	39	0.45	0.55	2.22						22.12
	44	0.425	0.575	2.35						21.70
	49	0.4	0.6	2.5						21.60
3X	58	0.375	0.625	2.67	8	2	5	0.34	31	20.28
	67	0.35	0.65	2.85						19.57
	75	0.325	0.675	3.08						19.54
3.5 X	90	0.3	0.7	3.33	9	2	5.5	0.37	30.7	18.30
	105	0.275	0.725	3.64						17.72
4X	130	0.25	0.75	4	9	3	6	0.40	30.4	16.29

(VRR=Volume Reduction Ratio, VRR= Final volume / Initial volume

VRF=Volume reduction factor, VRF =Initial VRR- Final VRR

CF=Concentration factor

Concentration Factor = 1/(1- Y) ; Y = Percentage recovery expressed as a decimal)

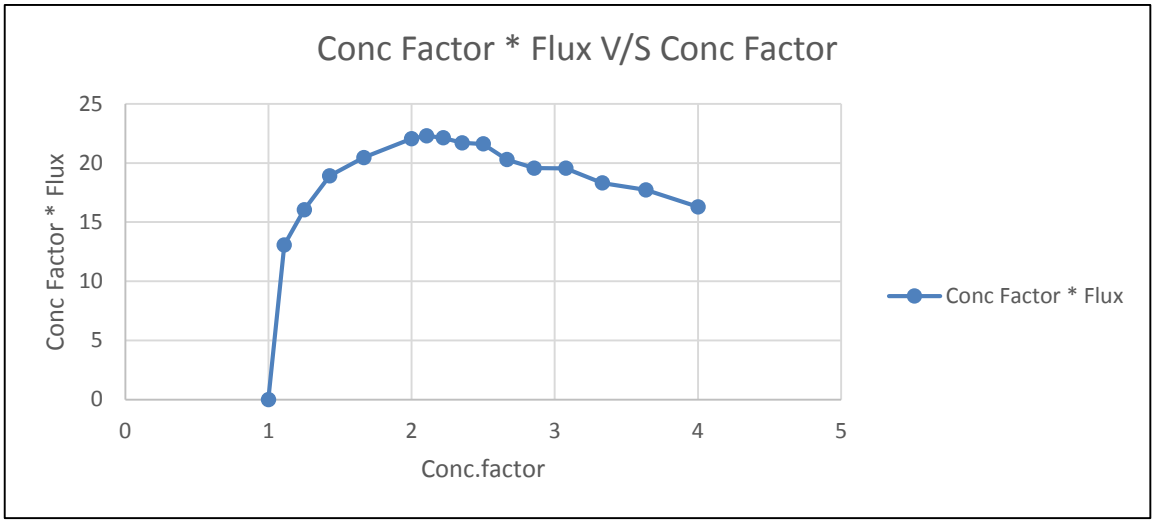


Figure. 12: Diafiltration time optimization.

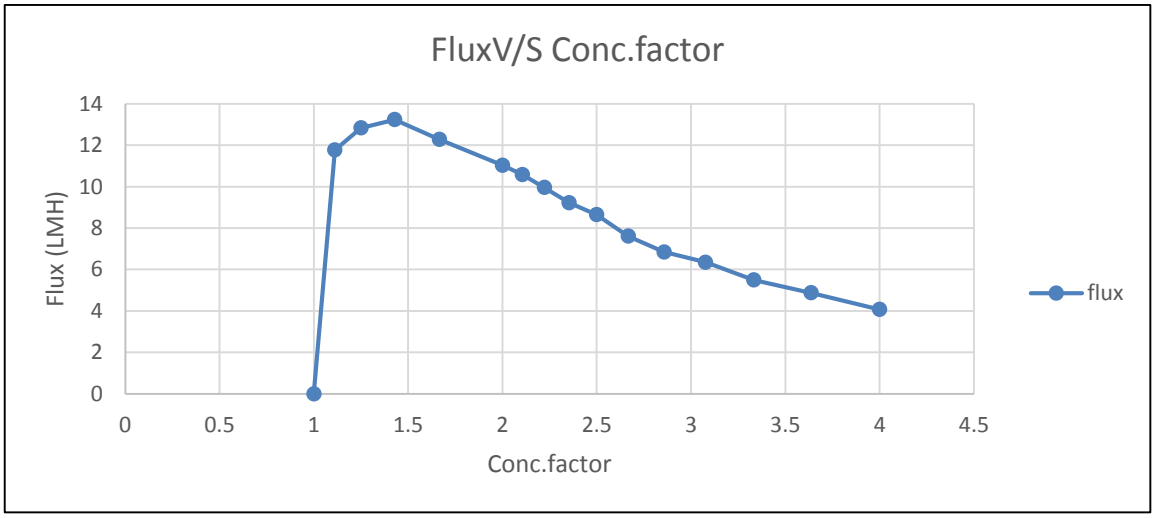


Figure. 13: Filtrate flux rate at the different concentration factor in the concentration mode

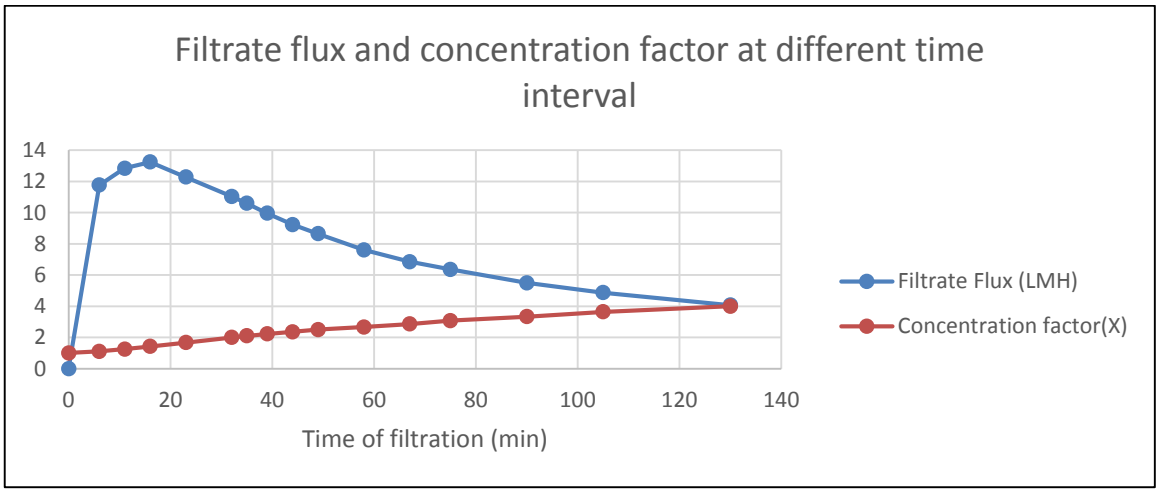


Figure. 14: Filtrate flux and concentration factor at different time interval

Result:

In the concentration mode of the TFF, flux was increased from the 1X to approximately 1.5 X concentration and then after it was subsequently decrease till 3X concentration, further drastically decrease till 4X concentration in this liposome case. Another parameter called as **concentration factor * flux** also important for the concentration mode. It was increased from 1X to 2X, then after steady state till 3X. After 3X it was decreased till 4X.

Conclusion:

The highest value on the y axis at the highest concentration represents the fastest diafiltration with the lowest buffer consumption. In this example, diafiltration takes the same time if performed at 2.5 times or 3 times concentration, because the decrease in retentate volume at 3 times concentration is offset by the decrease in flux.

5.5.5 Optimization of the Diafiltration mode

Procedure:

Run the TFF system with liposomal solution (1L) in the reservoir at the flow rate approximately 50 ml/min (Watson marlow pump about 200 rpm).Increasing the inlet pressure with the time period and subsequently increase the outlet pressure to maintain the TMP 5-8 psi. Optimize the Filtrate rate against TMP at particular time period. (Approximately 4-5 hr from the concentration factor 1X). Diafilter with the buffer solution (10 % sucrose solution).Calculate the filtrate flux of the sample at constant surface area of 500 kd cartridge (0.085 m^2).Measure the conductivity of the filtrate of the each Diafiltration step. Calculate the % salt removed from the feed solution. Plot the graph of Diafiltration Volume v/s % permeable salt removed of all the filtration process.

- **Initial concentration =1X ;Target concentration = 1X**
- **Initial Volume =1000 ml; Diafiltration Volume= 11 volume**
- **Initial Conductivity of feed (mS/cm) = 33.45**
- **Final Conductivity of feed (mS/cm) = 0.01**
- **Observation table:**

Table 5. 19 : ***Optimization of flow rate in diafiltration mode:***

DV (N)	Time (min)	Time (hr)	Duration (hr)	Filtrat (ml)	Total Filtrat (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrat Flux (LMH)	Averag Flux (LMH)	Retentate (ml)
0	0	0.00	0.00	0	0	0.00	0.00	0.00	0	1000
1	72	1.20	1.20	1000	1000	13.89	0.83	9.80		1000
2	145	2.42	1.22	1000	2000	13.79	0.83	9.74		1000
3	190	3.17	0.75	1000	3000	15.79	0.95	11.15		1000
4	225	3.75	0.58	1000	4000	17.78	1.07	12.55		1000
5	265	4.42	0.67	1000	5000	18.87	1.13	13.32		1000
6	285	4.75	0.33	1000	6000	21.05	1.26	14.86		1000
7	300	5.00	0.25	1000	7000	23.33	1.40	16.47	12.55	1000
8	320	5.33	0.33	1000	8000	25.00	1.50	17.65		1000
9	334	5.57	0.23	1000	9000	26.95	1.62	19.02	13.84	1000
10	340	5.67	0.10	1000	10000	29.41	1.76	20.76		1000
11	350	5.83	0.17	1000	11000	31.43	1.89	22.18	15.23	1000

Table 5. 20 : ***Optimization of Transmembrane Pressure (TMP)***

DV (N)	Time (min)	Time (hr)	Pressure (psi)		TMP (psi)	TMP (bar)	Conductivity of filtrate (mS/cm)	% salt in the filtrate	% salt reduction from the feed
			Inlet	Outlet					
0	0	0	0	0	0	0	0	0	0
1	72	1.20	8	2	5	0.340	24.5	73.24	26.76
2	145	2.42	8	2	5	0.340	11.5	34.38	65.62
3	190	3.17	10	5	7.5	0.510	7.8	23.32	76.68
4	225	3.75	10	5	7.5	0.510	2.3	6.88	93.12
5	265	4.42	10	5	7.5	0.510	0.65	1.94	98.06
6	285	4.75	10	5	7.5	0.510	0.32	0.96	99.04
7	300	5.00	10	5	7.5	0.510	0.16	0.48	99.52
8	320	5.33	10	5	7.5	0.510	0.08	0.24	99.76
9	334	5.57	12	3	7.5	0.510	0.05	0.15	99.85
10	340	5.67	12	3	7.5	0.510	0.03	0.09	99.91
11	350	5.83	13	2	7.5	0.510	0.02	0.06	99.94

DV= Diafiltration Volume

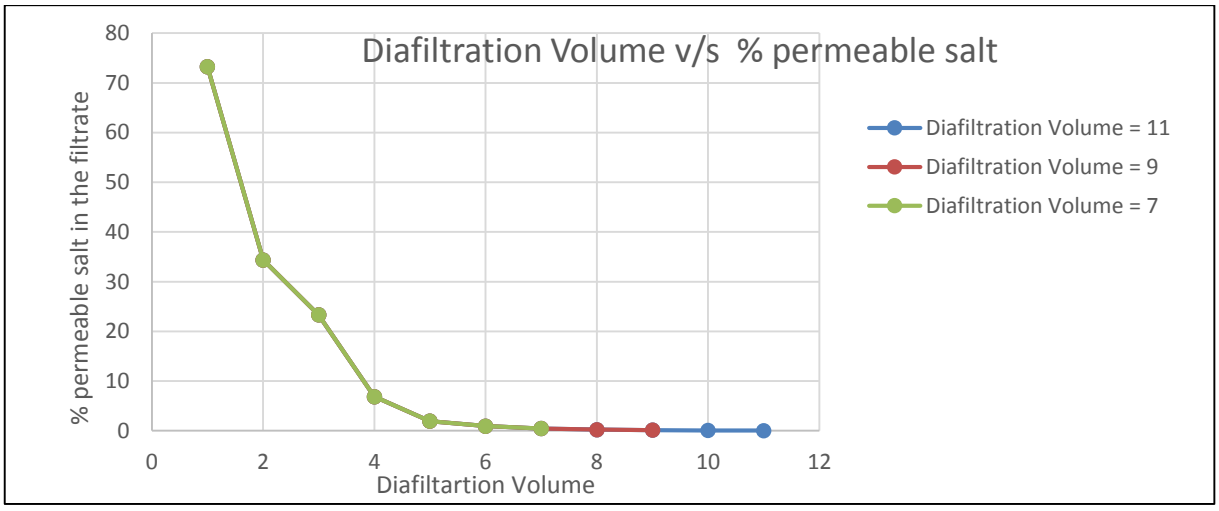


Figure. 15: ***Scale for the diafiltration volume v/s % permeable salts***

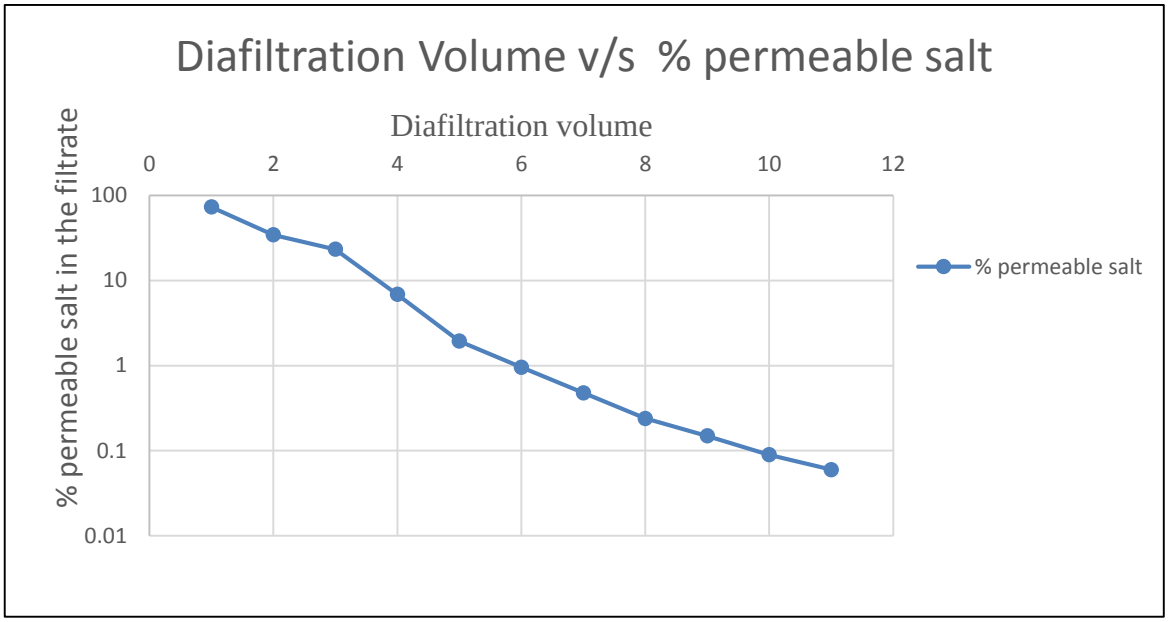


Figure. 16 : ***Semi logarithmic scale for the diafiltration volume v/s permeable salts***

Result:

In the Diafiltration mode of the TFF, Conductivity of the filtrate was decreased from the 1 DV to approximately 11 DV; it means the ammonium salt was reduced from the feed solution, furthermore after 8 DV salt reduced from the feed > 99.5 %.

Conclusion: After 6 diafiltration volume more than 99 % of the salt remove from the feed, and up to 10 diafiltration volume maximum 99.90 % of the salt reduced. So 8 DV was appropriate for the process to reduce the time and buffer consumption.

5.6 Risk assessment

Risk identification and risk analysis are two basic components of risk assessment as outlined in the ICH Q9 document. The goal of these two assessments is to obtain the highest risk factors that will be subjected to a more complex DOE study to establish a product or process design space. In the current study, Liposome encapsulation efficiency was the major response for very critical product qualities and an understanding and awareness of the potential risks is very important. To accomplish this, cause-and-effect diagrams (Ishikawa diagram) were constructed to identify the potential causes of product variability, as shown in Figs.

Selection of Variable for Design of Experiment

Table 5. 21 ***Experimental conditions for the screening.***

Sr No.	Independent Variable (Factor)	Type	Lower limit	Upper limit	Unit
1	Transmembrane Pressure (TMP)	Process	3	10	PSI
2	Cross-flow velocity	Process	40	60	ml/min
3	Buffer concentration	Formulation	8	12	% w/v
4	Number of Diafiltration Volume	Process	7	11	N
5	Concentration Factor	Process	1	3	X
6	Temperature	Process	10	25	^{0}C
7	Surface area of the membrane cartridge	Process	0.085	0.28	m^2

Risk analysis of the Encapsulation efficiency:

Ishikawa diagram was generated to identify quality attributes for the better performance of developed liposomal suspension formulation by injection method. Liposome concentration, Transmembrane pressure (TMP), Diafiltration volume, membrane molecular-weight cut of (MWCO), cross-flow velocity were found to be critical quality attributes of the ultrafiltration separation technique. Factors influencing these quality attributes were identified as critical process parameters (CPP) and further risk assessment of CPP were

evaluated using placket-burman screening design approach with the help of regression analysis and contour plots.

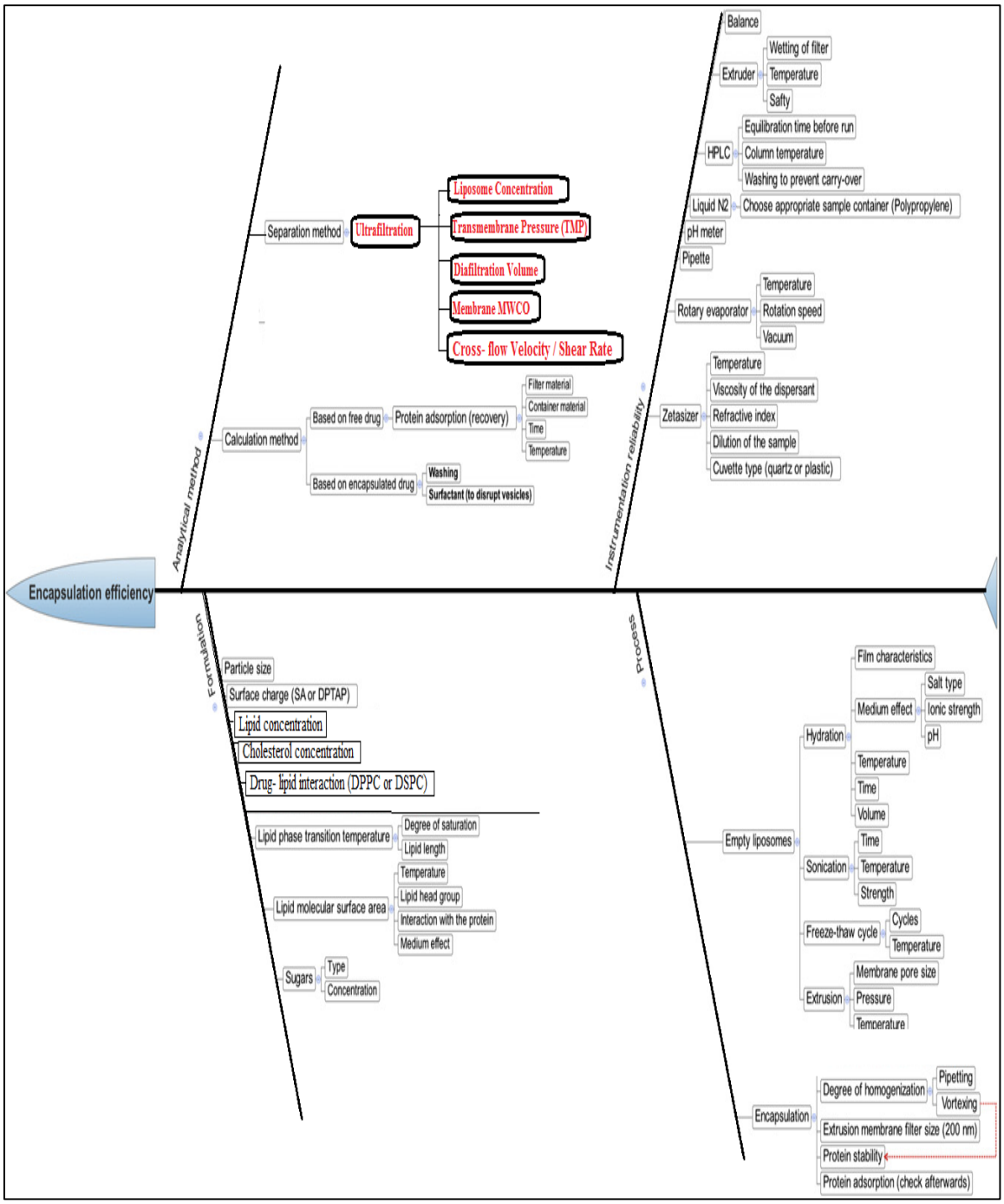


Figure. 17 : ***An Ishikawa diagram illustrating factors that may have impact on the Encapsulation efficiency of liposome*** [59]

5.7 Plackett-Burman screening study

This study was used to screen various factors including Transmembrane Pressure(TMP) (X1), Feed flow(X2), Concentration factor(X3), Surface Area (X4), Temperature (X5), Buffer Concentration (X6), Diafiltration Volume (X7).These factors were assessed to be of high importance (risk) compared with others based on the risk analysis. The first 7 columns of the Plackett–Burman design table were used for the screening study with each factor evaluated at low (−1) and high (+1) levels. The selection of the low and high values was based on the preliminary study results.

Plackett-Burman Design (PBD) with factor and responses

Plackett-Burman Design (PBD)			
Factors: 7	Response: 5	Base blocks: 1	Total blocks: 1
Base runs: 15	Total runs: 15	Center points: 3	Replicates: 1

Table 5. 22 : ***Placket-Burman study design table and results****.*

(X1: Transmembrane Pressure (TMP); X2: Feed flow rate; X3: Concentration factor of the feed; X4: Surface area of the cartridge; X5: temperature; X6: Buffer concentration; X7: Diafiltration volume.)

ID	X1 (psi)	X2 (ml/ min)	X3 (X)	X4 (m^2)	X5 (^{0}C)	X6 (% W/ V)	X7 (N)	Flux (LMH)	% Salt Reduced	Filtration time (hr)	Volume of Buffer (L)	Osmolarity (mOsmol)
PB-1	3	60	1	0.085	10	12	10	7.5	99.9	7.5	10	310
PB-2	3	60	3.5	0.085	25	8	7	4.5	98.9	5.2	2	277
PB-3	3	40	3.5	0.28	25	8	10	3.8	99	4.8	2	275
PB-4	10	40	3.5	0.085	10	8	10	10	99.4	4.2	3	270
PB-5	10	40	3.5	0.28	10	12	7	8.5	98.8	2	2	305
PB-6	6.5	50	2.3	0.085	17.5	10	8.5	10	99.6	4.6	4.2	285
PB-7	6.5	50	2.3	0.085	17.5	10	8.5	10	99.8	4.6	4.3	280
PB-8	3	60	3.5	0.28	10	12	10	4.1	99.8	5	3	315
PB-9	10	60	1	0.28	10	8	7	18.5	99.1	3.2	7	278
PB-10	3	40	1	0.085	10	8	7	5.8	99	5.5	7	270
PB-11	3	40	1	0.28	25	12	7	5	99.2	4.8	7	295
PB-12	10	60	3.5	0.085	25	12	7	12.5	98.6	2.5	2	312
PB-13	10	40	1	0.085	25	12	10	18.5	99.8	4.5	10	308
PB-14	10	60	1	0.28	25	8	10	19	99.6	4	10	280
PB-15	6.5	50	2.3	0.085	15	10	9	10.5	99.6	4.6	4.2	287

To evaluate the potential curvature, 3 center points were added. The responses evaluated include Filtrate flux (LMH) (Liter/m^2/hr) (Y1), % salt Reduced (Y2), Time filtration (hr) (Y3), Volume of buffer (L) (Y4), Osmolarity (mOsmol) (Y5). Multi-linear regression and ANOVA were performed to analyze the data, and a series of Pareto charts were constructed by the minitab17 to demonstrate the influence of each parameter on the responses.

Results:

Influence of various factors on Flux:

7 high risk factors were identified in a risk analysis study to have potential impact on liposomal filtrate flux As shown in Table, flux varied from 3.8 LMH (PB-3) to19 LMH (PB-14) for the various factor combinations. The most significant factors were TMP, Feed Flow and concentration factor ($p < 0.05$) relative to other factors influencing flux as shown in Table 5.23.

Table 5. 23 ***ANOVA table for the flux:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value (> 0.05)**	**Model Significant/Non-Significant**
Model	8	376.127	47.016	27.40	0.000	**Significant**
Linear	7	375.819	53.688	31.28	0.000	**Significant**
TMP	1	264.141	264.141	153.92	0.000	**Significant**
Feed Flow	1	17.521	17.521	10.21	0.019	**Significant**
Concentration factor	1	79.568	79.568	46.37	0.000	**Significant**
Surface Area	1	0.001	0.001	0.00	0.983	**Non-Significant**
Temperature	1	6.601	6.601	3.85	0.098	**Non-Significant**
Buffer Concentration	1	2.521	2.521	1.47	0.271	**Non-Significant**
Diafiltration Volume	1	5.467	5.467	3.19	0.125	**Non-Significant**
Curvature	1	0.292	0.292	0.17	0.694	**Non-Significant**
Error	6	10.297	1.716			
Lack-of-Fit	4	10.130	2.533	30.39	0.032	
Pure Error	2	0.167	0.083			
Total	14	386.424				

In Table 5.24, the “Effect” column determines each factor’s relative strength, the higher the absolute value the greater the effect of that factor on the response. A positive effect value indicates an effect that favors the response, and a negative value represents an inverse

relationship between the response and the factor. In this study, the results indicated that both increase in TMP and feed flow, and decrease in the concentration factor of the feed would contribute to higher flux value, and TMP had a more dominant effect.

The prediction confidence level of the model was 76.31% and a good correlation was obtained between the observed and predicted values as indicated by the r2 value of 0.9734. Further analysis using ANOVA indicated a significant effect of variables on the response (Flux) ($p < 0.05$) and no curvature was observed ($p > 0.05$). [60]

Table 5. 24 ***Estimated effects and coefficients for Flux:***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		9.808	0.378	25.94	0.000	
TMP	9.383	4.692	0.378	12.41	0.000	1.00
Feed Flow	2.417	1.208	0.378	3.20	0.019	1.00
Concentration factor	-5.150	-2.575	0.378	-6.81	0.000	1.00
Surface Area	0.017	0.008	0.378	0.02	0.983	1.20
Temperature	1.483	0.742	0.378	1.96	0.098	1.02
Buffer Concentration	-0.917	-0.458	0.378	-1.21	0.271	1.00
Diafiltration Volume	1.350	0.675	0.378	1.78	0.125	1.02
Ct Pt		0.389	0.943	0.41	0.694	1.24

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
1.31000	97.34%	93.78%	76.31%

Regression Equation in Uncoded Units

Flux (LMH) =

-3.59 + 1.340 TMP + 0.01208 Feed Flow

- 2.060 Concentration factor+ 0.09 Surface Area + 0.0989 Temperature

- 0.229 Buffer Concentration+ 0.450 Diafiltration Volume + 0.389 Ct Pt

Regression Equation in Coded Units

$Y_1=B_0 + B_1X_1+ B_2X_2 + B_3X_3 + B_4X_4 + B_5X_5 + B_6X_6 + B_7X_7 + \varepsilon$

Where, β_0 = intercept

$\beta_1$to β_7 = co – efficient; X_1 to X_7 = variable; ε = error

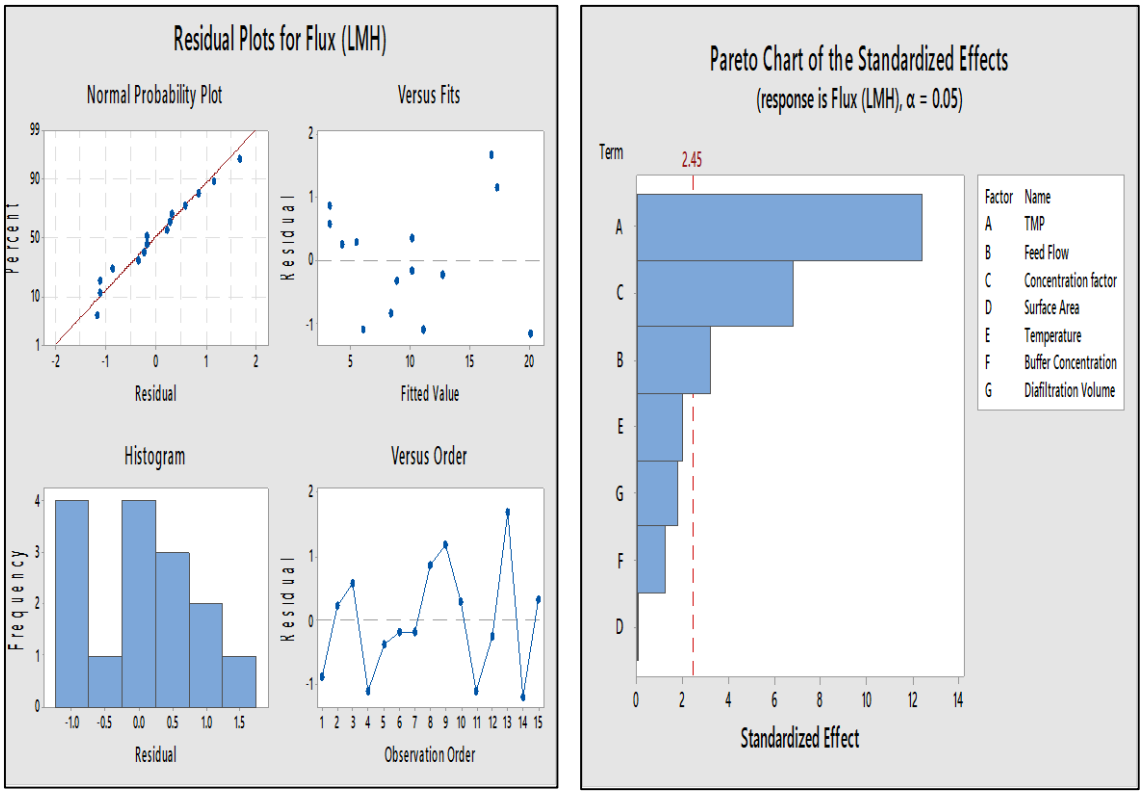


Figure. 18 : ***Residual plot and Pareto chart of the Flux (LMH)***

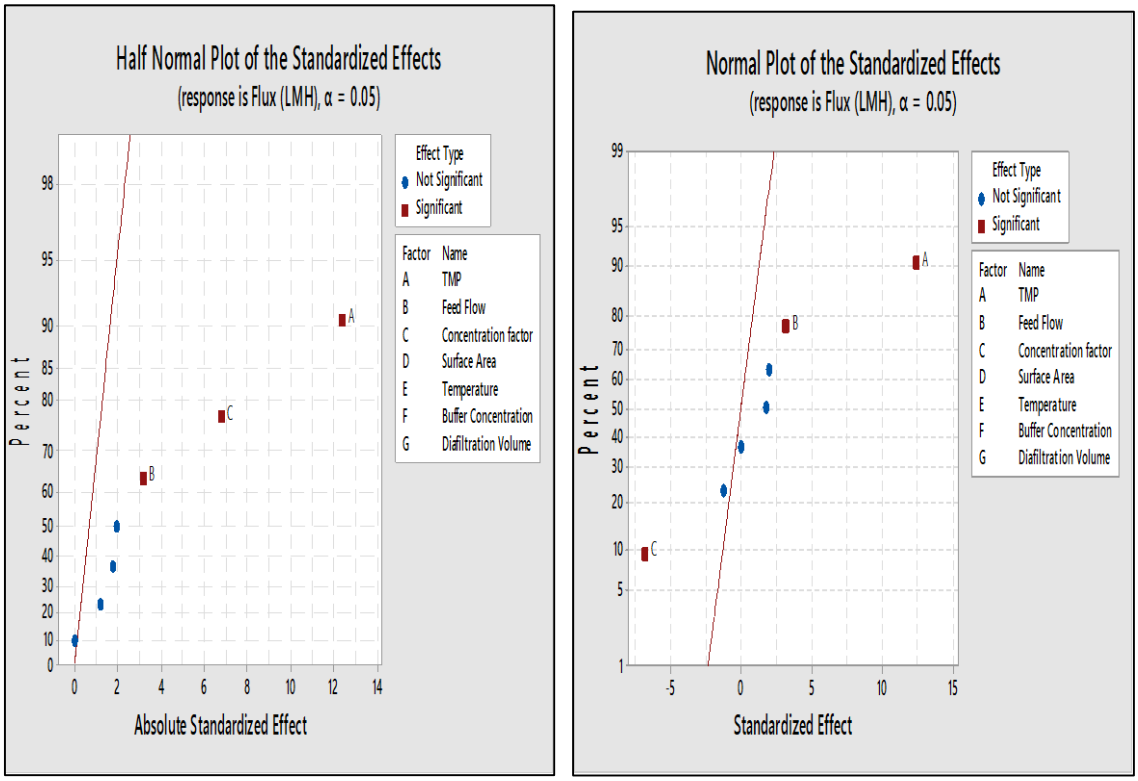


Figure. 19: ***Half Normal and Normal Effects Plot for Flux (LMH)***

Influence of various factors on % salt reduction:

The influence of various factors on **% salt reduction** was analyzed. The % salt reduced varied from 98.6 (PB-12) to 99.9 % (PB-1) for the various factor combination. The most significant factor was diafiltration volume and concentration factor ($p < 0.05$) compared with other factors which influenced the % salt reduced shown in Table 5.25. The salt reduction from all the trail was greater than 98.5 %. So the diafiltration volume minimum 7 would produce the satisfactory salt reduction, but for better result it should up to 8.

*Table 5. 25: **ANOVA table for the % salt reduction:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-value**	**P -Value**	**Model Significant/ Non-Significant**
Model	8	2.26600	0.28325	7.39	0.013	**Significant**
Linear	7	1.86583	0.26655	6.95	0.015	**Significant**
TMP	1	0.02083	0.02083	0.54	0.489	**Non-Significant**
Feed Flow	1	0.04083	0.04083	1.07	0.342	**Non-Significant**
Concentration factor	1	0.36750	0.36750	9.59	0.021	**Significant**
Surface Area	1	0.00083	0.00083	0.02	0.888	**Non-Significant**
Temperature	1	0.06750	0.06750	1.76	0.233	**Non-Significant**
Buffer Concentration	1	0.10083	0.10083	2.63	0.156	**Non-Significant**
Diafiltration Volume	1	1.26750	1.26750	33.07	0.001	**Significant**
Curvature	1	0.13714	0.13714	3.58	0.107	**Non-Significant**
Error	6	0.23000	0.03833			
Lack-of-Fit	4	0.20333	0.05083	3.81	0.218	
Pure Error	2	0.02667	0.01333			
Total	14	2.49600				

In table 5.26 the “Effect” column show that both increase in diafiltration volume and decrease in concentration factor of the feed would contribute to increase the % salt reduced, and diafiltration volume had a more dominant effect to the salt reduction process.

The prediction confidence level of the model was 24.28 % and a good correlation was obtained between the observed and predicted values as indicated by the r2 value of 0.9079. Further analysis using ANOVA indicated a significant effect of variables on the response (% salt reduction) ($p < 0.05$) and no curvature was observed ($p > 0.05$).

*Table 5. 26 : **Estimated effects and coefficients for % salt reduction:***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		99.2583	0.0565	1756.18	0.000	
TMP	-0.0833	-0.0417	0.0565	-0.74	0.489	1.00
Feed Flow	0.1167	0.0583	0.0565	1.03	0.342	1.00
Concentration factor	-0.3500	-0.1750	0.0565	-3.10	0.021	1.00
Surface Area	-0.0167	-0.0083	0.0565	-0.15	0.021	1.00
Temperature	-0.1500	-0.0750	0.0565	-1.33	0.233	1.02
Buffer Concentration	0.1833	0.0917	0.0565	1.62	0.156	1.00
Diafiltration Volume	0.6500	0.3250	0.0565	5.75	0.001	1.02
Ct Pt		0.267	0.141	1.89	0.107	1.24

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R-sq (pred)**
0.195789	90.79%	78.50%	24.28%

Regression Equation in Uncoded Units

% salt reduced =

97.250 - 0.0119 TMP + 0.000583 Feed Flow - 0.1400 Concentration factor +

0.085 Surface Area - 0.01000 Temperature + 0.0458 Buffer Concentration

+ 0.2167 Diafiltration Volume + 0.267 Ct Pt

Regression Equation in Coded Units

$Y_2 = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_5X_5 + B_6X_6 + B_7X_7 + \varepsilon$

Where, β_0 = intercept

β_1 to β_7 = co – efficient; X_1 to X_7 = variable; ε = error

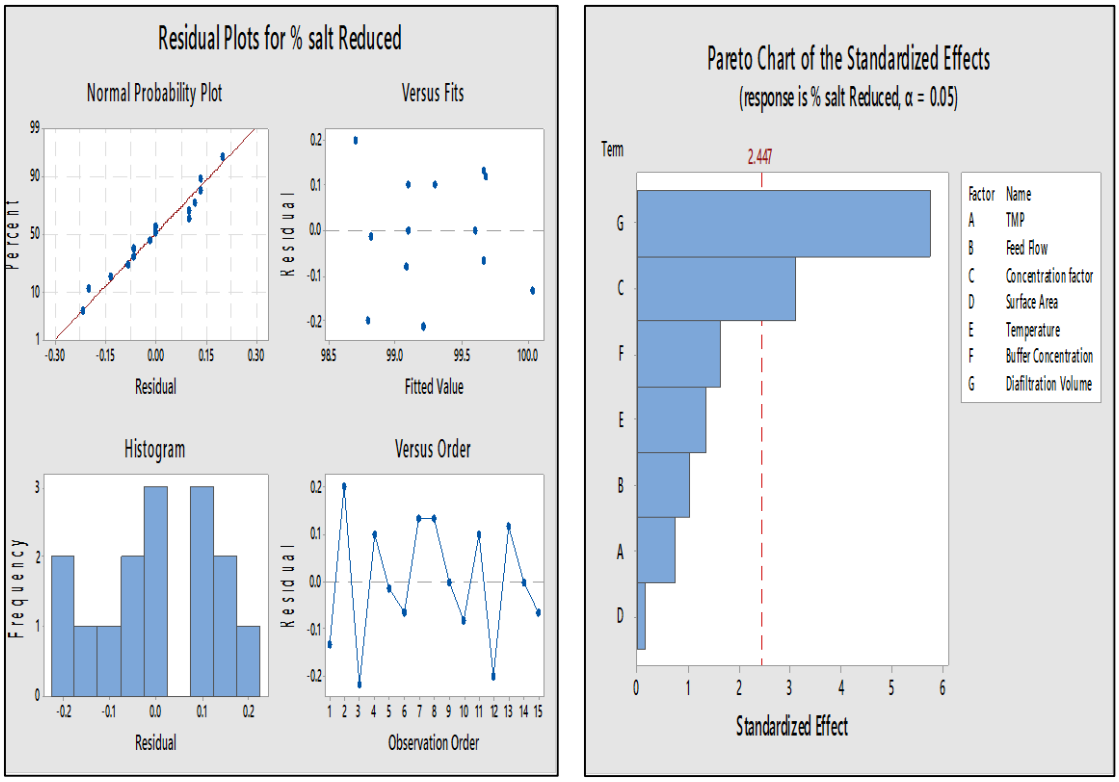


Figure. 20 : ***Residual plot and Pareto chart of the % salt reduced:***

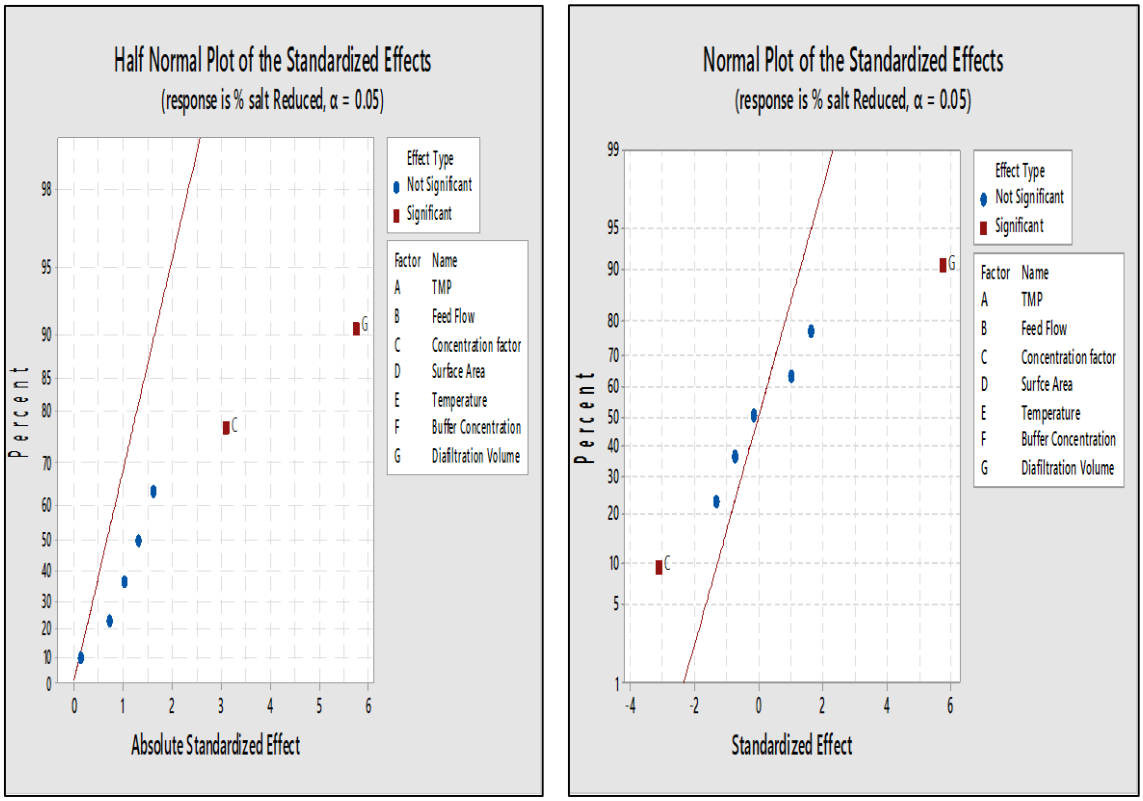


Figure. 21: ***Half Normal and Normal Effects Plot for % salt Reduced:***

Influence of various factors on Filtration time:

The influence of various factors effect on the total filtration timewas analyzed. The filtration time varied from 2 (PB-5) to 5.5 (PB-10) for the various factor combination. The most significant factor was Transmembrane Pressure (TMP), diafiltration volume concentration factor and surface area ($p < 0.05$) compared with other factors which influenced the filtration time shown in Table. The filtration time from all the trail was greatly affected by the TMP, diafiltration volume, concentration factor of the feed solution and surface area. So the concentration factor from 2 to 3 would produce the desire filtration time, but for better result it should be about 2.5. Surface area of cartridge filter was also affect the filtration time, but for better filtration time for further process it should done at $0.085M^2$ (500 kD).

Table 5. 27 ***ANOVA table for the Filtration time:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/Non-Significant**
Model	8	22.6067	2.8258	27.95	0.000	**Significant**
Linear	7	22.5400	3.2200	31.85	0.000	**Significant**
TMP	1	12.8133	12.8133	126.73	0.000	**Significant**
Feed Flow	1	0.2133	0.2133	2.11	0.197	**Non-Significant**
Concentration factor	1	2.8033	2.8033	27.73	0.002	**Significant**
Surface Area	1	2.6133	2.6133	25.85	0.002	**Significant**
Temperature	1	0.2133	0.2133	2.11	0.197	**Non-Significant**
Buffer Concentration	1	0.0300	0.0300	0.30	0.606	**Non-Significant**
Diafiltration Volume	1	3.8533	3.8533	38.11	0.001	**Significant**
Curvature	1	0.5486	0.5486	5.43	0.059	**Non-Significant**
Error	6	0.6067	0.1011			
Lack-of-Fit	4	0.6067	0.1517			
Pure Error	2	0.0000	0.0000			
Total	14	23.213				

In table 5.28 the “Effect” column show that increase in concentration factor of the feed would contribute to decrease the buffer volume, and diafiltration volume also affect the vice versa. Concentration factor had a more dominant effect to the volume of buffer in all the process.

The prediction confidence level of the model was 76.48 % and a good correlation was obtained between the observed and predicted values as indicated by the r2 value of 0.9739. Further analysis using ANOVA indicated a significant effect of variables on the response (% filtration time) ($p < 0.05$) and no curvature was observed ($p > 0.05$).

Table 5. 28 ***Estimated effects and coefficients for Filtration time:***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		4.4333	0.0918	48.30	0.000	
TMP	-2.0667	-1.0333	0.0918	-11.26	0.000	1.00
Feed Flow	0.2667	0.1333	0.0918	1.45	0.197	1.00
Concentration factor	-0.9667	-0.4833	0.0918	-5.27	0.002	1.00
Surface Area	-0.9333	-0.4667	0.0918	-5.08	0.002	1.20
Temperature	-0.2667	-0.1333	0.0918	-0.54	0.606	1.00
Buffer Concentration	-0.1000	-0.0500	0.0918	-0.54	0.606	1.00
Diafiltration Volume	1.1333	0.5667	0.0918	6.17	0.001	1.02
Ct Pt		-0.533	0.229	-2.33	0.059	1.24

Model Summary			
S	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
0.317980	97.39%	93.90%	76.48%

Regression Equation in Uncoded Units

Time filtration (Hr) =

4.779 - 0.2952 TMP + 0.001333 Feed Flow - 0.3867 Concentration factor

- 4.786 Surface Area - 0.0178 Temperature - 0.0250 Buffer Concentration

+ 0.3778 Diafiltration Volume - 0.533 Ct Pt

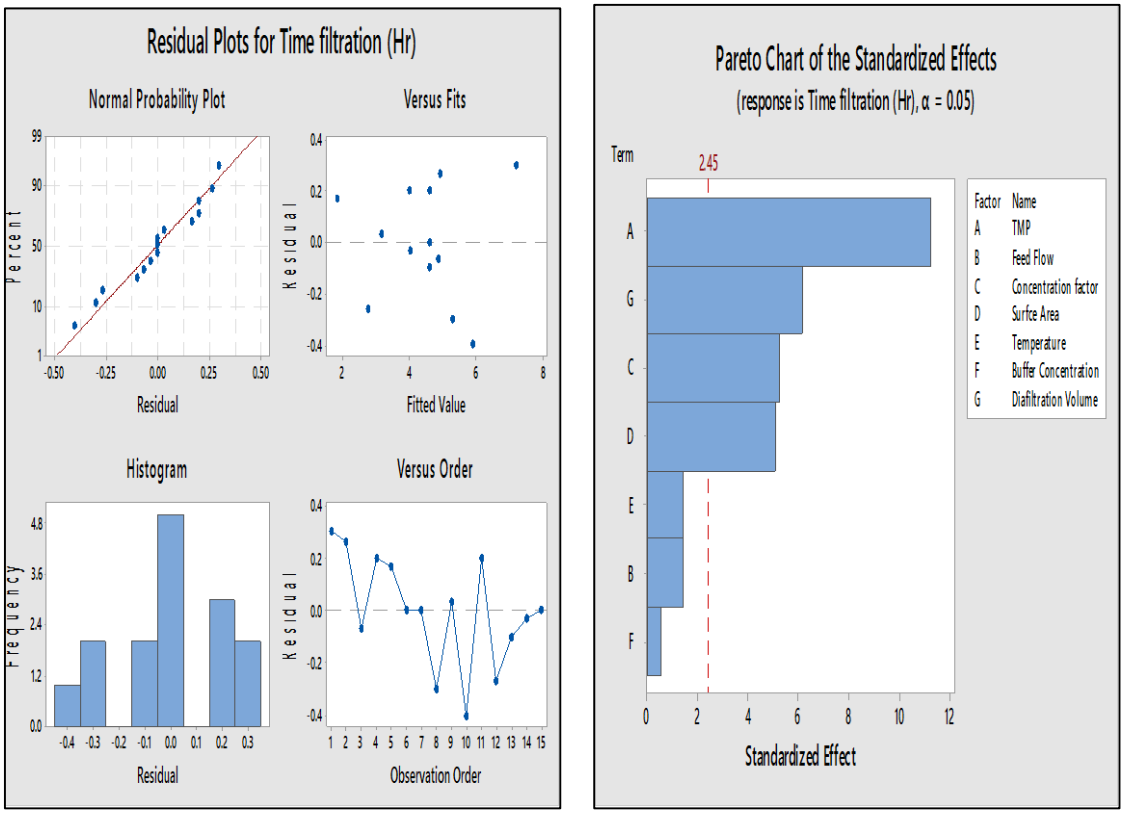


Figure. 22: ***Residual plot and Pareto chart of the filtration time:***

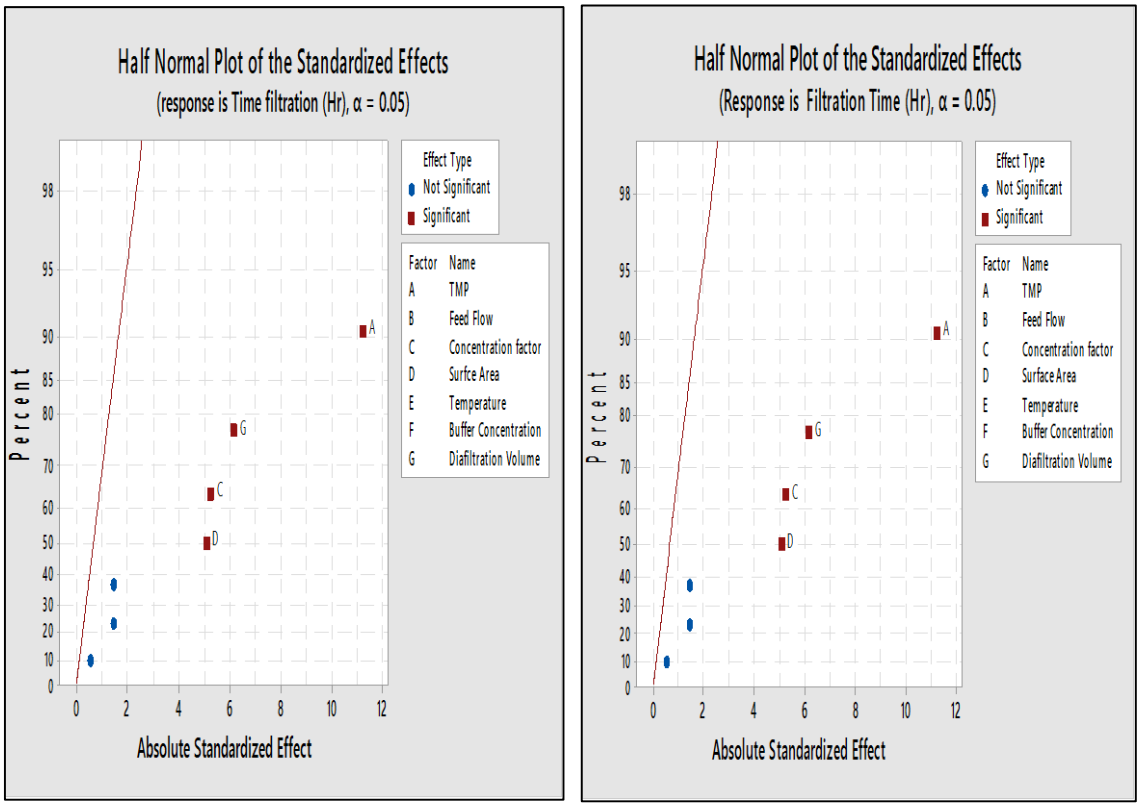


Figure. 23: ***Half Normal and Normal Effects Plot for filtration time:***

Influence of various factors on Volume of buffer:

The influence of various factors effect on the buffer volume was analyzed. The buffer volume varied from 10 (PB-1, PB-13 & PB-14) to 2 (PB-2, PB-3, PB-5, PB-12) for the various factor combination. The most significant factor was concentration factor and diafiltration volume ($p < 0.05$) compared with other factors which influenced the buffer volume shown in Table. The volume of buffer from all the trail was greatly affected by the concentration factor of the feed solution .So the concentration factor from 2 to 3 would produce the economical buffer volume consumption , but for better result it should be about 2.5. Diafiltration volume was also affect the buffer volume consumption, but for better % salt reduction for further process it should kept constant at 8.

*Table 5. 29 : **ANOVA table for the Volume of buffer:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/ Non-Significant**
Model	8	130.611	16.326	58.54	0.000	**Significant**
Linear	7	127.250	18.179	65.18	0.000	**Significant**
TMP	1	0.750	0.750	2.69	0.152	**Non-Significant**
Feed Flow	1	0.750	0.750	2.69	0.152	**Non-Significant**
Concentration factor	1	114.083	114.083	409.06	0.000	**Significant**
Surface Area	1	0.750	0.750	2.69	0.152	**Non-Significant**
Temperature	1	0.083	0.083	0.30	0.604	**Non-Significant**
Buffer Concentration	1	0.750	0.750	2.69	0.152	**Non-Significant**
Diafiltration Volume	1	10.083	10.083	36.16	0.001	**Significant**
Curvature	1	5.647	5.647	20.25	0.004	**Significant**
Error	6	1.673	0.279			
Lack-of-Fit	4	1.667	0.417	125.00	0.008	
Total	14	132.284				

In table 5.30 the “Effect” column show that increase in concentration factor of the feed would contribute to decrease the buffer volume, and diafiltration volume also affect the vice versa. Concentration factor had a more dominant effect to the volume of buffer in all the process.

The prediction confidence level of the model was 88.65 % and a good correlation was obtained between the observed and predicted values as indicated by the r2 value of 0.9874. Further analysis using ANOVA indicated a significant effect of variables on the response (% volume of buffer) ($p < 0.05$) and curvature was observed ($p < 0.05$).

Table 5. 30 : ***Estimated effects and coefficients for Volume of buffer:***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P- Value**	**VIF**
Constant		5.417	0.152	35.53	0.000	
TMP	0.500	0.250	0.152	1.64	0.152	1.00
Feed Flow	0.500	0.250	0.152	1.64	0.152	1.00
Concentration Factor	-6.167	-3.083	0.152	-20.23	0.000	1.00
Surface Area	-0.500	-0.250	0.152	-1.64	0.152	1.20
Temperature	0.167	0.083	0.152	0.55	0.604	1.02
Buffer Concentration	0.500	0.250	0.152	1.64	0.152	1.00
Diafiltration Volume	1.833	0.917	0.152	6.01	0.001	1.02
Ct Pt		-1.711	0.380	-4.50	0.004	1.24

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
0.528099	98.74%	97.05%	88.65%

Regression Equation in UncodedUnits

Volume of buffer (L) =

3.08 + 0.0714 TMP + 0.00250 Feed Flow- 2.467 Concentration factor - 2.56 Surface Area + 0.0111 Temperature + 0.1250 Buffer Concentration + 0.611 Diafiltration Volume - 1.711 Ct Pt

Regression Equation in Coded Units

$Y_2 = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_5X_5 + B_6X_6 + B_7X_7 + \varepsilon$

Where, β_0 = intercept

β_1 to β_7 = co - efficient

X_1 to X_7 = variable ; ε = error

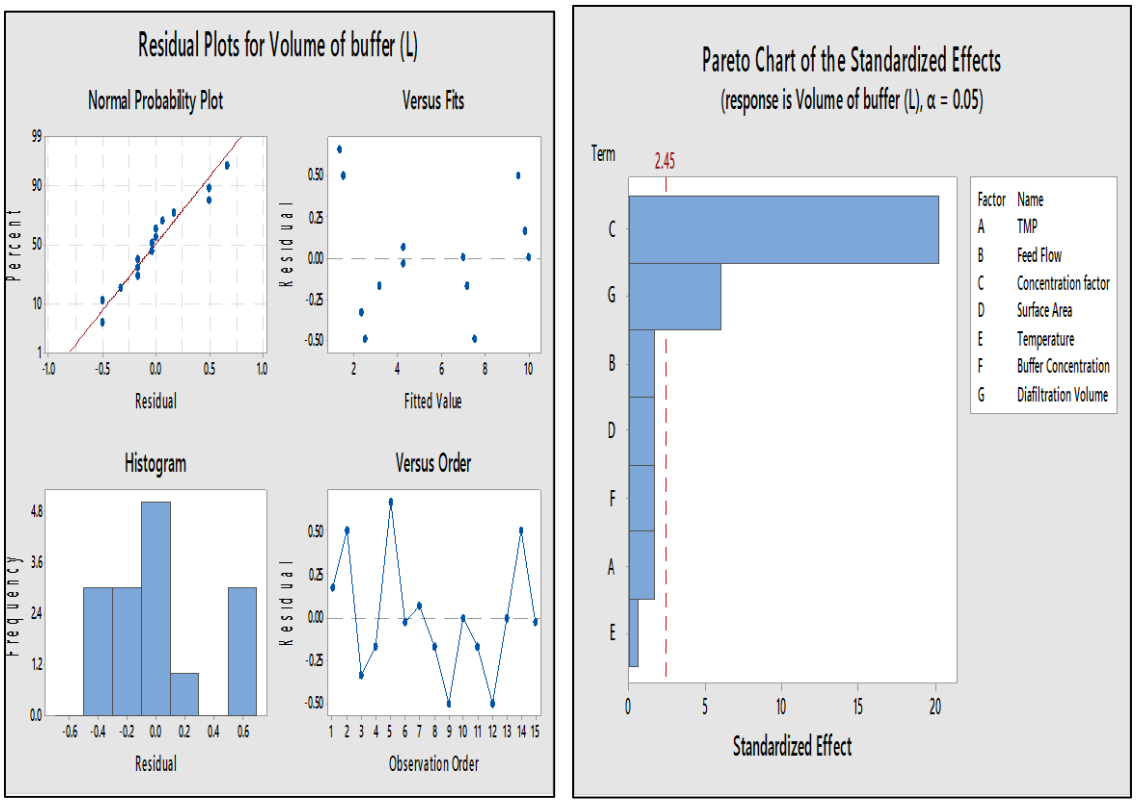


Figure. 24: ***Residual plot and Pareto chart of the filtration time:***

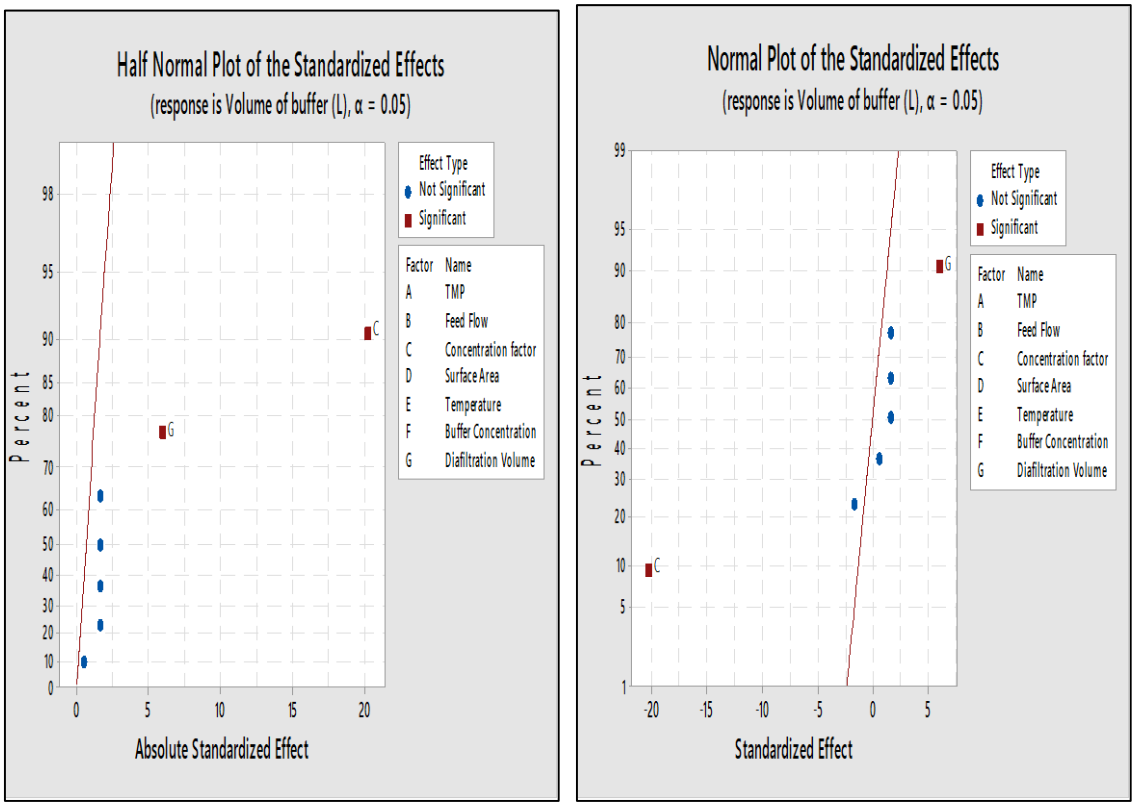


Figure. 25: ***Half Normal and Normal Effects Plot for filtration time:***

Influence of various factors on Osmolarity:

The influence of various factors effect on the osmolaritywas analyzed. The osmolarity varied from 315 (PB-8) to 270 (PB-4, PB-10) for the various factor combination. The most significant factor was buffer concentration and feed flow ($p < 0.05$) compared with other factors which influenced the osmolarity shown in Table 5.31. The osmolarity from all the trail was greatly affected by the buffer concentration .So the buffer concentration from 8 to 12 (%w/v) would produce the effective osmolarity of the solution, but for better result it should be about 10 (%w/v). Feed flow was also affect the osmoalrity of the solution, but for better prediction of the process it should kept constant at 50 ml/min.

Table 5. 31 ***ANOVA table for the osmolarity:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant / Not significant**
Model	8	3556.07	444.51	27.12	0.000	**Significant**
Linear	7	3429.92	489.99	29.90	0.000	**Significant**
TMP	1	10.08	10.08	0.62	0.463	**Significant**
Feed Flow	1	200.08	200.08	12.21	0.013	**Significant**
Concentration factor	1	14.08	14.08	0.86	0.390	**Not significant**
Surface Area	1	0.08	0.08	0.01	0.945	**Not significant**
Temperature	1	0.08	0.08	0.01	0.945	**Not significant**
Buffer Concentration	1	3168.75	3168.75	193.35	0.000	**Significant**
Diafiltration Volume	1	36.75	36.75	2.24	0.185	**Not significant**
Curvature	1	116.67	116.67	7.12	0.037	**Significant**
Error	6	98.33	16.39			
Lack-of-Fit	4	72.33	18.08	1.39	0.459	
Pure Error	2	26.00	13.00			
Total	14	3654.40				

In table 5.32 the “Effect” column show that increase the buffer concentration and feed flow would contribute to increase the osmolarity. Buffer concentration had a more dominant effect to the osmolarity of the solution in all the process.

The prediction confidence level of the model was 80.59% and a good correlation was obtained between the observed and predicted values as indicated by the r2 value of 0.9731. Further analysis using ANOVA indicated a significant effect of variables on the response (% volume of buffer) ($p < 0.05$) and curvature was observed ($p < 0.05$).

*Table 5. 32 **Estimated effects and coefficients for Osmolarity:***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		291.25	1.17	249.22	0.000	
TMP	1.83	0.92	1.17	0.78	0.463	1.00
Feed Flow	8.17	4.08	1.17	3.49	0.013	1.00
Concentration factor	2.17	1.08	1.17	0.93	0.390	1.00
Surface Area	0.17	0.08	1.17	0.07	0.945	1.20
Temperature	-0.17	-0.08	1.17	-0.07	0.945	1.02
Buffer Concentration	32.50	16.25	1.17	13.90	0.000	1.00
Diafiltration Volume	3.50	1.75	1.17	1.50	0.185	1.02
Ct Pt		-7.78	2.92	-2.67	0.037	1.24

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
4.04832	97.31%	93.72%	80.59%

Regression Equation in Uncoded Units

Osmolarity (mOsmol) =

176.1 + 0.262 TMP + 0.0408 Feed Flow + 0.867 Concentration factor + 0.9 Surface Area - 0.011 Temperature + 8.125 Buffer Concentration + 1.167 Diafiltration Volume - 7.78 Ct Pt

Regression Equation in Coded Units

$Y_2 = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_5X_5 + B_6X_6 + B_7X_7 + \varepsilon$

Where, β_0 = intercept

β_1 to β_7 = co - efficient

X_1 to X_7 = variable ; ε = error

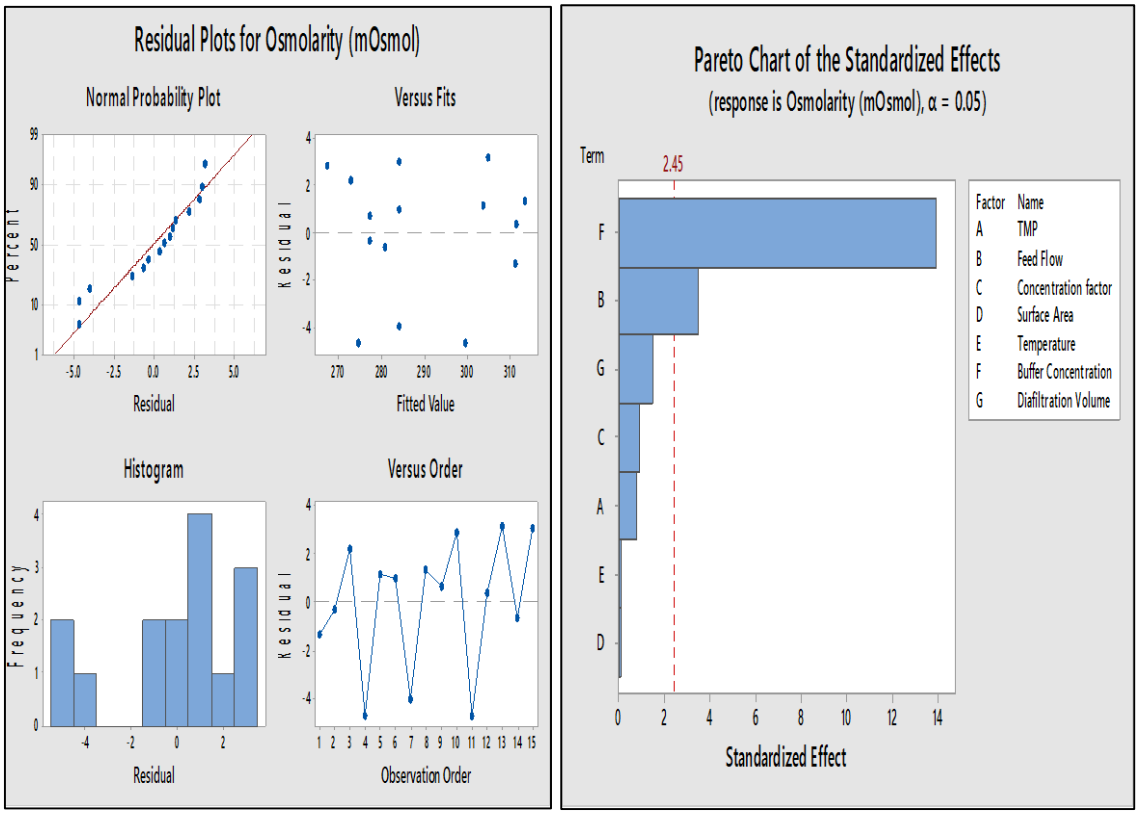


Figure. 26: ***Residual plot and Pareto chart of the osmolarity:***

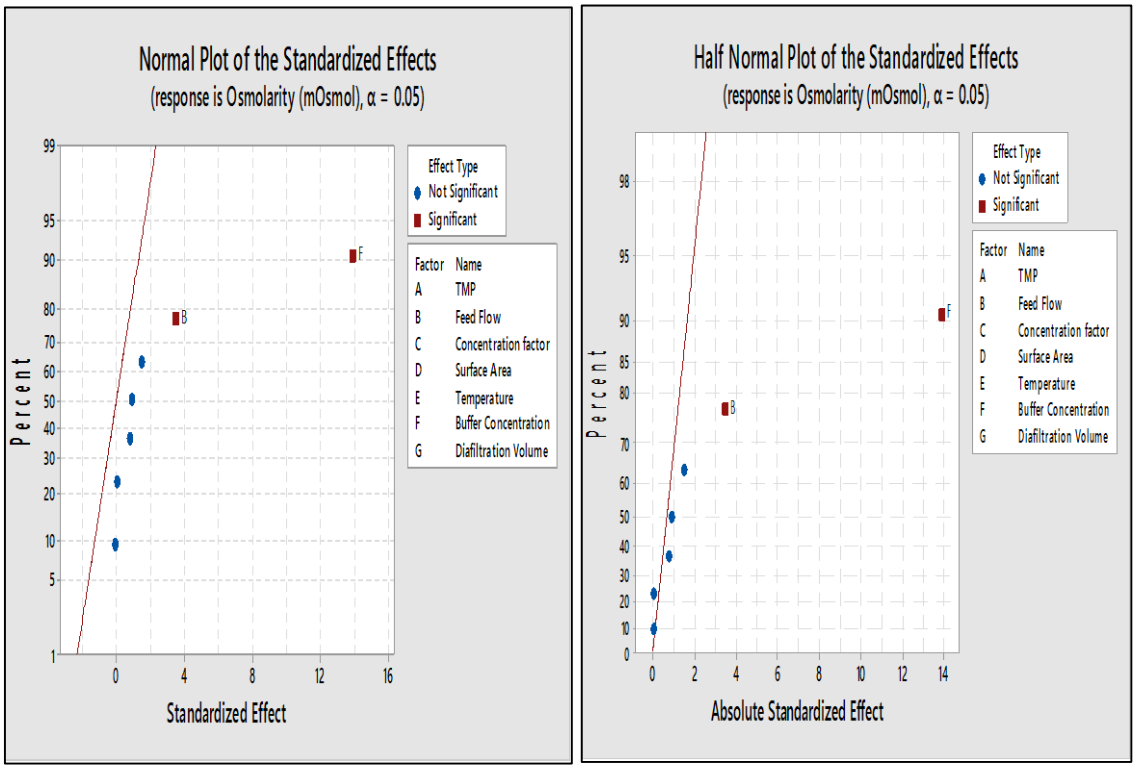


Figure. 27: ***Half Normal and Normal Effects Plot for filtration time:***

Table 5. 33: Model Summary of the all responses with respect to S, ***R-sq, R-sq (adj, R-sq (pred)***

Model Summary					
Sr.No	**Factor**	**S**	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
1	Flux	1.31000	97.34%	93.78%	76.31%
2	% salt reduction	0.195789	90.79%	78.50%	24.28%
3	Filtration time	0.317980	97.39%	93.90%	76.48%
4	Volume of Buffer	0.528099	98.74%	97.05%	88.65%
5	Osmolarity	4.04832	97.31%	93.72%	80.59%

Conclusion:

R^2 value of the individual response was above to 95 %, so the model was fit for the particular responses; except for the % salt reduction having 90.79 %.Flux, filtration time and volume of buffer were the most important response for the further optimization of the process. Other responses like % salt reduction and the osmolarity of the product not significantly changed; by changing the major variable of the process. Therefore it was not included in the further optimization.

5.8 Central Composite Design

Based on the screening study results, two variables (TMP and Concentration Factor) were selected for the optimization study, using response surface method (RSM), and more specifically a central composite design. In this two factor CCD design, four axial points were selected so that the distance, α from the center of the design to any axial point is = $2^{2/4}$ = 1.414.

Central Composite Design	
Factors: 2	Replicates: 1
Base runs: 12	Total runs: 12
Base blocks: 1	Total blocks: 1

Two-level factorial: Full factorial	
Cube points:	4
Center points in cube:	4
Axial points:	4
Center points in axial:	0
α:	1.41421

12 Trail batch of the TFF process at the constant diafiltration Volume = 8

Table 5. 34 : ***Design table of the central composite design and results for flux (LMH), filtration time (Hr), Buffer consumption (L).***

CENTRAL COMPOSITE DESIGN (CCD) FOR 2 FACTOR								
ID	Type	Coded Value (Factor)		Uncoded Value (Factor)		Responses		
						Y_1	Y_2	Y_3
		X1	X2	TMP	CF	Flux (LMH)	Time of Filtration (Hr)	Buffer Consumption (L)
CCD-1	Center	0	0	6.5	2.25	12	4.7	3.8
CCD-2	Center	0	0	6.5	2.25	13	4.5	3.8
CCD-3	Axial	0	1.414	6.5	3.31	6.2	3.8	2.5
CCD-4	Center	0	0	6.5	2.25	12.5	4.8	4
CCD-5	Fact	1	-1	8	1.5	15.5	3.8	5.5
CCD-6	Axial	0	-1.414	6.5	1.18	12	5.2	7.2
CCD-7	Center	0	0	6.5	2.25	12	5	4
CCD-8	Axial	-1.414	0	4.37	2.25	9.5	6.66	3.7
CCD-9	Fact	1	1	8	3	9.5	4	2.8
CCD-10	Fact	-1	-1	5	1.5	13.5	4.75	5.5
CCD-11	Fact	-1	1	5	3	5.5	3.91	2.8
CCD-12	Axial	1.414	0	8.62	2.25	16.5	3.36	3.6

As shown in Table 5.34, each of the two factors was tested at 5 different levels and 4 center points were included. Minitab 17.0 software was used for the design and analysis, and to plot the various 3D and contour graphs.The contour plot for prediction of flux. Every single

point corresponds to a combination of TMP and CF. The space inside the dashed circle is the testing domain of the central composite design, and the imbedded square is the full factorial design domain with center point. Based on the ANOVA result of the particular response predicted model was created.

Response Surface Regression: Flux (LMH) versus TMP, Concentration Factor

Table 5. 35 : ***ANOVA table for the Flux:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/ Non-Significant**
Model	5	113.676	22.7352	21.49	0.000	**Significant**
Linear	2	93.218	46.6089	44.05	0.000	**Significant**
TMP	1	31.599	31.5992	29.86	0.001	**Significant**
CF	1	61.619	61.6185	58.23	0.000	**Significant**
Square	2	19.458	9.7290	9.19	0.011	**Significant**
TMP*TMP	1	1.045	1.0446	0.99	0.354	**Non- Significant**
CF*CF	1	16.984	16.9837	16.05	0.005	**Significant**
2-Way Interaction	1	1.000	1.0000	0.95	0.363	**Non- Significant**
TMP*CF	1	1.000	1.0000	0.95	0.363	
Error	7	7.407	1.0582			
Lack-of-Fit	3	6.107	2.0357	6.26	0.054	**Non- Significant**
Pure Error	4	1.300	0.3250			
Total	12	121.083				

Table 5. 36: ***Estimated regression coefficients for Flux (Quadratic model)***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		12.200	0.460	26.52	0.000	
TMP	3.975	1.987	0.364	5.46	0.001	1.00
CF	-5.551	-2.775	0.364	-7.63	0.000	1.00
TMP*TMP	0.775	0.387	0.390	0.99	0.354	1.02
CF*CF	-3.125	-1.563	0.390	-4.01	0.005	1.02
TMP*CF	1.000	0.500	0.514	0.97	0.363	1.00

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R-sq (pred)**
1.02868	93.88%	89.51%	62.46%

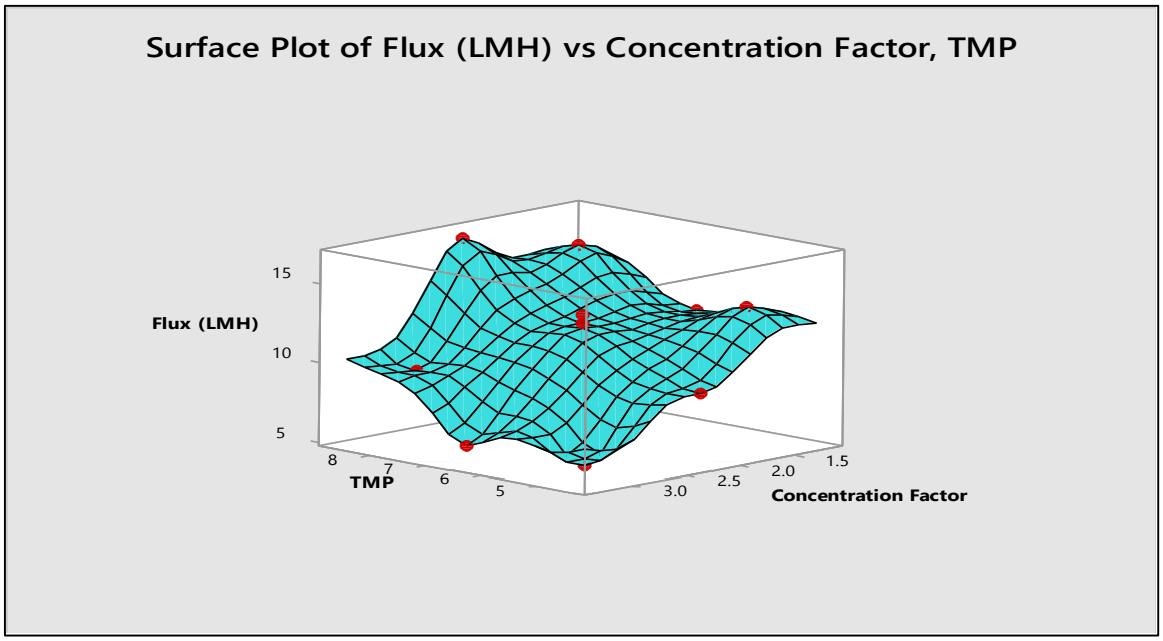


Figure. 28: ***(Response Surface Plots for predicting Flux (LMH).Red point was flux responses within the 3D Surface.)***

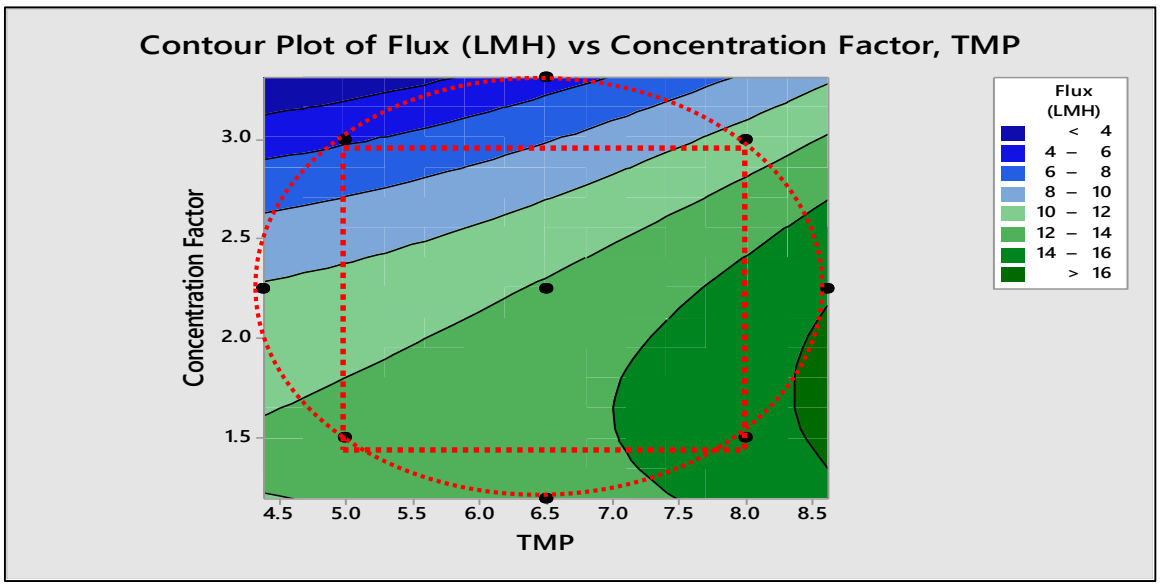


Figure. 29: ***(Contour plot for Flux with respect to Concentration Factor, Transmembrane pressure. Black stars are central composite design points. Within the circle (red) is the design space for prediction of flux.)***

Regression Equation in Uncoded Units

Flux (LMH) =

11.6 - 1.91 TMP + 5.91 CF + 0.172 TMP*TMP - 2.778 CF*CF + 0.444 TMP*CF

$Y_1 = \beta_0$ (constant) + $\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2$

Where, β_0 = intercept; β_1 to β_5 = co –efficient; X_1 and X_2 = variable

Central composite design to obtain the response surface for Flux:

As shown in table 5.34, filtrate flux varied from 5.5 (CCD-11) to 16.5 (CCD-12) and rest of the points were distributed evenly across this range. To fully utilize the central composite design and to able to make accurate prediction for future process, three mathematical models were evaluated in order to obtain the highest prediction power. These three models were: a linear model (only main effects); a quadratic model (main effects, interactions, and squared terms); and a linear model with interaction terms. Among these three models, the first and second model has significant lack of fit ($p < 0.05$), but the third model having not significant effect on the flux. Based on the R^2- predictive value of the different model, Linear and squared terms model having the most suitable for the flux.

A Linear Model: (**Main effects):** $Y = b_o + b_1X_1 + b_2X_2$ (first order)

A Quadratic Model: (**Main effects, Squared Terms, Interaction)**

$Y = b_o + b_1X_1 + + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$ (second order)

(**Main effects, Interaction)**: $Y = b_o + b_1X_1 + + b_2X_2 + b_{12}X_1X_2$

*Table 5. 37 **ANOVA of Reduced model for Flux (Linear +Squared terms)***

Analysis of Variance of Reduced model (Linear +Squared terms)						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/ Non-Significant**
Regression	3	111.631	37.2104	35.43	0.000	Significant
TMP	1	31.599	31.5992	30.09	0.000	Significant
CF	1	9.143	9.1426	8.71	0.016	Significant
CF *CF	1	18.414	18.4135	17.53	0.002	Significant
Error	9	9.452	1.0502			
Lack-of-Fit	5	8.152	1.6304	5.02	0.072	
Pure Error	4	1.300	0.3250			
Total	12	121.083				

*Table 5. 38 : **Estimated regression coefficients for Flux (Linear +Squared terms)***

Coefficients					
Term	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant	-2.33	3.76	-0.62	0.550	
TMP	1.325	0.242	5.49	0.000	1.00
CF	9.20				
CF*CF	-2.868	0.685	-4.19	0.002	41.70

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
1.02479	92.19 %	89.59 %	75.96 %

Regression Equation for Reduced model

Flux (LMH) =

-2.33 + 1.325 TMP + 9.20 CF - 2.868 CF*CF

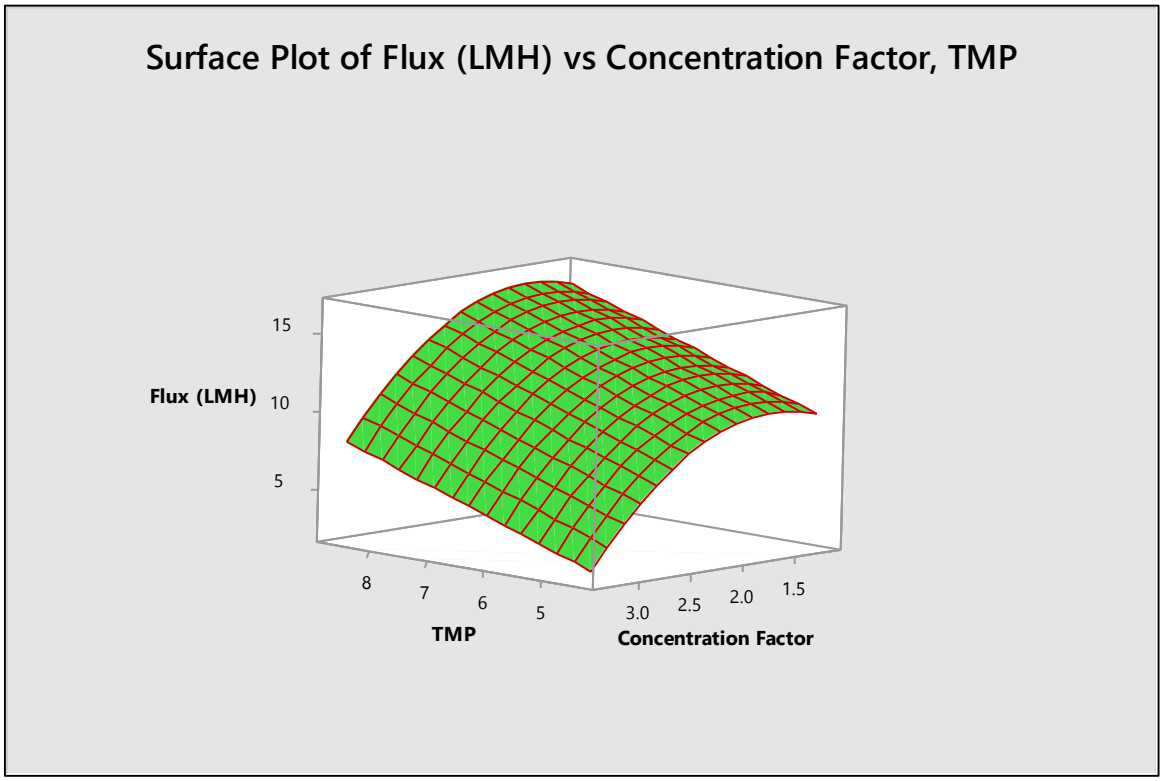


Figure. 30: ***Reduced model of the flux (Y1)***

Conclusion:

(Linear + squared terms) model was significant effect on the flux response. Main effect of the variable (TMP & Concentration factor) was the greatest effect on the flux. So, the quadratic model fit for the flux.

Response Surface Regression: Time of Filtration (Hr) versus TMP, CF

*Table 5. 39 : **ANOVA table for the time of filtration:***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/ Non-Significant**
Model	5	5.53182	1.10636	2.59	0.123	
Linear	2	4.67632	2.33816	5.48	0.037	**Significant**
TMP	1	3.81833	3.81833	8.95	0.020	**Significant**
CF	1	0.85798	0.85798	2.01	0.199	**Non-Significant**
Square	2	0.58510	0.29255	0.69	0.535	**Non-Significant**
TMP*TMP	1	0.00852	0.00852	0.02	0.892	
CF*CF	1	0.58504	0.58504	1.37	0.280	
2-Way Interaction	1	0.27040	0.27040	0.63	0.452	**Non-Significant**
TMP*CF	1	0.27040	0.27040	0.63	0.452	**Non-Significant**
Error	7	2.98718	0.42674			
Lack-of-Fit	3	2.85518	0.95173	28.84	0.004	
Pure Error	4	0.13200	0.03300			
Total	12	8.51900				

*Table 5. 40: **Estimated regression coefficients for time of filtration (Quadratic model)***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		4.760	0.292	16.29	0.000	
TMP	-1.382	-0.691	0.231	-2.99	0.020	1.00
CF	-0.655	-0.327	0.231	-1.42	0.199	1.00
TMP*TMP	-0.070	-0.035	0.248	-0.14	0.892	1.02
CF*CF	-0.580	-0.290	0.248	-1.17	0.280	1.02
TMP*CF	0.520	0.260	0.327	0.80	0.452	1.00

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R- sq(pred)**
0.653254	64.94%	39.89%	0.00%

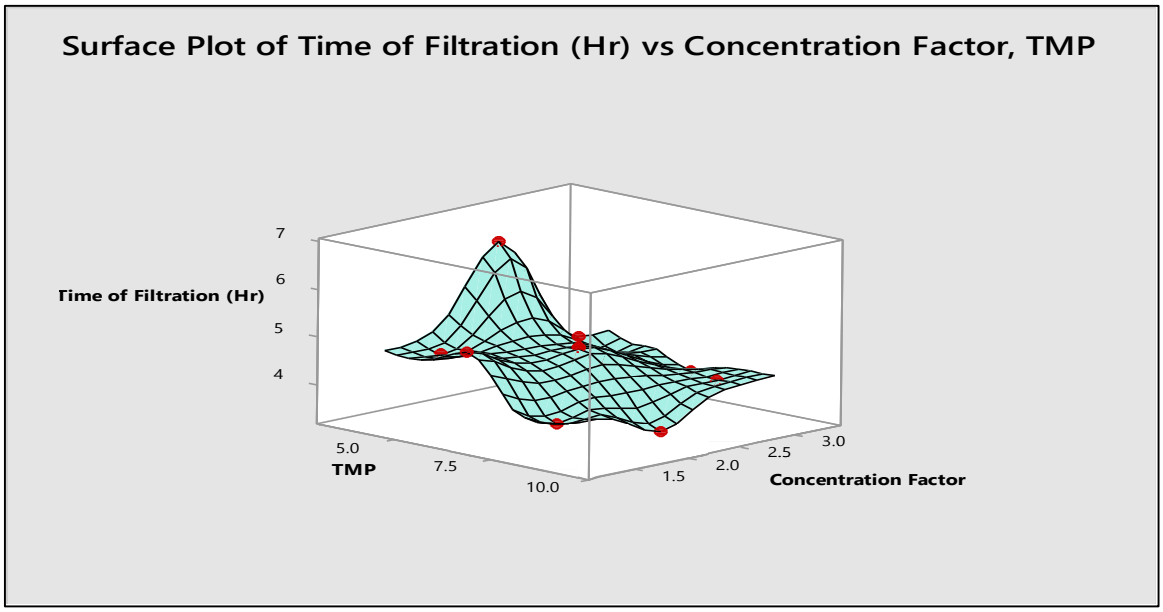


Figure. 31: ***(Response Surface Plot for predicting Time of filtration (Hr). Red point was filtration time responses within the 3D Surface.)***

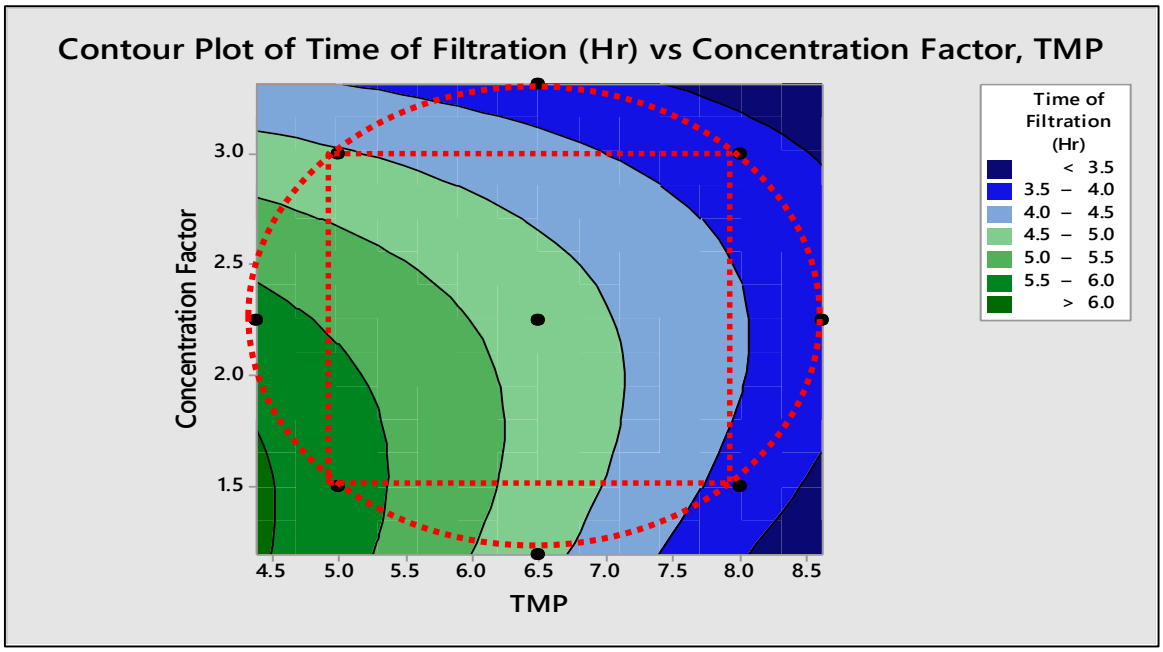


Figure. 32: ***(Contour plot for time of filtration with respect to Concentration Factor, Transmembrane pressure. Black stars are central composite design points. Within the circle (red) is the design space for prediction of filtration time.)***

Regression Equation in Uncoded Units

Time of Filtration (Hr) =

8.85 - 0.78 TMP + 0.38 CF - 0.016 TMP*TMP - 0.516 CF*CF + 0.231 TMP*CF

$Y_2 = \beta_0$ (constant) + $\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2$

Where, β_0 = intercept β_1 to β_5 = co - efficient; X_1 and X_2 = variable

Central composite design to obtain the response surface for time of filtration:

As shown in table 5.34, filtration time varied from 3.36 (CCD-12) to 6.66 (CCD-8) and rest of the points were distributed evenly across this range. Among the three models, the first model has significant lack of fit ($p < 0.05$), the second and third having not fits the data ($p > 0.05$). So, filtration time of the process having only fit the linear model. Based on the R^2- predictive value of the different model, Linear model having the most suitable for the filtration time.

Regression Analysis: Time of Filtration (Hr) versus TMP

*Table 5. 41: **ANOVA of Reduced model for time of filtration (Linear)***

Analysis of Variance Reduced model (Linear)						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/ Non-Significant**
Regression	1	3.8183	3.81833	8.94	0.012	**Significant**
TMP	1	3.8183	3.81833	8.94	0.012	**Significant**
Error	11	4.7007	0.42733			
Lack-of-Fit	7	4.5687	0.65267	19.78	0.006	
Pure Error	4	0.1320	0.03300			
Total	12	8.5190				

*Table 5. 42 : **Estimated regression coefficients for time of filtration (Linear)***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		7.55	1.02	7.42	0.00	
TMP		-0.461	0.154	-2.99	0.012	1.00

Model Summary			
S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
0.653707	44.82%	39.81 %	9.70 %

Regression Equation

Time of Filtration (Hr) = 7.55 - 0.461 TMP

Conclusion:

Linear model (main effect) only significant for the filtration time response. Second and third model not fit the filtration time response. Only TMP affect the filtration time at the particular membrane surface of the filter cartridge.

Response Surface Regression: Buffer Consumption (L) versus TMP, CF

*Table 5. 43 : **ANOVA table for the buffer consumption***

Analysis of Variance						
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Model Significant/ Non-Significant**
Model	5	19.8244	3.9649	0104.81	0.000	**Significant**
Linear	2	18.1432	9.0716	239.79	0.000	**Significant**
TMP	1	0.0025	0.0025	0.07	0.805	**Non-Significant**
CF	1	18.1407	18.1407	479.52	0.000	**Significant**
Square	2	1.6812	0.8406	22.22	0.001	**Significant**
TMP*TMP	1	0.1781	0.1781	4.71	0.067	**Non-Significant**
CF*CF	1	1.3468	1.3468	35.60	0.001	**Significant**
2-Way Interaction	1	0.0000	0.0000	0.0000	1.000	**Non-Significant**
TMP*CF	1	0.0000	0.0000	0.0000	1.000	
Error	7	0.2648	0.0378			
Lack-of-Fit	3	0.2168	0.0723	6.02	0.058	
Pure Error	4	0.0480	0.0120			
Total	12	20.0892				

*Table 5. 44 : **Estimated regression coefficients for Buffer consumption (Quadratic model)***

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		3.9200	0.0870	45.07	0.00	
TMP	-0.0354	-0.0177	0.0688	-0.26	0.805	1.00
CF	-3.0117	-1.5059	0.0688	-21.90	0.000	1.00
TMP*TMP	-0.3200	-0.1600	0.0737	-2.17	0.067	1.02
CF*CF	0.8800	0.4400	0.0737	5097	0.001	1.02
TMP*CF	0.0000	0.0000	0.0973	0.00	1.000	1.00

Model Summary			
S	**R-sq**	**R-sq(adj**	**R-sq(pred)**
0.194501	98.68%	97.74%	91.95%

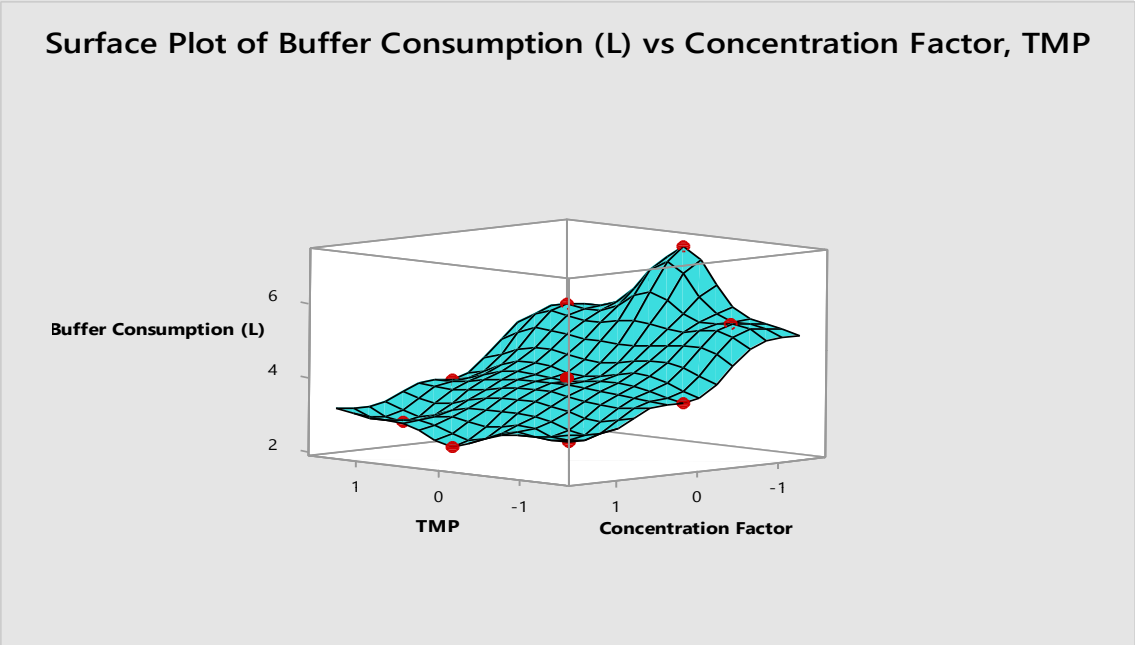


Figure. 33: ***(Response Surface Plot for predicting Buffer consumption (L).Red point was Buffer consumption responses within the 3D Surface.)***

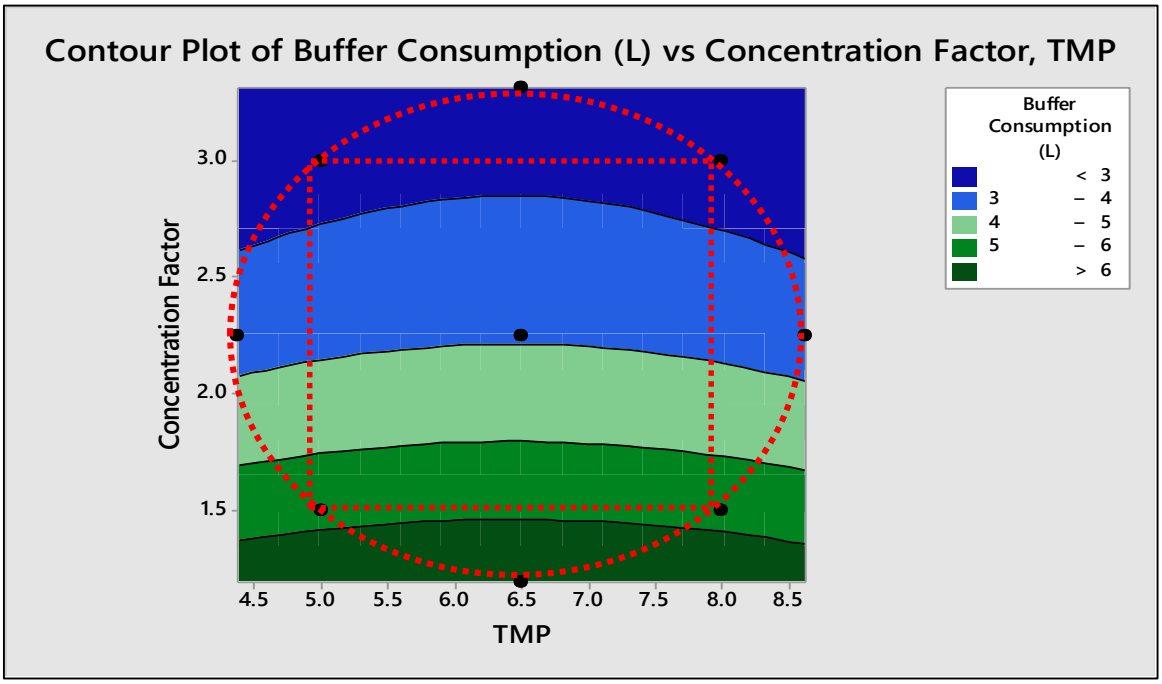


Figure. 34: ***Contour Plot of Buffer Consumption (L) vs CF, TMP***

Regression Equation in Uncoded Units

Buffer Consumption (L) =

9.47 + 0.913 TMP - 5.528 CF - 0.0711 TMP*TMP + 0.782 CF*CF - 0.0000 TMP*CF

$Y_3 = \beta_0$ (constant) + $\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2$

Where, β_0 = intercept; $\beta_1$to β_5 = co – efficient; X_1 and X_2 = variable

Central composite design to obtain the response surface for buffer consumption (L)

As shown in table 5.34, buffer consumption varied from 3.36 (CCD-12) to 6.66(CCD-8) and rest of the points were distributed evenly across this range. Among the three models, the first model and second model has significant lack of fit ($p < 0.05$), but third having not fits the data ($p > 0.05$).. Based on the R^2- predictive value of the different model, Linear and squared terms model having the most suitable for the buffer consumption. Ideally quadratic model fit for the buffer consumption.

Regression Analysis: Buffer Consumption (L) versus Concentration Factor

*Table 5. 45: **ANOVA table for reduced model of the buffer consumption (Linear +Squared model)***

Analysis of Variance for Reduced model (Linear +Squared model)					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Regression	2	19.6438	9.82191	220.52	0.000
CF	1	3.5000	3.50003	78.58	0.000
CF* CF	1	1.5031	1.50314	33.75	0.000
Error	10	0.4454	0.04454		
Lack-of-Fit	6	0.3974	0.06623	5.52	0.060
Pure Error	4	0.0480	0.01200		
Total	12	20.0892			

*Table 5. 46 : **Estimated regression coefficients for buffer consumption***

Coded Coefficients					
Term	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant	12.474	0.704	17.71	0.000	
CF	-5.695	0.642	-8.86	0.000	41.70
CF*CF	0.819	0.141	5.81	0.000	41.70

Model Summary			
S	**R-sq**	**R-sq(adj**	**R-sq(pred)**
0.211045	97.78 %	97.34 %	93.77 %

Regression Equation

Buffer Consumption (L) = 12.474 - 5.695 *CF + 0.819*CF*CF

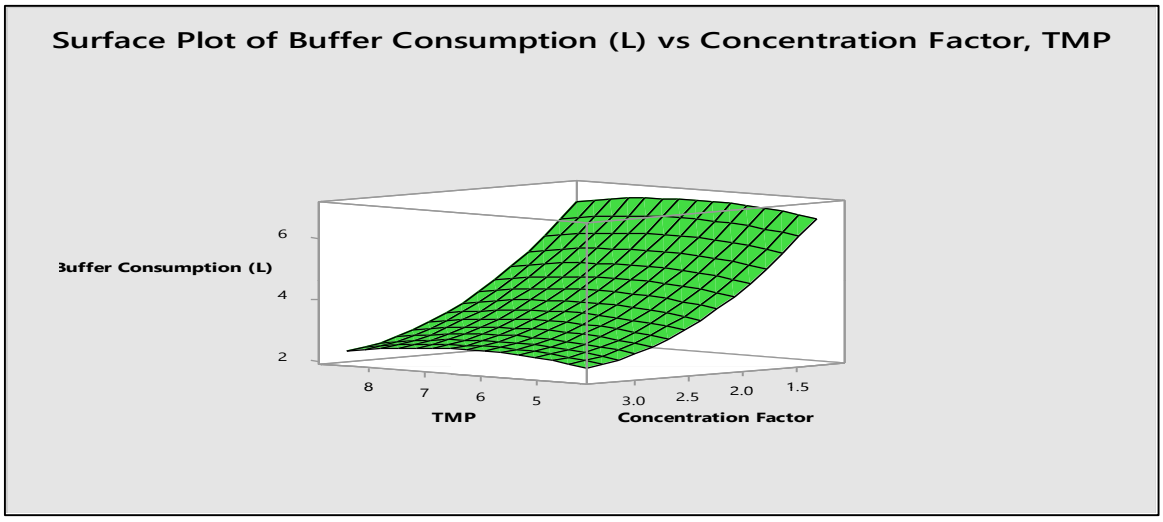


Figure. 35: ***Reduced model of the Buffer consumption (Y3)***

Table 5. 47 Over all model summary for the Flux, Time of filtration and Buffer consumption

Model Summary					
Sr.No	**Factor**	**S**	**R-sq**	**R-sq (adj)**	**R-sq (pred)**
1	Flux	1.02868	93.88%	89.51%	62.46%
2	Flux (Linear model)	1.66929	76.99%	72.38%	52.54%
3	Flux (Quadratic model)	1.02514	93.06%	89.58%	71.82%
4	Flux (Linear + Squared terms)	1.02479	92.19 %	89.59 %	75.96 %
5	Time of Filtration	0.653254	64.94%	39.89%	0.00%
6	Time of filtration (Linear model)	0.619894	54.89 %	45.87 %	4.46 %
7	Time of filtration (Linear-TMP)	0.653707	44.82%	39.81%	9.70%
8	Buffer Consumption	0.194501	98.68%	97.74%	91.95%
9	Buffer Consumption (Quadratic model)	0.221836	97.80%	97.06%	92.92%
10	Buffer Consumption (Linear +squared terms)	0.211045	97.78 %	97.34 %	93.77 %

5.9 Design Space with check point in the design space & outside the design space

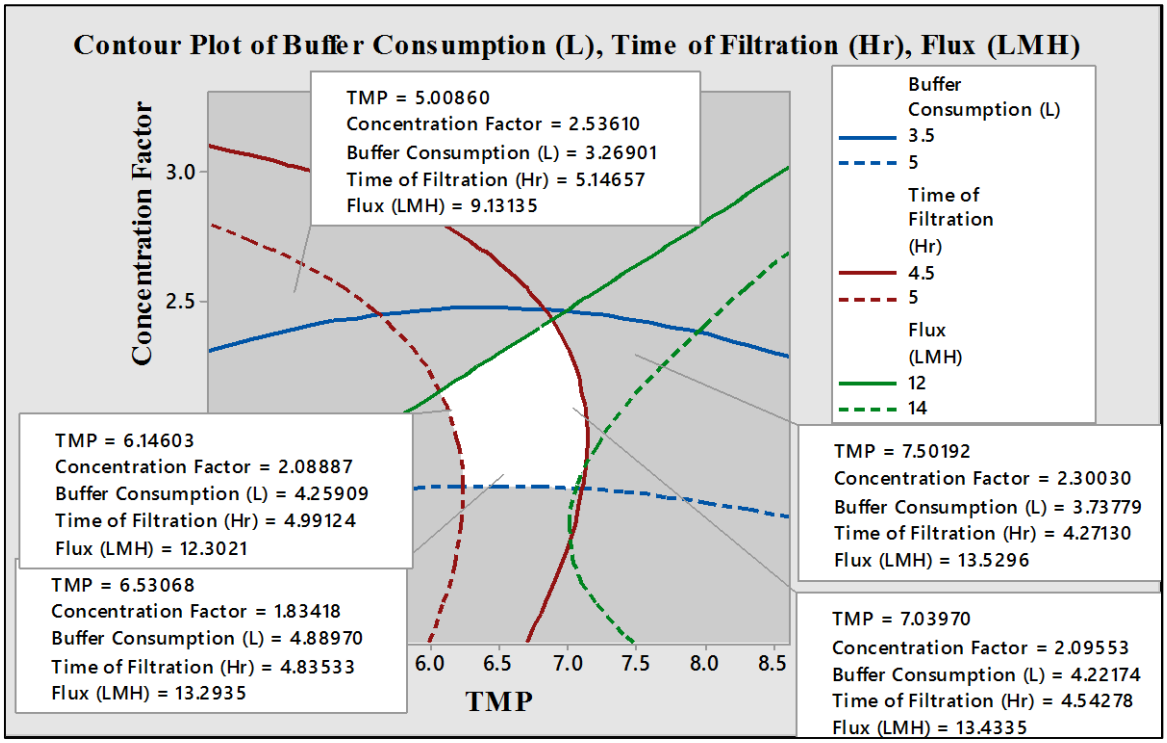


Figure. 36: ***Design Space with check point in the design space & outside the design space***

Table 5. 48 ***Comparison of the predicted and experimental values for the additional data points inside and outside the design space.***

ID	Uncoded Value (Factor)		Actual Responses			Predicted Response (Design Space)		
	TMP (psi)	Concentration Factor (X)	Flux (LMH)	Time of Filtration (Hr)	Buffer Consumption (L)	Flux (LMH)	Time of Filtration (Hr)	Buffer Consumption (L)
CP-1	5	2.5	9.5	5	3.2	9.13	5.15	3.27
OP-2	6	2	12.5	4.8	4.1	12.30	4.99	4.25
CP-3	6.5	1.8	13.5	4.7	5.0	13.29	4.83	4.88
OP-4	7	2	14	4.5	4.1	13.43	4.54	4.22
CP-5	7.5	2.3	13.80	4.0	3.5	13.53	4.27	3.73

Table 5. 49 ***Accuracy of the design Space for the different Responses:***

ID	TMP	CF	Actual value			Quadratic model			Reduced model		
	X1	X2	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
CCD-1	6.5	2.25	12	4.7	3.8	12.179	4.725	3.921	12.463	4.554	3.806
CCD-2	6.5	2.25	13	4.5	3.8	12.179	4.725	3.921	12.463	4.554	3.806
CCD-3	6.5	3.31	6.2	3.8	2.5	5.131	3.678	2.671	5.312	4.554	2.597
CCD-4	6.5	2.25	12.5	4.8	4	12.179	4.725	3.921	12.463	4.554	3.806
CCD-5	8	1.5	15.5	3.8	5.5	15.271	3.767	5.691	15.617	3.862	5.774
CCD-6	6.5	1.18	12	5.2	7.2	12.963	4.606	6.966	13.145	4.554	6.894
CCD-7	6.5	2.25	12	5	4	12.179	4.725	3.921	12.463	4.554	3.806
CCD-8	4.37	2.25	9.5	6.66	3.7	10.137	5.650	3.623	9.641	5.535	3.806
CCD-9	8	3	9.5	4	2.8	10.712	3.626	2.678	10.058	3.862	2.760
CCD-10	5	1.5	13.5	4.75	5.5	12.295	5.692	5.725	11.642	5.245	5.774
CCD-11	5	3	5.5	3.91	2.8	5.738	4.511	2.712	6.083	5.245	2.760
CCD-12	8.62	2.25	16.5	3.36	3.6	15.761	3.661	3.578	15.272	3.576	3.806
CP-1	5	2.5	9.5	5	3.2	9.313	5.163	3.325	9.370	5.245	3.355
OP-2	6	2	12.5	4.8	4.1	12.368	5.062	4.460	12.548	4.784	4.360
CP-3	6.5	1.8	13.5	4.7	5	13.284	4.819	4.984	13.550	4.554	4.877
OP-4	7	2	14	4.5	4.1	13.582	4.536	4.449	13.873	4.323	4.360
CP-5	7.5	2.3	13.8	4	3.5	13.506	4.229	3.741	13.596	4.093	3.708

Accuracy of the Response Y1(Flux):

Graph of experimental value of the flux(x-axis) v/s predicted value(y-axis) can give the better idea about the response Y1 in the particular design space. Two model was generated on the basis of the significant terms for the particular response Y1 :1) Quadratic model (significant + non significant terms) 2) Reduced model (significant terms only). The prediction of the response was based upon the R^2 value of the model. For the response Y1,R^2 value of quadratic and reduced model was 0.9503 and 0.9398, respectivly. Quadratic model give the best idea about the Response Y1(Flux) for the independent variable like X1 and X2 (TMP and CF).

Accuracy of the Response Y2 (Filtration time):

For the response Y2 (filtration time), R^2 value of quadratic and reduced model was 0.653 and 0.4755, respectivly. R^2 value of the both model was not sufficient. Quadrtic model give the idea about the Response Y2(Filtration time) for the independent variable

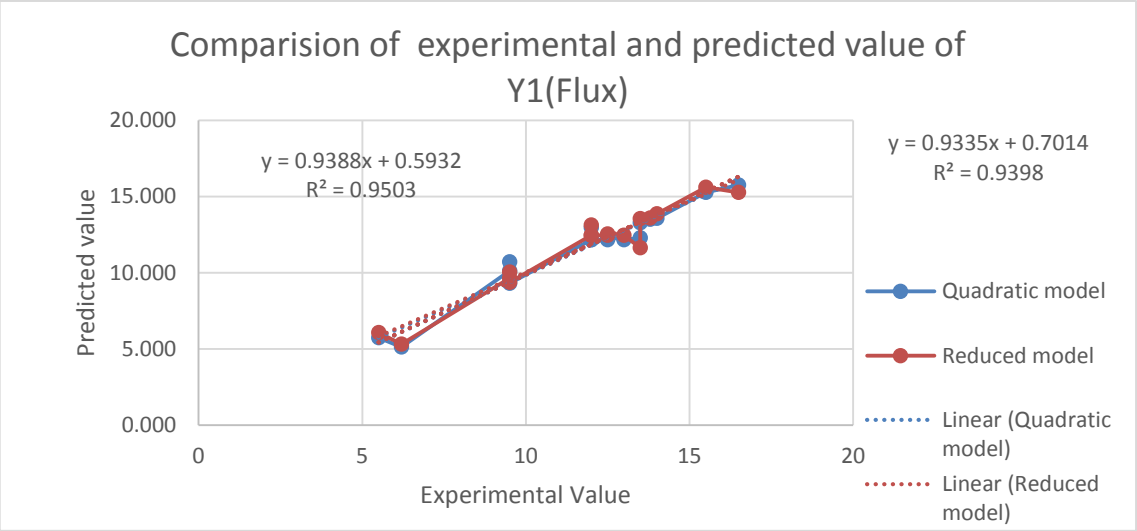


Figure. 37: ***Accuracy of the Response Y1 (Flux)***

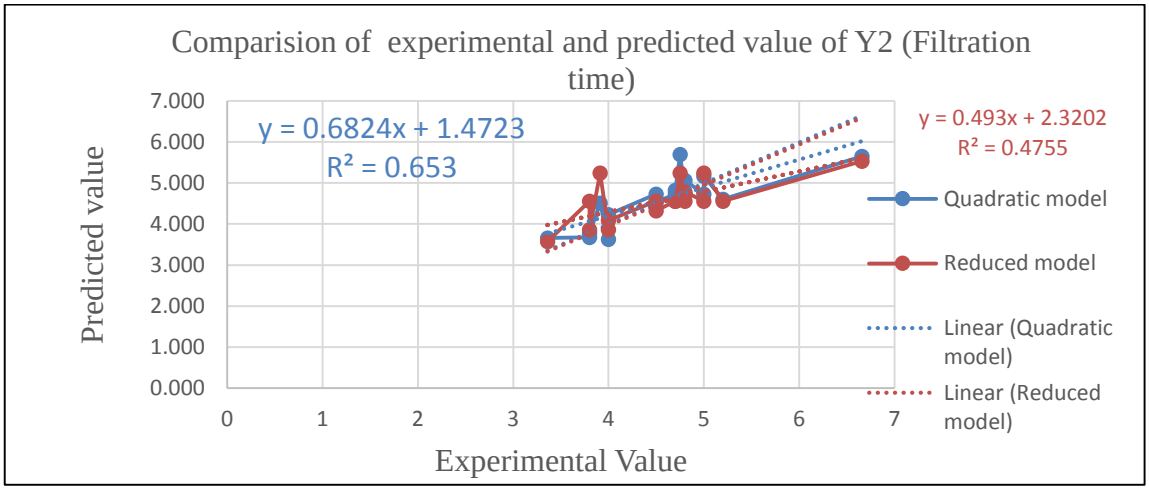


Figure.49: ***Accuracy of the Response Y2 (Filtration time)***

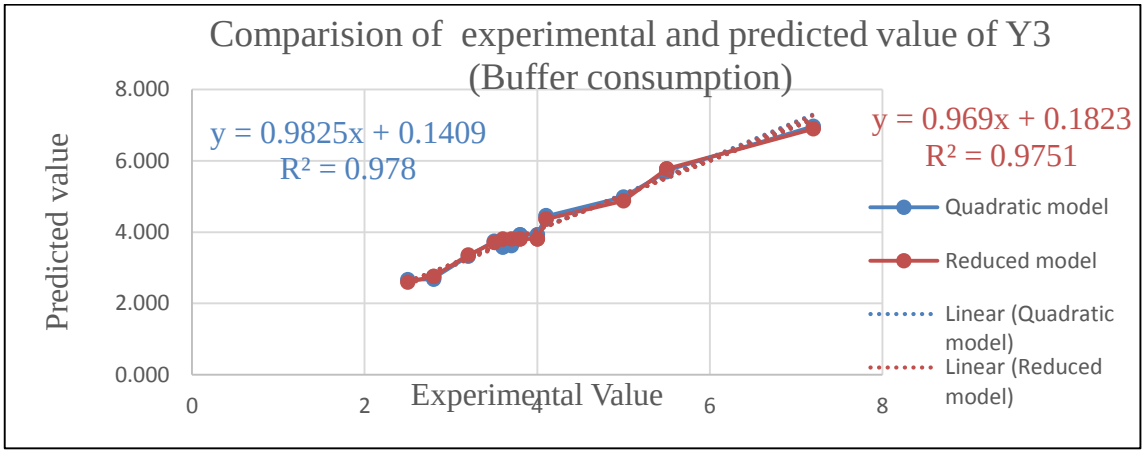


Figure. 38: ***Accuracy of the Response Y3 (Buffer consumption)***

like X1 and X2 (TMP and CF).But the Filtration time not sufficiently predicted from the design space.

Accuracy of the Response Y3 (Buffer consumption) :

For the response Y3, R^2 value of quadratic and reduced model was 0.978 and 0.9751, respectivly. Quadrtic model give the best idea about the Response Y3(Flux) for the independent variable like X1 and X2 (TMP and CF).R^2 value of the both the model was good, So design space give the best idea about the reponse Y3.

Conclusion :

It was observed, all the points were very close to their predicted values even though, those data points outside the testing domain and the model was robust and accurate for flux and buffer consumption and slightly significant for filtration time as per different plot. Due to its high prediction accuracy, the countour plot that was obtained here for responses also serves as the design space for predicting and controlling filtrate flux, filtration time and buffer consumption.

5.10 Case study:

For the optimization of the process different design mode of the filtration process was analyzed.

5.10.1 Concentration*Diafiltration Trail

Procedure:

Conduct the diafiltration process at concentrate first to the (2.5X) and then after the difiltration at the 8 DV. Maintaining the Transmembrane Pressure (TMP) in the desire range throughout the process.

Table 5. 50 ***Concentration mode:***

Concentration mode	Time (min)	Cu. Time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Retentate (ml)	Inlet (psi)	Outlet (psi)	Pressure drop (psi)	TMP (psi)	TMP (bar)	Conductivity (mS/cm)
	0	0	0	0	0	0	0	1000	0	0	0	0	0	33.41
	10	10	200	200	20	1.200	14.12	800						
2 X	6	16	100	300	18.75	1.125	13.24	700						
	7	23	100	400	17.3913	1.043	12.28	600	7.5	2	5.5	4.75	0.323	31.85
	7	30	100	500	16.6667	1.000	11.76	500						
2.5 X	15	45	100	600	13.3333	0.800	9.41	400						
Total		**0.75**		**600**	**17.2283**	**1.034**	**12.16**	**400**						

Table 5. 51 ***Diafiltration mode:***

DV	Time (min)	Cu. time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Inlet (psi)	Outlet (psi)	Pressure Drop	TMP (psi)	TMP (bar)	Conductivity (mS/cm)
0	0	0	0	0	0	0	0	0	0	0	0	0.00	31.85
1	25	25	400	400	16.00	0.96	11.29	7.5	2	5.5	4.75	0.32	23.93
2	25	50	400	800	16.00	0.96	11.29	8	2	6	5	0.34	12.40
3	26	76	400	1200	15.79	0.95	11.15	10	5	5	7.5	0.51	4.50
4	26	102	400	1600	15.69	0.94	11.07	10	5	5	7.5	0.51	1.24
5	24	126	400	2000	15.87	0.95	11.20	10	5	5	7.5	0.51	0.58
6	19	145	400	2400	16.55	0.99	11.68	10	5	5	7.5	0.51	0.23
7	17	162	400	2800	17.28	1.04	12.20	10	5	5	7.5	0.51	0.11
8	16	178	400	3200	17.98	1.08	12.69	10	4.5	5.5	7.25	0.49	0.07
Total		**2.97 hr**		**3200**	**16.40**	**0.98**	**11.57**						

5.10.2 Diafiltration* Concentration Trail

Procedure:

Conduct the diafiltration process at first difiltration at the 8 DV and then after then after concentrate to the 2.5X.Maintaining the Transmembrane Pressure (TMP) in the desire range throughout the process.

Table 5. 52 ***Diafiltration mode:***

DV	Time (min)	Cu. Time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Retentate (ml)	Inlet (psi)	Outlet (psi)	Presure drop (psi)	TMP (psi)	Conductivity (mS/cm)
(1000 ml)	0	0	0	0	0.00	0.00	0.00	1000	0	0	0	0	0
1	72	72	1000	1000	13.89	0.83	9.80	1000	8	2	6	5	24.5
2	73	145	1000	2000	13.79	0.83	9.74	1000	8	2	6	5	11.5
3	45	190	1000	3000	15.79	0.95	11.15	1000	10	5	5	7.5	7.8
4	35	225	1000	4000	17.78	1.07	12.55	1000	10	5	5	7.5	2.3
5	40	265	1000	5000	18.87	1.13	13.32	1000	10	5	5	7.5	0.65
6	20	285	1000	6000	21.05	1.26	14.86	1000	10	5	5	7.5	0.32
7	15	300	1000	7000	23.33	1.40	16.47	1000	10	5	5	7.5	0.16
8	20	320	1000	8000	25.00	1.50	17.65	1000	10	5	5	7.5	0.08
Total		5.33		8000	18.69	1.00	13.19	1000					

Table 5. 53 ***Concentration mode:***

Concentration mode	Time (min)	Cu. Time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Retentate (ml)	Inlet (psi)	Outlet (psi)	Presure drop (psi)	TMP (psi)	TMP (bar)	Conductivity (mS/cm)
	0	0	0	0	0.00	0	0	1000	0	0	0	0	0	0.08
	10	10	200	200	20.00	1.200	14.12	800						0.072
2 X	6	16	100	300	18.75	1.125	13.24	700						0.048
	8	24	100	400	16.67	1.000	11.76	600	7.5	2	5.5	4.75	0.323	0.02
	8	32	100	500	15.63	0.938	11.03	500						0.018
2.5 X	16	48	100	600	12.50	0.750	8.82	400						0.015
Total		0.80		600	16.71	1.003	11.79	400						

5.10.3 Concentration * Diafiltration * Concentration Trail

Procedure:

Conduct the diafiltration process at concentrate first to the (2X) and then after the difiltration at the 8 DV. Again the concentrate to the feed solution at the 2.5 X. Maintaining the Transmembrane Pressure (TMP) in the desire range throughout the process.

Table 5. 54 ***Concentration mode:***

Concentration mode	Time (min)	Cu.time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Retentate (ml)	Inlet (psi)	Outlet (psi)	Pressure Drop (psi)	TMP (psi)	TMP (bar)	Conductivity (mS/cm)
2X	0	0	0	0	0	0	0	1000	0	0	0	0	0	32.41
	10	10	200	200	20.00	1.20	14.12	800	7.5	2	5.5	4.75	0.323	31.56
	6	16	100	300	18.75	1.13	13.24	700						
	8	24	100	400	16.67	1.00	11.76	600						
	8	32	100	500	15.63	0.94	11.03	500						
Toatal		**0.53**		**500**	**17.76**	**1.07**	**12.54**	**500**						

Table 5. 55 ***Diafiltration mode:***

DV	Time (min)	Cu. Time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Inlet (psi)	Outlet (psi)	Pressure drop	TMP (psi)	TMP (bar)	Conductivity (mS/cm)
0	0	0	0	0	0	0	0	0	0	0	0	0	32.410
1	33	33	500	500	15.16	0.910	10.70	7.5	2	5.5	4.75	0.323	23.930
2	38	71	500	1000	14.18	0.851	10.01	8	2	6	5	0.340	12.400
3	41	112	500	1500	12.3	0.738	8.68	10	5	5	7.5	0.510	4.500
4	38	150	500	2000	13.21	0.793	9.68	10	5	5	7.5	0.510	1.239
5	34	184	500	2500	14.88	0.893	10.68	10	5	5	7.5	0.510	0.576
6	28	212	500	3000	18.52	1.111	11.68	10	5	5	7.5	0.510	0.232
7	25	237	500	3500	20.33	1.220	12.68	10	5	5	7.5	0.510	0.110
8	25	262	500	4000	20.33	1.220	13.68	10	4.5	5.5	7.25	0.493	0.070
Total		**4.367 Hr**		**4000**	**16.114**	**0.967**	**10.98**						

Table 5. 56 ***Concentration mode:***

Comcentration mode	Time (min)	Cu. Time (min)	Filtrate (ml)	Cu. Filtrate (ml)	Flow Rate (ml/min)	Flow Rate (L/Hr)	Filtrate Flux (LMH)	Retentate (ml)	Inlet (psi)	Outlet (psi)	TMP (psi)	TMP (bar)	Conductivity (mS/cm)
2 X	0	0	0	4000		0.000	0.0000	500	0	0	0	0	0.025
2.5 X	5	5	100	4100	20.00	1.200	14.12	400	10	5	7.5	0.5102	0.02
3.3 X	8	13	100	4200	7.69	0.462	5.43	300	11	5	8	0.5442	0.017
Total		**0.217 Hr**		**4200**	**13.85**	**0.831**	**9.77**	**300**					

Result: On the basis of the different mode of filtration process Diafiltration operation optimized by total filtrate time for the filtration and the total permeate volume for further

analysis. First case A, concentration mode then after Diafiltration mode produce less total filtration time and total permeate volume compare to other mode. While in the case B first Diafiltration and then after concentration mode produce more filtration time and permeate volume compare with other mode. Case C having the intermediate filtration time and permeate volume. All the results described in the table.

Table 5. 57 ***Comparison of Different Concentration/Diafiltration combinations for dialysis processing of the liposome. (Objective: 2.5 X Concentration and 8 (N) Diafiltration Volume)***

	Batch Volume (ml)	Liposome concentration (X)	Filtrate Rate (L/h)	Filtrate Flux (LMH)	Permeate Volume (ml)	Filtrate Time (hr)
CASE A						
Initial	1000	1				
Conc. X 2.5	400	2.5	1.034	12.16	600	0.75
Diaf. X 8	400	2.5	0.98	11.57	3200	2.97
Total					3800	3.72
CASE B						
Initial	1000	1				
Diaf. X 8	1000	1	1.00	13.19	8000	5.33
Conc. X 2.5	1000	2.5	1.003	11.79	600	0.80
Total					8600	6.13
CASE C						
Initial	1000	1				
Conc. X 2.0	500	2	1.07	12.54	500	0.53
Diaf. X 8	500	2	0.967	10.98	4000	4.367
Conc. X 2.5	400	2.5	1.2	14.12	100	0.083
Conc X 3.3	300	3.3	0.831	9.77	200	0.217
Total(2.5X)					4600	4.98
Total(3.3X)					4700	5.114

Conclusion: For better purification of the nanoparticle and maintaining all the physical parameter of the process, Case C was ideal mode of the Diafiltration process; Although Case A having less filtration time and permeate volume.

Ideal Mode:

5.11 Impact of purification process on the physical parameter of the product

In process to check the physical parameters like Particle size, Zeta potential, Viscosity, Lipid content, pH, conductivity determine.

- Particle size & zeta potential measured by the Malvern zetasizer.
- pH & conductivity measured by the pH & conductivity meter. (Thermo scientific)
- Lipid content determine by the lipid assay.
- Osmolarity determine by the Advanced Instrument Osmometer.
- Viscosity determine by the viscometer.

Table 5. 58 **Before Process:**

Sr No.	TMP	Particle size (nm)	Zeta potential (mV)	pH	Conductivity (mS/cm)	Lipid content (% w/v)	Osmolarity (mOsmol)
1	**5 psi**	**96.54 ±0.26**	**-16.4 ± 0.35**	**5.5**	**32.41**	**98.56**	**265 ± 5**
2	**8 psi**	**99.43 ±3.15**	**-13.0 ± 5.57**	**5.5**	**33.56**	**97.87**	**275± 6**

Table 5. 59 ***After Process (Particle size and Zeta potential):***

Sr No.	Mode	Particle size (nm)		Zeta potential (mV)	
		TMP		TMP	
		5 psi	8 psi	5 psi	8 psi
1	Concentration (2X)	94.27± **4.3**	97.89 ± **0.4**	-9.72± **0.45**	-15.9± **1.19**
2	Concentration (3X)	97.40± **0.3**	97.40± **0.2**	-9.19 ± **0.27**	-10.4± **0.52**
3	Diafiltration (7DV)	92.45 ± **0.7**	88.21± **4.6**	-5.39 ± **0.63**	-8.73± **0.44**
4	Diafiltration(10DV)	91.88± **1.0**	90.25 ± **0.5**	-4.08 ± **0.52**	-10.6± **0.20**

Table 5. 60 ***After Process (pH, conductivity and PDI):***

Sr No.	Mode	pH		Conductivity (mS/cm)		PDI	
		TMP		TMP		TMP	
		5 psi	8 psi	5 psi	8 psi	5 psi	8 psi
1	Concentration (2X)	5.45	5.5	30.75	31.56	0.036	0.077

2	Concentration (3X)	5.40	5.42	29.32	30.25	0.072	0.072
3	Diafiltration (7DV)	5.5	5.32	0.085	0.056	0.056	0.058
4	Diafiltration(10DV)	5.8	5.45	0.023	0.025	0.067	0.084

Table 5. 61 ***After Process (Lipid content and Osmolarity):***

Sr No.	**Mode**	**Lipid content (% w/v)**		**Osmolarity (mOsmol)**	
		TMP		**TMP**	
		5 psi	**8 psi**	**5 psi**	**8 psi**
1	Concentration (2X)	93.56	94.56	298	302
2	Concentration (3X)	92.23	92.31	287	295
3	Diafiltration (7DV)	91.56	91.58	285	297
4	Diafiltration(10DV)	89.98	90.24	283	284

Result:

Diafiltration process performing at the 5 psi and 8 psi no significant change occur in the particle size in both the process concentration and Diafiltration. Sometime there is slightly decreased in the particle size in the process. No significant change occur in the PDI and pH of the formulation. Lipid content of the formulation slightly change in the diafiltration process, it was based on the cartridge membrane filter pore size. Small amount of the lipid loss in the process which was absorbed on the filter membrane. Conductivity of the formulation change in the concentration mode and drastically change in the diafiltration mode at the TMP of 5 psi and 8 psi.

Conclusion:

There was not significant changes occur in physical parameters of the final formulation during the diafiltration process which occur at the trans-membrane pressure 5 psi and 8 psi, respectively. Only conductivity was decreased during the concentration and diafiltration mode of the process, due to elimination of ammonium and sulphate ions from the external medium of the liposomal formulation.

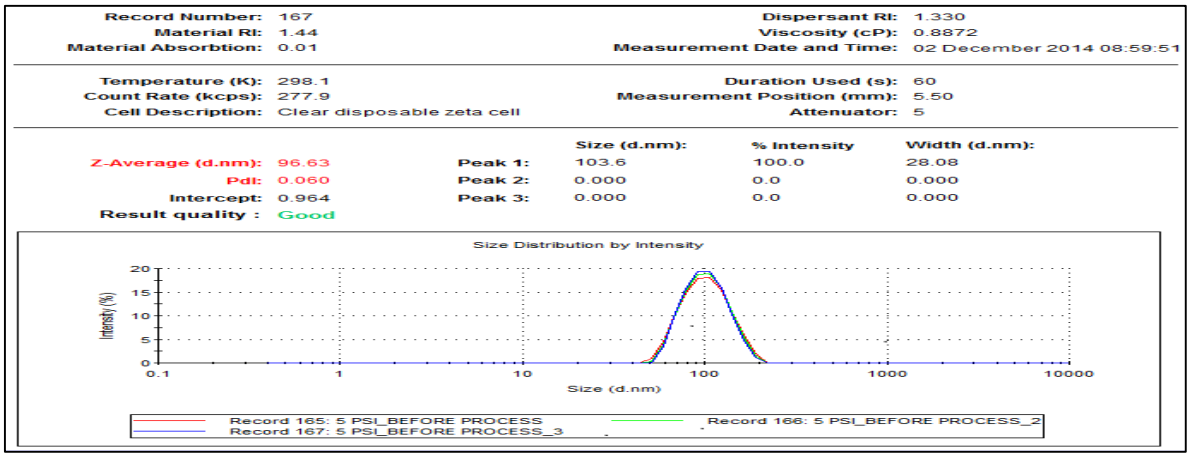


Figure. 39: ***Before Process at 5 psi***

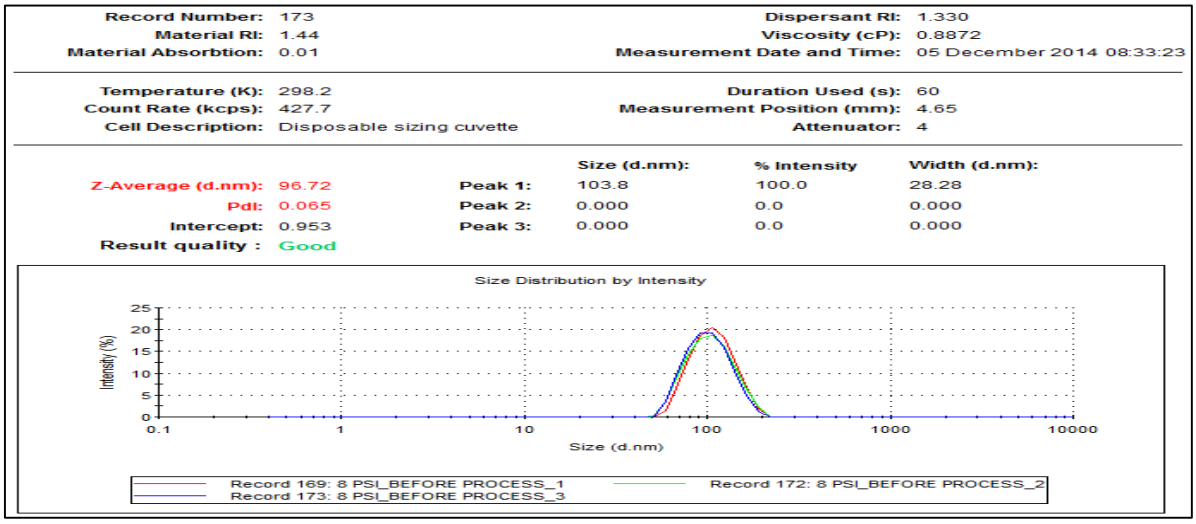


Figure. 40: ***Before Process at 8 psi***

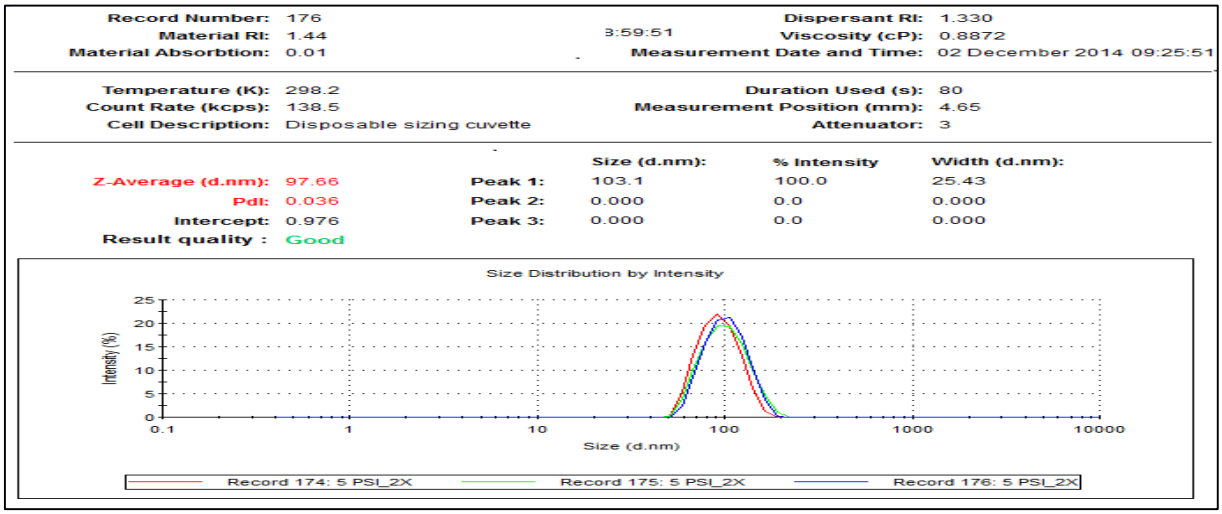


Figure. 41: ***Concentration (2X) at 5 psi***

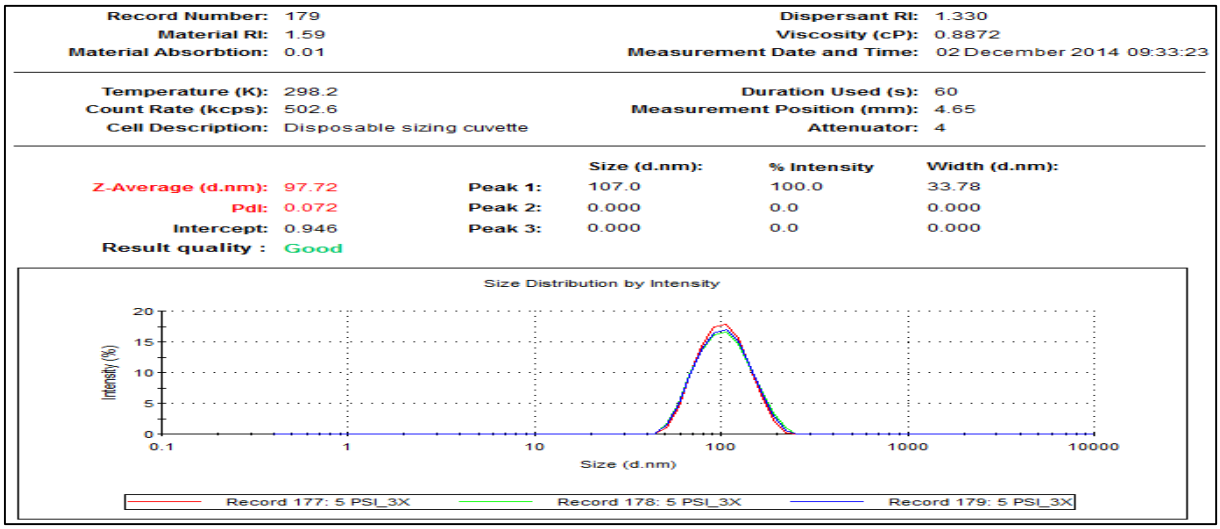


Figure. 42: ***Concentration (3X) at 5 psi***

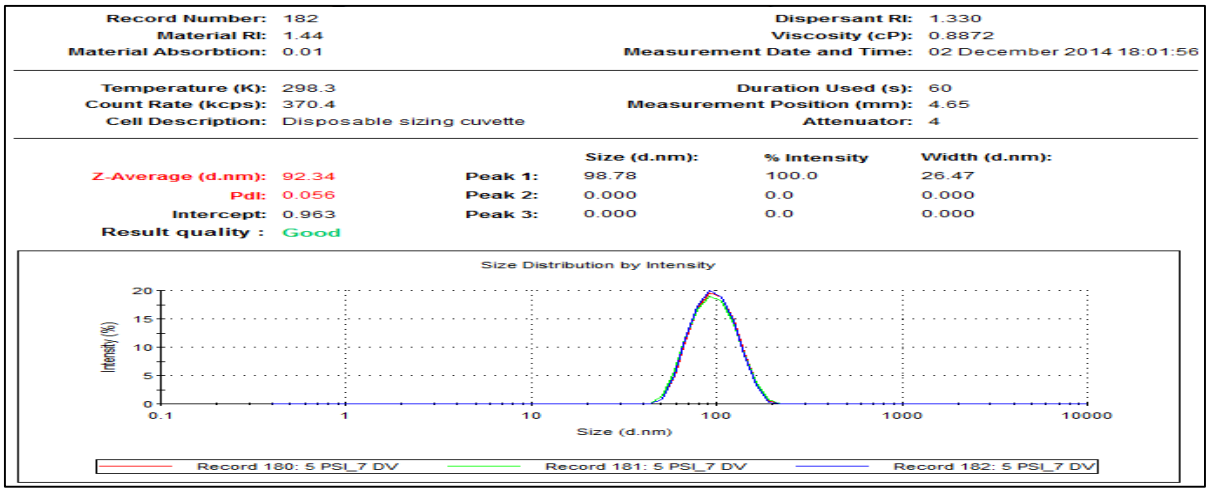


Figure. 43: ***Diafiltration (7 DV) at 5 psi***

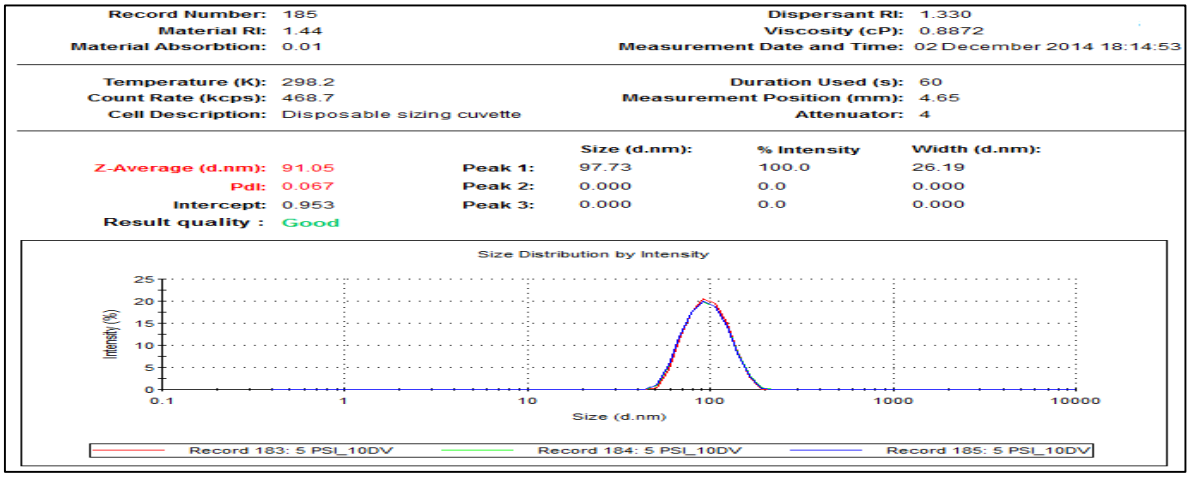


Figure. 44: ***Diafiltration (10 DV) at 5 psi***

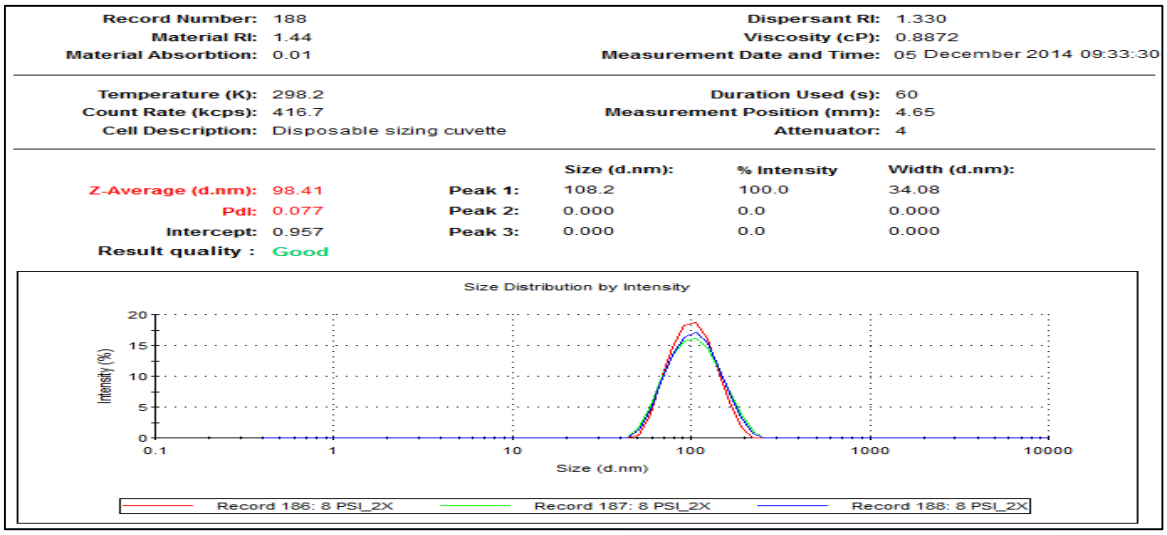


Figure. 45: ***Concentration (2X) at 8 psi***

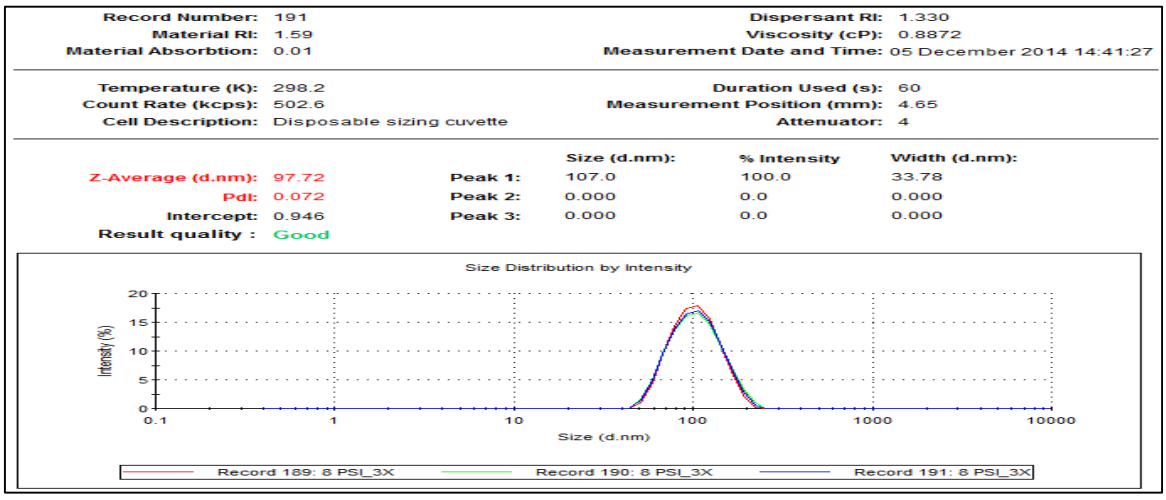


Figure. 46: ***Concentration (3X) at 8 psi***

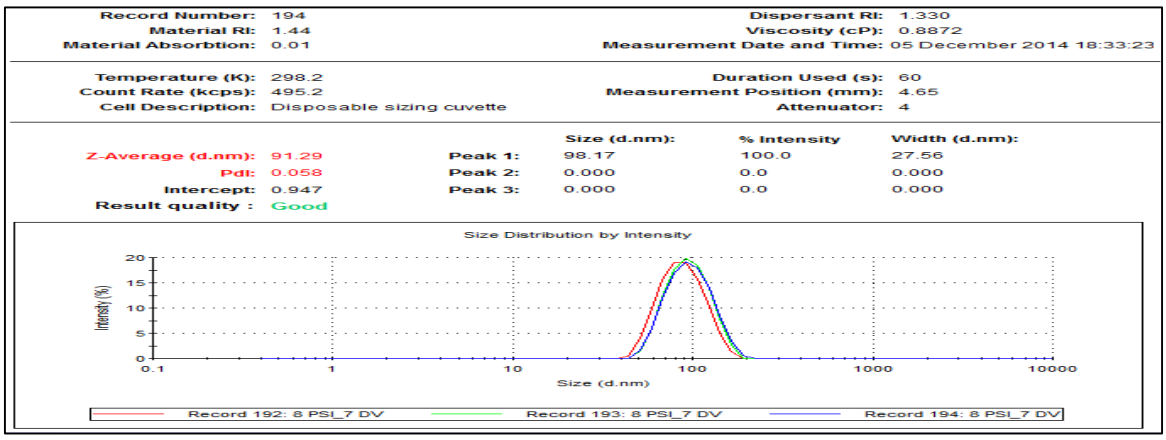


Figure. 47: ***Diafiltration (7 DV) at 8 psi***

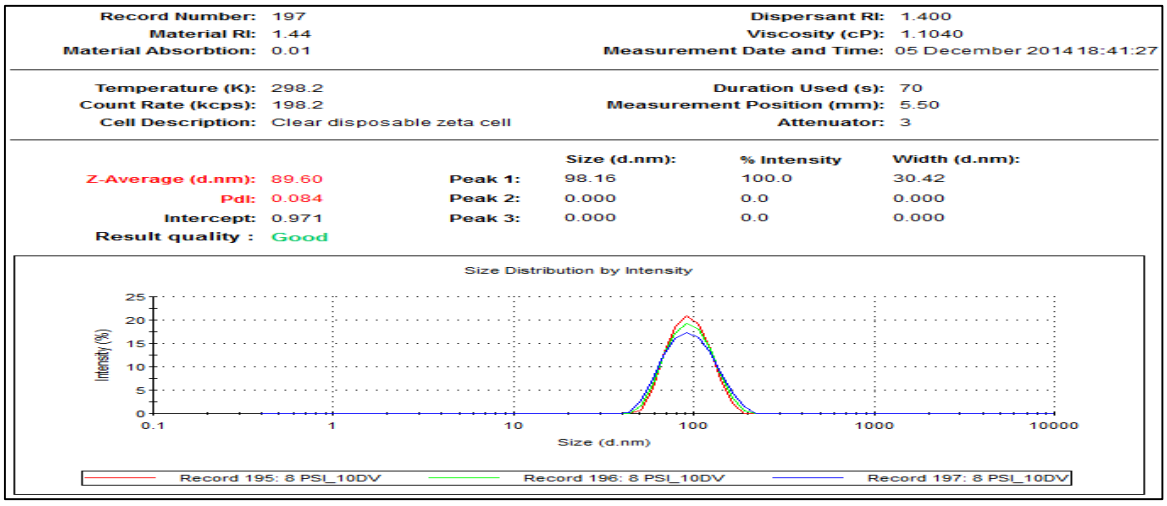


Figure. 48: ***Diafiltration (10 DV) at 8 psi***

Zeta potential graph:

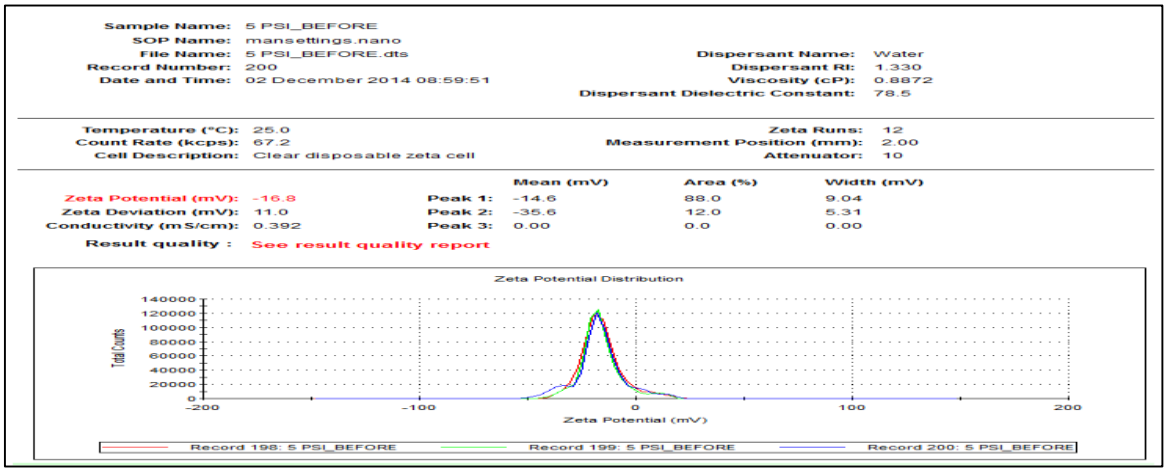


Figure. 49: ***Before Process at 5 psi***

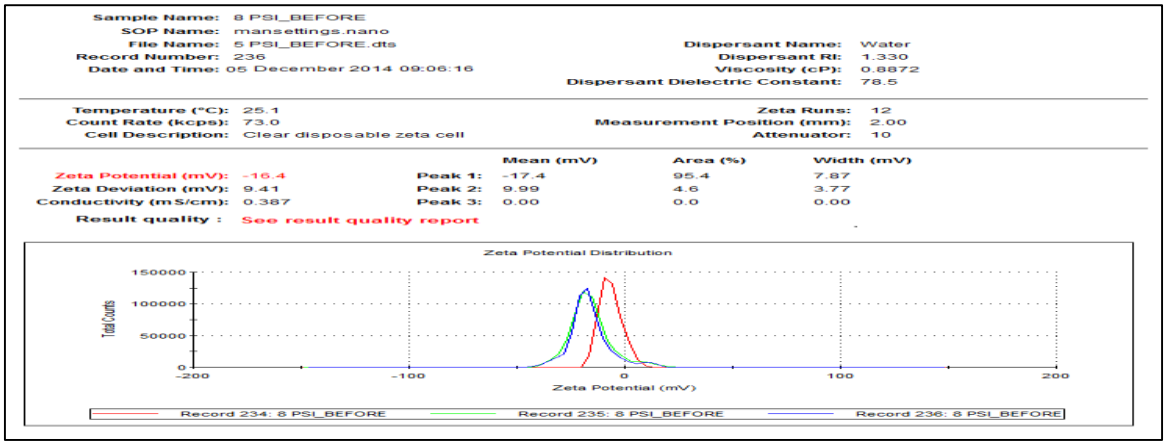


Figure. 50: ***Before Process at 8 psi***

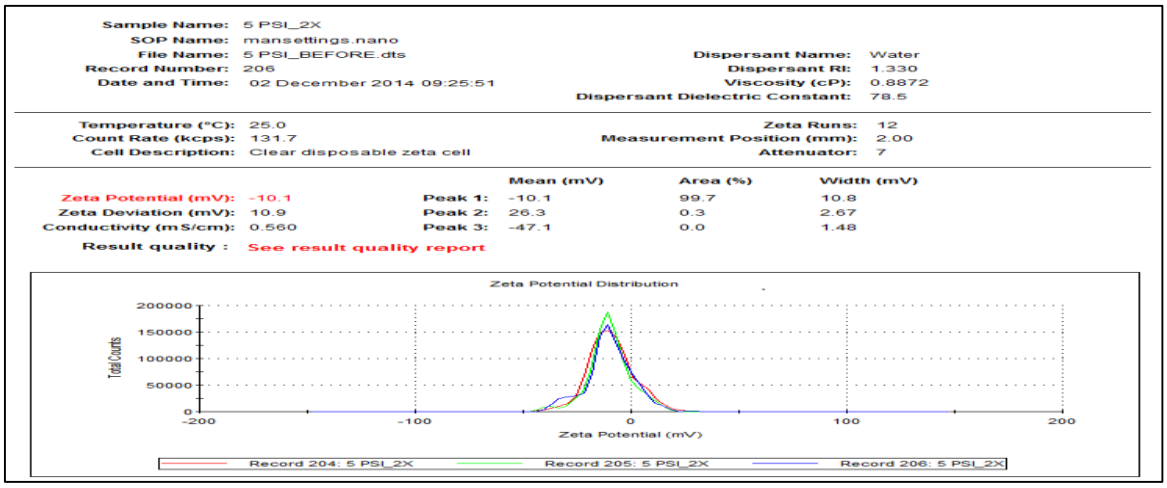


Figure.651: ***Concentration (2X) at 5 psi***

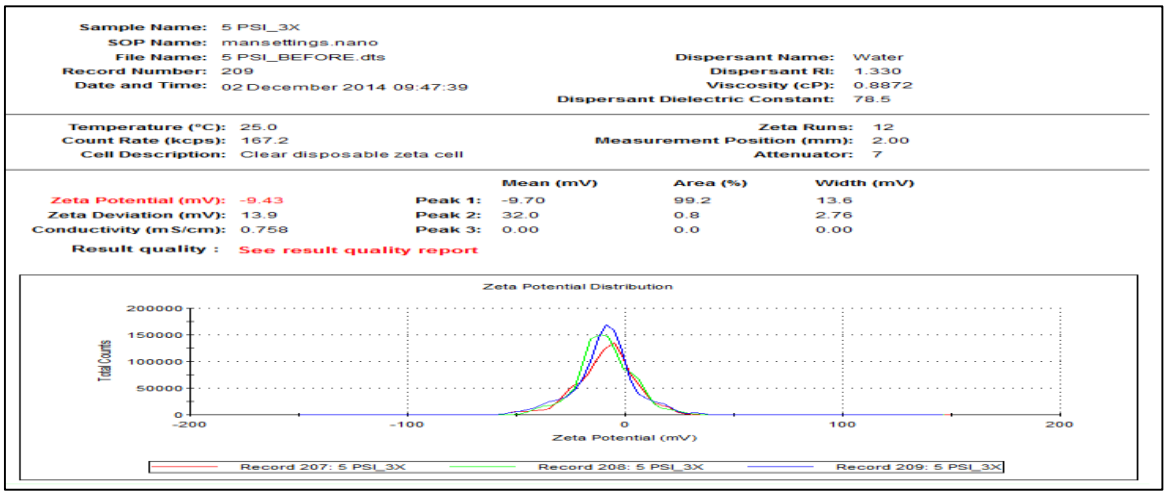


Figure. 52: ***Concentration (3X) at 5 psi***

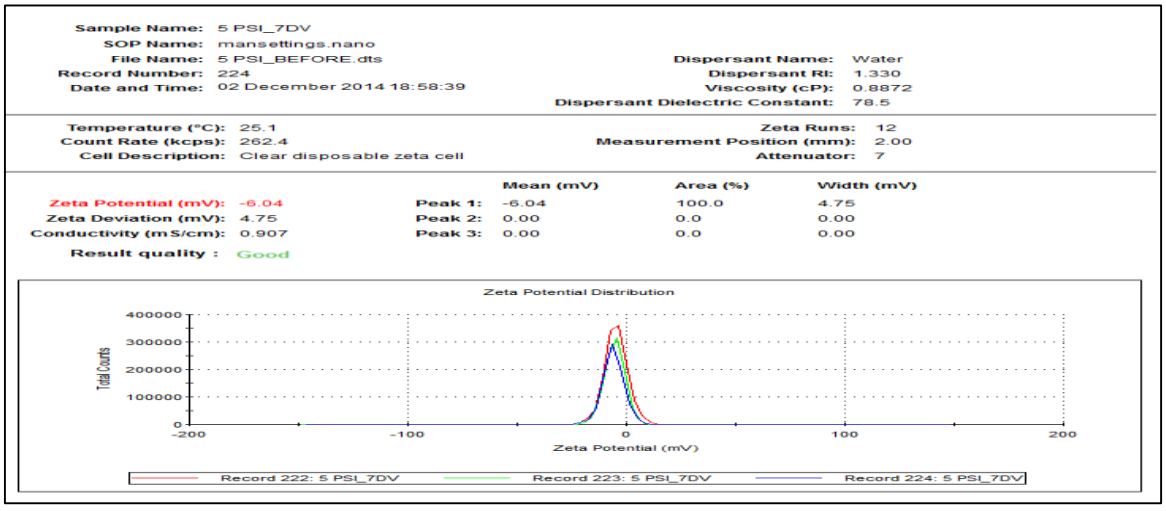


Figure. 53: ***Diafiltration (7 DV) at 5 psi***

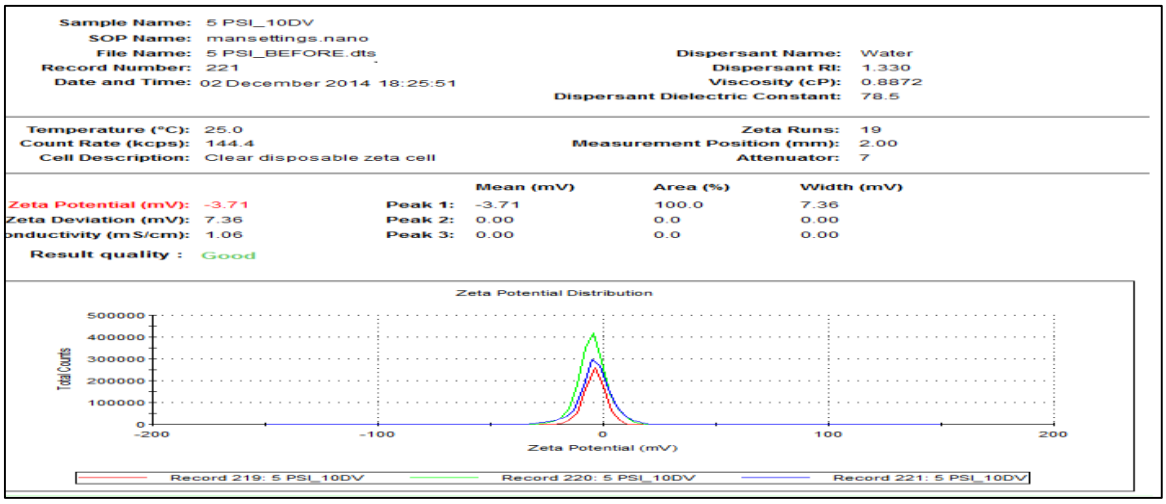


Figure. 54: ***Diafiltration (10 DV) at 5 psi***

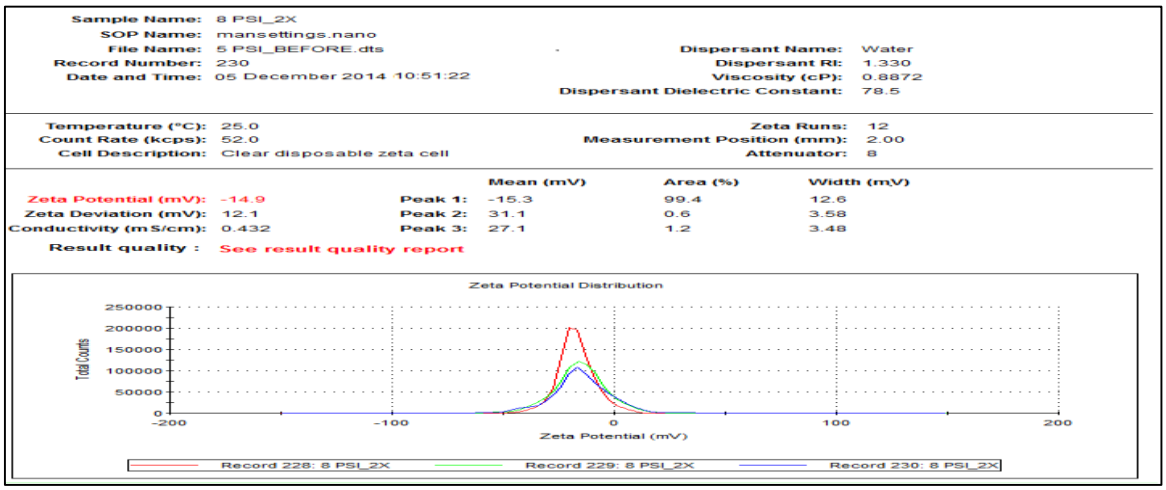


Figure. 55: ***Concentration (2X) at 8 psi***

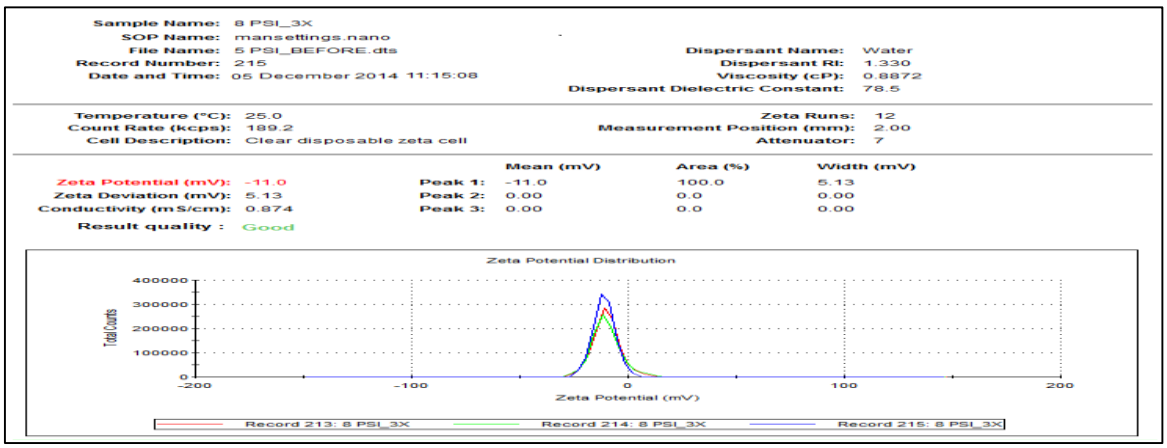


Figure. 56: ***Concentration (3X) at 8 psi***

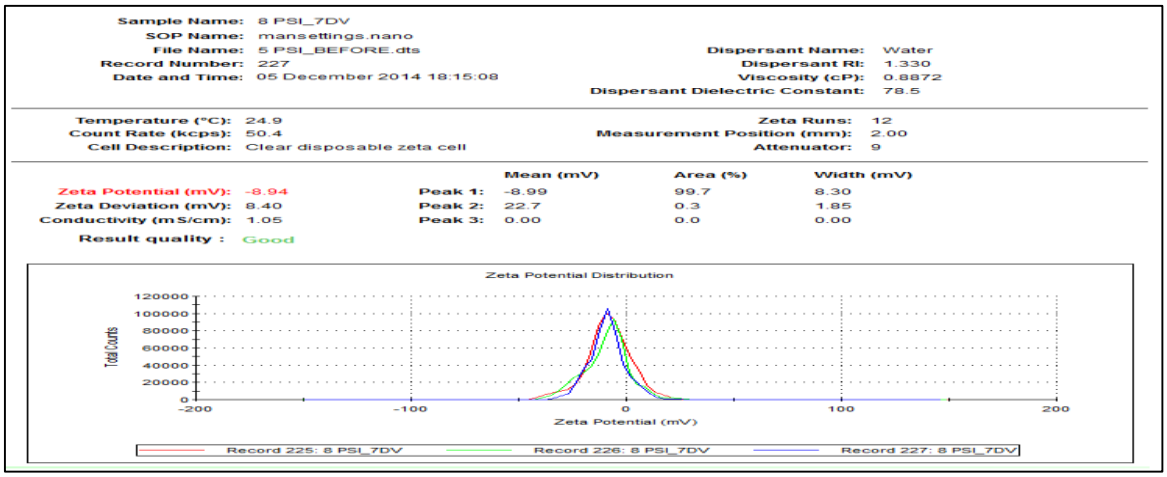


Figure. 57: ***Diafiltration (7 DV) at 8 psi***

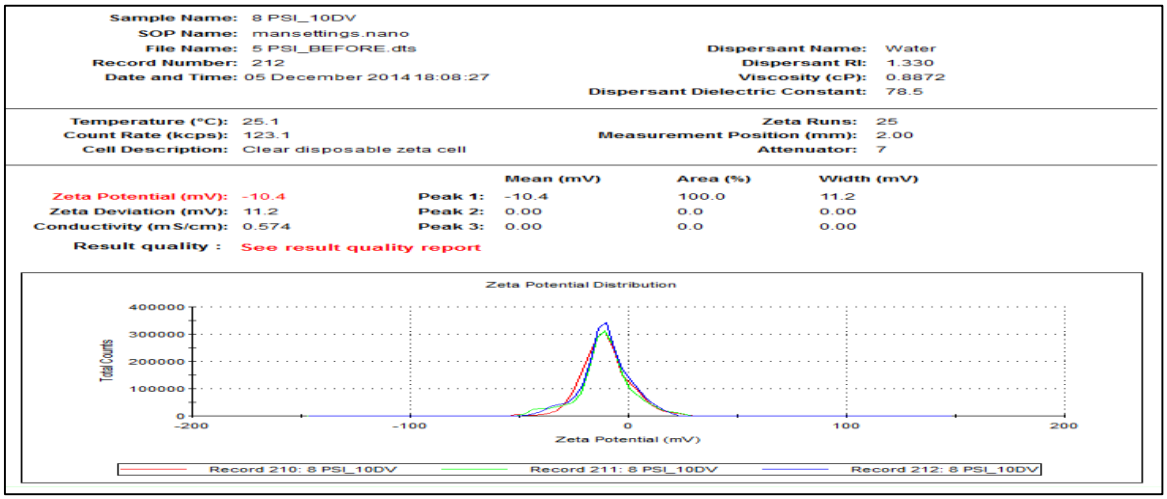


Figure. 58 ***Diafiltration (10 DV) at 8 psi***

5.12 Drug loading:

Table 5. 62: Drug loading in the different sucrose concentration

Sr No.	Drug (mg/ml)	Sucrose solution (% w/v)	EE %	Osmolarity (mOsmol)	pH	Partic-le size (nm) Mean ± S.D	PDI	Zeta Potential (mV) Mean ± S.D
1	2	8	95.12	260	5.65	84.17 ± 5.343	0.067	-22.6 ± 5.00
2	2	10	97.42	295	5.75	91.88 ± 1.014	0.058	-26.4 ± 1.65
3	2	12	96.54	320	5.65	88.21 ± 4.618	0.004	-19.5 ± 5.03

Procedure:

- Drug was dissolved in a glass container with 10 % sucrose solution at a temperature of 60^0 C. A sufficient quantity of histidine was added to the drug solution and after transferred to the liposomal suspension obtained after dialysis process.
- Suspension was maintained at then maintained at a temperature at 60^0 C for about 30 min.After that suspension was cooled at the temperature of 2^0 C to 4^0 C and incubate for 30 min. Final volume make up with the 10 % sucrose solution.

Result:

Drug loaded with the solution of the histidine buffer in the different concentration of the sucrose solution (8, 10, 12 % w/v). 10 % sucrose solution produce the desire osmolarity of the formulation compare with other concentration. Encapsulation efficiency and particle size result show that no significant changes occur due to different concentration of the sucrose solution.

Drug loading in the 8 % sucrose solution:

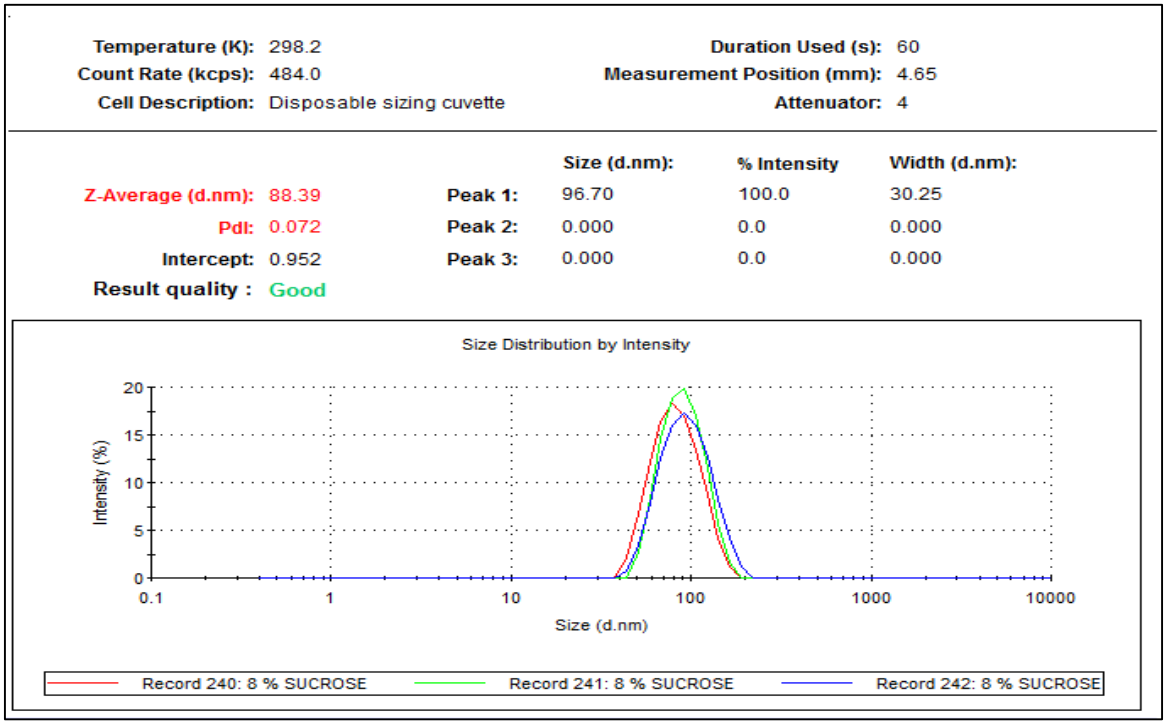


Figure. 59: ***Particle size report***

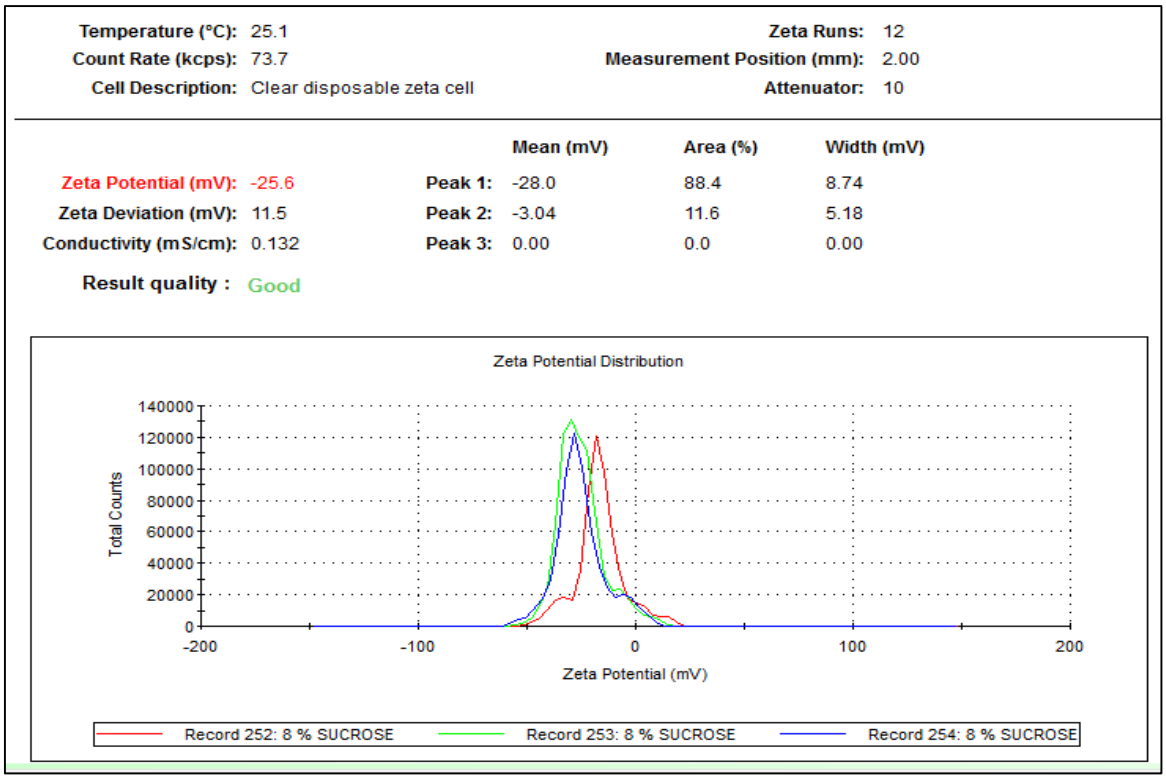


Figure. 60: ***Zeta potential report:***

Drug loading in the 10 % sucrose solution:

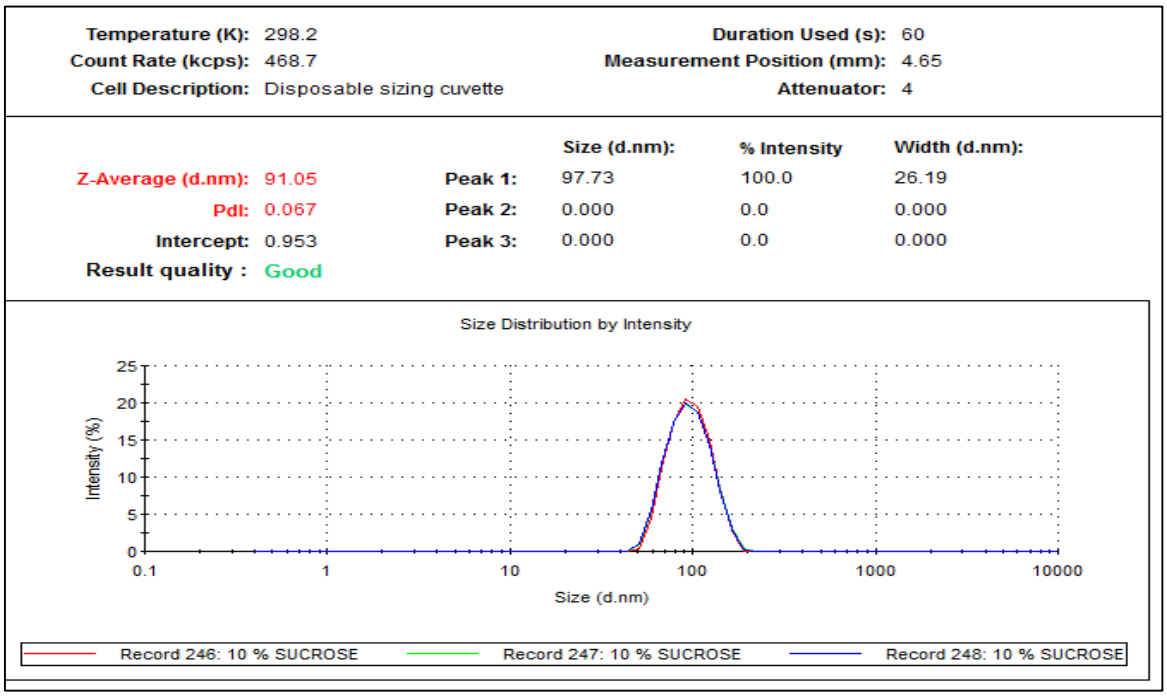


Figure. 61: ***Particle size report:***

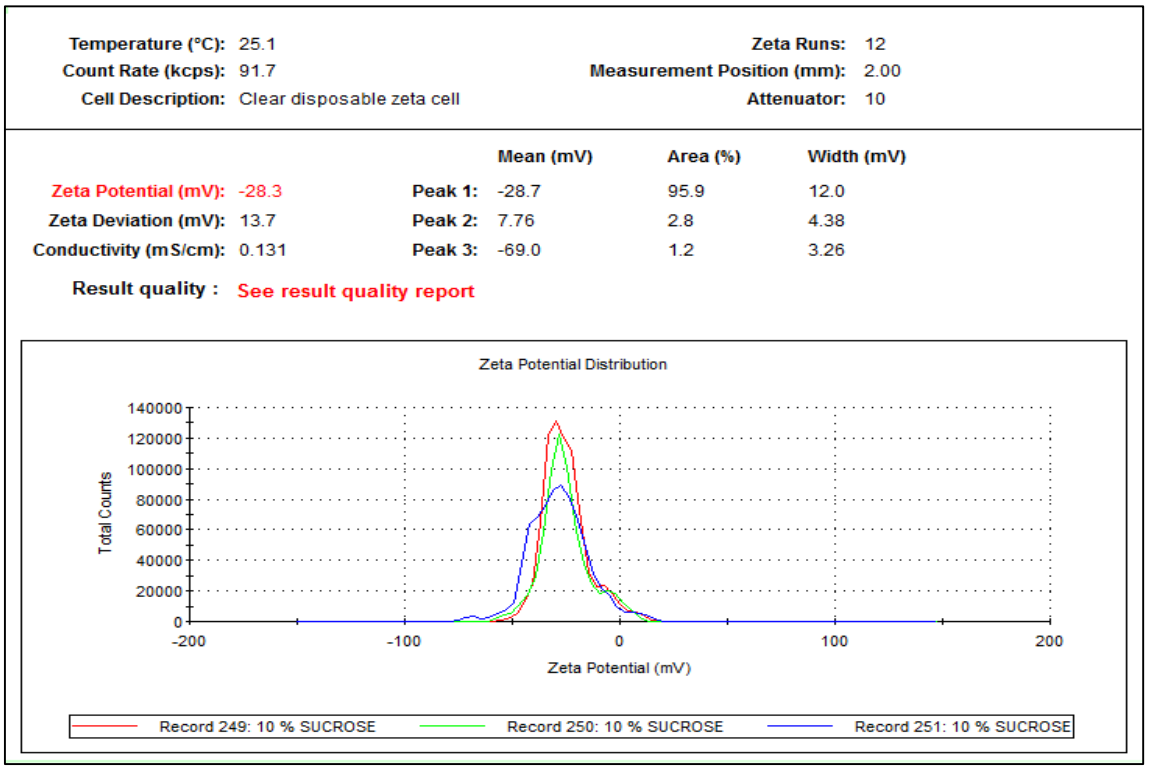


Figure. 62: ***Zeta potential report:***

Drug loading in the 12 % sucrose solution:

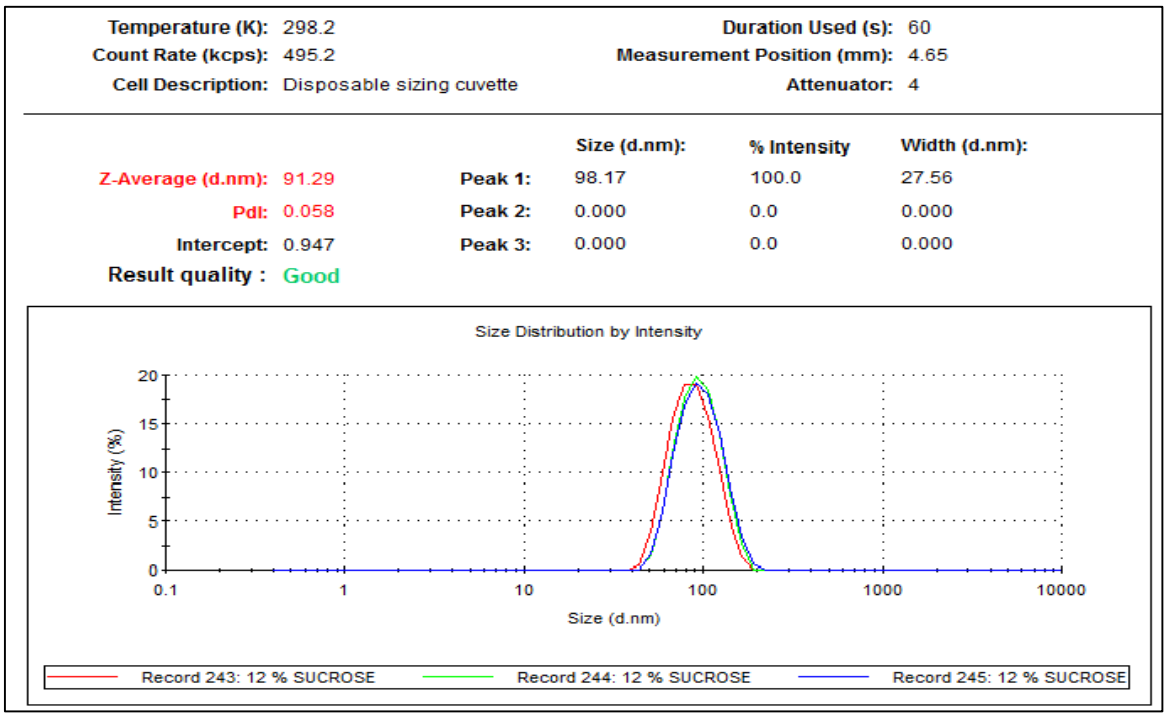


Figure. 63: ***Particle size report:***

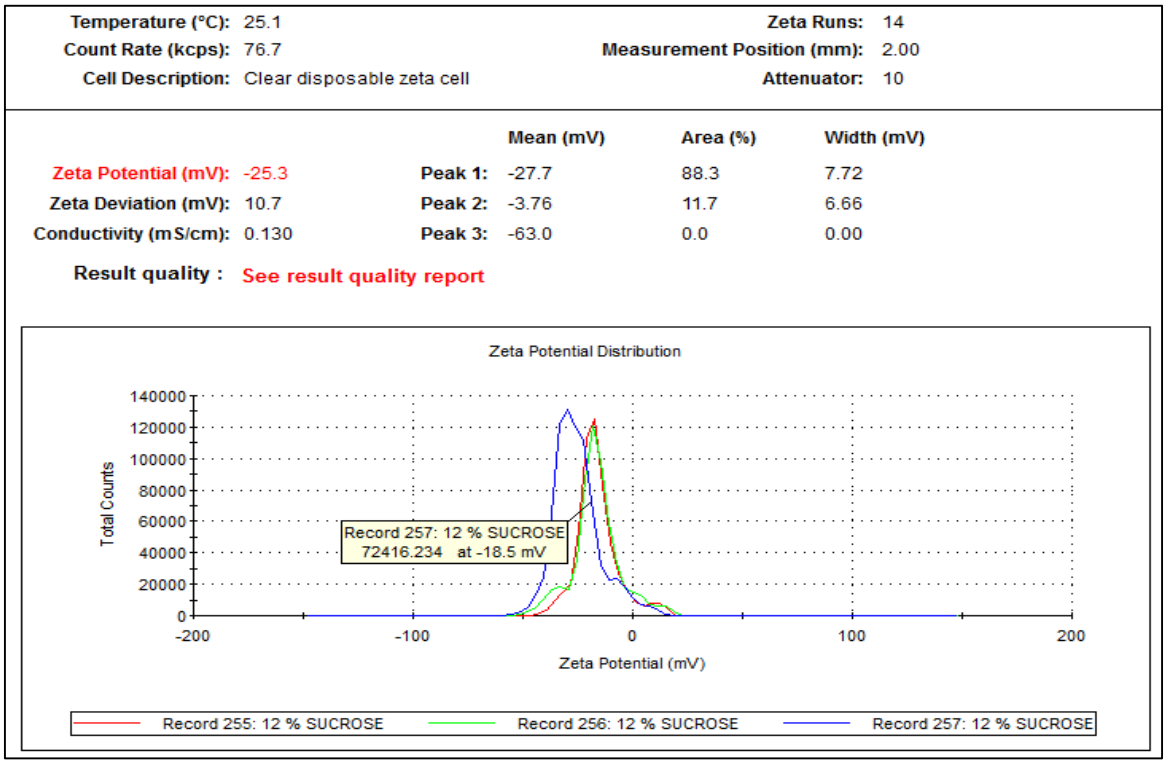


Figure. 64: ***Zeta potential report:***

5.13 Effect of external ion concentration on the free drug content

Procedure:

During the dialysis process, a sample of 10 ml was withdrawn at different external ion concentration level of permeate at 25,100, 150 and 200 ppm. Calculate the conductivity of the sample in the triplicate.The samples having different external ion concentration were then subjected to drug loading and the final sample were subjected to % assay, % EE and % free drug content by the analysis.% Assay determined by the Steward Assay and Berlet assay of the lipid analysis by the analytical department**.** % EE were determine by the extraction method of the HLB Cartridge.Determine the optimum external ion concentration for the effective drug encapsulation. (> 95%)

Table: *5. 63 : Effect of different concentration of the external buffer to the Encapsulation*

Sr No.	Conductivity (mS /cm)	External ion concentration (ppm) (approximately)	Assay (%) of drug content	Entrapment (%)	Free drug content (%)
1	30.98 ± 1.2	200	98.79	85.45	14.55
2	22.08 ± 2.7	150	99.50	88.00	12.00
3	16.86 ± 2.1	100	99.80	95.12	4.88
4	4.56 ± 1.2	25	99.46	96.06	2.28

Observation 5. 64 : Absorbance of the sample

Sr No.	Sample ID	Absorbance	Mean ± S.D
1	Blank	0.000	0.00
2	Standard-1	0.873	0.8817 ± 0.0076
3	Standard-2	0.885	
4	Standard-3	0.887	
5	Total-1	1.016	1.0223 ± 0.0055
6	Total-2	1.025	
7	Total-3	1.026	
8	Entrapped-1	1.016	1.0097 ± 0.0071
9	Entrapped-2	1.002	
10	Entrapped-3	1.011	
11	Free drug-1	0.043	0.0403 ± 0.0023
12	Free drug-2	0.039	
13	Free drug-3	0.040	
14	Std _Bkt	0.868	

Table 5. 65 : Calculation of encapsulation efficiency

Sample	Absorbance	ppm	ppm/10ml	mg/0.2 ml	mg /ml	EE %
Total drug	1.0223	46.37	463.79	0.464	2.319	96.06
Entrapped drug	1.0097	45.80	458.07	0.458	2.290	
Free drug	0.0403	1.82	18.28	0.018	0.091	

Result:

External ion concentration was determine approximately from the conductivity of the feed solution at the different time interval by the calibration curve of the conductivity. Assay % of the drug was determined at the different stages by the analytical department and the free drug content % was determine by the above method. Approximately less than 100 ppm of the external ion concentration was required for the higher encapsulation efficiency.

Conclusion:

The minimum external ion concentration was the major tool for the higher amount of the drug loading. In this case it was noted that the less than 100 ppm of the external ion concentration was beneficial for the higher encapsulation (> 95 %)

5.14 Unentrapped drug removed from the external buffer by the TFF:

Diafiltration step

Procedure:

After drug loading analyze the encapsulation efficiency of the liposome. Find out the free drug content of the liposome by the analytical technique. Free drug remove by the same buffer (10% sucrose) solution with the TFF system. Optimized the diafiltration volume for the desire drug removal. Calculate the free drug by the analytical method. (HLB cartridge SPE)

Table 5. 66 ***Unentrapped drug remove from the external buffer by the diafiltration step***

Sr No	Diafiltration volume (N)	Free drug content (%)			% drug remove
		Before	After	Difference	
1	1	5.6	5.2	0.4	7.14
2	2		4.4	1.2	21.42
3	4		3.0	2.6	46.42
4	6		1.8	3.8	67.85
5	8		0.8	4.8	85.71

Unentrapped drug remove from the external buffer by the TFF:

Concentration step

Procedure:

Repeat the removal process with the TFF Concentration step only and analyze the how much % free drug remove. Compare the diafiltration and concentration step for the TFF.

Table 5. 67 : Unentrapped drug remove from the external buffer by the concentration step

Sr No	Concentration mode (X)	Free drug content (%)			% drug remove
		Initial	Final	Difference	
1	1	5.6	5.3	0.3	5.35
2	1.5		4.9	0.7	12.5
3	2		4.2	1.4	25
4	2.5		3.5	2.1	37.5
5	3		2.7	2.9	51.78

Result:

In diafiltration mode, free drug remove from the 5.6 % to 0.8 % up to 8 diafiltration volume of the same buffer (10% sucrose) solution. In concentration mode, free drug remove from the 5.6 % to only 2.7 % up to 3 concentration factor. So, it was not effective than diafiltration step.

6. DISCUSSION

Optimization of the Tangential flow filtration including optimizing various process parameters like TMP effect on the flux, different flow rate effect on the flux, TMP scouting, concentration mode and diafiltration mode.

Optimizing the **first parameter**, at high TMP a significant gel layer formed on the cartridge and flux was constant, independent of the TMP. The optimal TMP range for efficient and economic operation is just before the gel layer starts influencing the flux. For the **second parameter**, it can be concluded that the optimum combination of the highest cross flow and TMP, just before gel layer accumulation, the highest flux rate will be achieved. At the low concentration, flux increases with TMP at all cross flow rates and there is no clear optimal space. For the **third parameter**, At the high liposomal concentration, the curves flatten out at high TMP values, indicating that the formation of a concentration gradient is start to restrict flux across the membrane. For the **fourth parameter**, Plotting flux*concentration factor against concentration factor enables the optimization of diafiltration time. For the **fifth parameter**, Diafiltration step was essential to remove the buffer & salt ions from the feed. Approximately 8 Diafiltration Volume was sufficient to remove majority of the salt ions from the feed.

Experimental design minimizes the number of experiments required to identify the most critical factors affecting the response. Experimental design is the ideal screening of the factor which affect the process response. To best use the screening design, a careful examination of all the potential high impact factors was analyzed. For this reason, it is essential to study a risk analysis of all the factor which affect the particle size, encapsulation efficiency and stability of the formulation. Ishikawa diagram describe the major independent variables which are affected the liposomal encapsulation efficiency.

Major seven factors were identified which affect the Diafiltration process at very high process response and accuracy, but it cannot separate the main effects from the possible interactions. The goal of this **Plackett–Burman Design (PBD)** was to quickly reduce the high risk factors, such a design is sufficient. As shown in **Table 5.22**, out of seven factors only two are statistically significant, namely the **Transmembrane pressure (TMP)** and

Concentration Factor (CF). Number of Diafiltration volume having the important variable for the Diafiltration process. It was mainly affect the % salt reduced; but slightly affect the filtration time and volume of buffer consumption. If the Diafiltration volume fix at the level (DV=8) for further optimization, which give the desired % salt reduction (>99.9 %) from the external medium. Therefore, the other factor was easily optimized and better prediction achieved.

Feed flow of the formulation lesser extent affect the filtrate flux and osmolarity of the product. Concentration of the Diafiltration buffer solution was only affect the osmolarity of the product. Therefore feed flow and buffer concentration was fix at the level, 50 ml/min and 10% sucrose solution, respectively. Surface area of the membrane only affect the total filtration time. In general, increasing the surface area of the membrane, faster the filtration process and lesser the filtration time. So for better prediction of the other independent variable, 500 kD membrane cartridge (surface area=0.085m^2) was fix. Temperature of the process not significantly affect the Diafiltration responses; but for the stability of the product, temperature of the filtration process was set at 2^0 C to 10 ^{0}C. Higher temperature 50^0C to 60 ^{0}C (>Tg of the lipid) produce the degradation of the product and premature release of the liposome content.

In the **Placket-Burman design** all the response was analyzed based on the different chart like Residual, Pareto, Normal and Half normal chart which was generated by the Minitab.17.Pareto chart mainly describe the effect of the independent variable on the major response. Major independent variable TMP and concentration factor (CF) affect the diafiltration process.

Two factors, transmembrane pressure (TMP) and concentration factor (CF) was mainly identified as the key process parameter of the diafiltration process. Therefore, secondary screening design (**Central-Composite Design-CCD)** was based on these two independent variable and possible responses for these variable was filtrate flux, filtration time and buffer consumption. Based on the CCD, filtrate flux and buffer consumption fit the Quadratic model and the total filtration time fit the linear model of the ANOVA. ANOVA model give the best result of the filtrate flux (response Y1) and buffer consumption (response Y3)

because of the good r^2 value of the model; But it wasn't appropriate for the total filtration time (response Y2).

For the **filtrate flux (Y1)**, transmembrane pressure (TMP) and concentration factor (CF) inversely affect the flux. Increasing the TMP, produce the higher flux of the filtrate. But the increasing the TMP was in the desire range (10 -20 psi) for the product stability. Initially, starting concentration of the feed increase the flux of the filtrate and high volume of the filtrate passed out through the filter till the optimum concentration of the feed; then after achieving the desired level of the concentration, flux was decreased or stable. Identifying the optimum concentration of the feed was the most critical point in the diafiltration process for the constant flux. In this case, it should be 2X or 2.5X, approximately 500 ml to 400 ml of the starting volume (1L).

Only TMP affect the **total filtration time (Y2)** period. Increasing the TMP by increase the inlet or outlet pressure of the cartridge, it would increase the filtrate flux and decrease the filtration time at the particular surface area of the filter. If the membrane area of the cartridge increase (Higher MWCO cartridge), it would reduce the total filtration time.
Concentration factor of the feed not significantly affect the filtration time. But the higher the CF, lesser the diafiltration volume (DV) and ultimately decreased the diafiltration time period.

Only CF affect the **total buffer consumption (Y3)** in the process. TMP was not significantly affect the buffer consumption amount. Increasing the concentration of the feed by the concentration step likewise 1X (1Liter) to 2X (500 ml) or 2.5X (400 ml); ultimately decreased buffer consumption capacity. Concentrate the product up to desired point or C_G point of the product for the optimum buffer consumption. In this it should be around 375 ml for the optimum buffer consumption.

Impact of the diafiltration process on the various physical parameters of the product was analyzed at the two different TMP stage 5 psi and 8 psi, respectively. Only conductivity of the formulation was drastically changed during the overall diafiltration process, but the

other parameters like pH, particle size, zeta potential, PDI, lipid content and osmolarity was not significant changed in the process.

Optimization of the process time and buffer consumption, **three possible mode** of the diafiltration process was done. First concentrate the product and after that diafilter with the buffer solution produce the less filtration time and permeate volume compare with other mode of the filtration. First diafiltration step and after that concentration step produce maximum time and permeate volume. But for achieving the higher product yield, all the diafiltration procedure was performed at the sequence like first concentration, after diafiltration, again concentration to desire level and final flushing with the appropriate buffer solution. Flushing step was essential for the product recovery from the tube and other parts of the equipment; generally equipment design was such that the holdup volume of the product (product in the Bioprene tube-18 and filtration cartridge) was as much as minimum.

After all the optimization of the diafiltration process, **drug loading** to the liposomal formulation. Drug loading into the liposome formulation in the desired concentration of the sucrose solution. Osmolarity of the formulation was greatly affected by the concentration of the sucrose solution. 10 % sucrose solution produce the osmolarity which was in the range (280-300 mosmol).So, finally in all the formulation, final volume make up with the 10 % sucrose solution.

The **minimum external buffer ion concentration** for the better encapsulation efficiency of the drug. In this case it was noted that the less than 100 ppm of the external ion concentration was beneficial for the higher encapsulation (> 95 %).

Another application of this process was to reduce the free drug content from the formulation. So that decreased the free drug content from the external medium of the liposome and reduce the toxicity of the drug. By this way we can formulate the better chemotherapeutic formulation having less free drug content. **Unentrapped drug removal process by the TFF** system was effective to reduce the free drug content from the formulation. In TFF system, particular diafiltration mode produce greater drug removal process than the concentration.

7. SUMMARY

Depending on the method of preparation, certain impurities, certain of which may be toxic, could be present in the final product. These impurities include organic solvents such as ethanol, polyvinyl alcohol (PVA), dichloromethane (DCM) emulsifiers or stabilizer, salts, and large polymer aggregates. In these preparation, there is an absolute requirement for nanoparticles to be free of toxic impurities.

A range of methods have been used for purification of nanoparticles. Simple filtration through mesh or filters is often employed for removal large aggregates, centrifugation or ultracentrifugation techniques are commonly used for removal of organic solvents, free drug, or free stabilizers and electrolytes. Dialysis techniques, gel filtration, ultrafiltration, and more recently, Diafiltration and cross flow microfiltration have been used for the purification of nanoparticles. Centrifugation or ultracentrifugation widely used for remove large quantities of process impurities; But the effect of the centrifugation force was produce a low yield of nanoparticles.

Usually, high speed centrifugation at 50,000– 300,000 × *g*-forces up to 2-3 hr is normally used to isolate the solvents from the nanoparticles. This process produce the creation of pellets of the material which are usually difficult to redisperse due to aggregate formation. Use of lower g-forces can be recognized to avoid aggregate formation of the particle, but lower than 50,000 × *g* force conditions during centrifugation has produced incomplete separation of the submicron particles from the supernatant layer especially for particle sizes less than 100 nm.

In the current project, the use of PEGylated liposome resulted in a very small nanoparticles (80–120 nm) compared to that of simple liposome. These did not resolve from the free supernant liquid under the proposed ultracentrifugation conditions; therefore an alternative process was developed to purify the nanoparticles from excess solvent and free drug.

Purification by the dialysis technique was a time-consuming process with a high risk of microbial contamination, can potentially result in premature release of nanoparticle payload during the long purification time. Gel filtration also faster process, but it was limited

because only a relatively small volume of sample can be processed at a particular time. In addition, irreversible adsorption between nanoparticles and the column stationary phase and poor resolution of the large impurities and small nanoparticles can restrict this technique. Ultrafiltration, although more efficient than dialysis and gel filtration, can cause nanoparticles to stick together or adhere to the membrane surface, thus leading to a considerable decrease in filtrate flux. Concentration polarization, fouling, and cake formation are major problems in the ultrafiltration but can be overcame by cross flow microfiltration. Recently, ultrafiltration techniques have been expanded for the separation of a variety of macromolecules from complex matrices. The use of cross- flow microfiltration as a purification technique for nanoparticles has been investigated. Although the research into this process is limited, the technique has potential as an efficient purification technique with minimal harmful effects on nanoparticle size and drug-loading capacity.

The High pressure homogenization technique was successfully adopted to obtain the desired size and polydispersity. Tangential flow filtration is an easy, fast, and efficient method for separation and purification of biomolecules. TFF can be used to concentrate and desalt sample solutions ranging in volume from a few milliliters up to thousands of liters.Selection of the appropriate TFF equipment and operating conditions requires a thorough understanding of the process requirements and parameters.

8. CONCLUSION

Major goal of this project was to utilize the design of experiment approach on the Liposomal drug delivery system for an anticancer drug. In this direction efforts have been made to formulate crude liposome by using HSPC, m-PEG, Cholesterol, and Ethanol. For creating the ionic gradient across the liposomal bilayer ammonium Sulphate buffer used.

Once established, a broad range of membranes, formats and equipment are available to handle almost any application.The independent variables like Transmembrane pressure (TMP), feed flow rate, concentration factor, diafiltration volume, buffer concentration were studied to optimize the formulation with major respect to EE% , and also determined the impact on the particle size, pH and polydispersity as a response variables.

This was done by using Minitab 17.software selecting the appropriate design having levels low, medium, and high. First set the Plackett Burman screening design for optimize the independent variable and their major response. Obtained data will be analyzed by using (ANOVA) then optimize the formulation. Then after major independent variable like transmembrane pressure (TMP) and concentration factor was optimized using the central composite design for better prediction of the process. Design space was created according to its accuracy. The Plackett-Burman and central composite statistical designs were shown to be beneficial in these experiments as highly predictive models were obtained from small numbers of experiments. Using these models, formulation scientists can obtain a design space for the Diafiltration process of the liposomal formulation.

Drug loading was done after the creation of efficient ionic gradient across the liposomal bilayer. Significant amount of the external buffer concentration for the acceptable encapsulation efficiency was determined using the drug loading at the different concentration of the external ion concentration. Another application of this tangential flow filtration was to eliminate the free drug content from the external medium. Encapsulation efficiency of the liposomal formulation was determined by the HLB cartridge based on the extraction principle.

Compare with the other method tangential flow filtration most effective dialysis technique for the purification of the lipidic nanoparticles.

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10. GLOSSARY

Concentration Polarization: The accumulation of retained molecules (gel layer) on the upstream surface of the membrane.

Crossflow Rate (CF) (Retentate Flow Rate): The recirculating volumetric flow rate of the feed solution through the cassette assembly. Flow rate is measured at the retentate, and is typically recorded as liters/minute.

Crossflow Flux Rate (CFF): The recirculating flow rate of the feed solution per unit time per unit membrane area. Typically measured as liters/minute/ft^2 or liters/minute/m^2.

Diafiltration: The fractionation process that washes smaller molecules through a membrane and leaves larger molecules in the retentate (concentrate). It can be used to remove salts or exchange buffers, remove ethanol or other small molecules such as detergents, small peptides or nucleic acids.

Filtrate (Permeate): The portion of sample that has flowed through the membrane.

Filtrate Flux Rate: The flow rate at which sample passes through the membrane per unit area per unit time. Typically recorded at liters/m^2/hour or LMH.

Gel Layer: The microscopically thin layer of molecules that forms on the upstream side of the membrane. It causes a reduction in the filtrate flow rate and may increase the retention of molecules that would normally cross into the filtrate.

Molecular Weight Cut Off (MWCO): The molecular weight cutoff of a membrane, sometimes called Nominal Molecular Weight Limit (NMWL), is defined by its ability to retain a given percentage of a globular solute of a defined molecular weight. Solute retention can vary due to molecular shape, structure, solute concentration, presence of other solutes and ionic conditions. Different membrane manufacturers use different criteria to assign MWCO ratings to a family of membranes.

Normalized Water Permeability (NWP): The water filtrate flux rate at 20°Cover the transmembrane pressure for a given membrane.

$$\mathbf{NWP} = \frac{\mathbf{Filtrate\ Flux\ (LMH)}}{\boldsymbol{TMP}} \times \boldsymbol{TCF}\ \mathbf{20}^{\circ}\mathrm{C}$$

Where TCF 20^0C = Temperature Correction Factor

The primary NWP of the membrane cassette is essential to calculate because it is used as the basis to determine membrane recovery, i.e. how effectively the membranes were cleaned back to their original state.

Membrane Water Permeability: The water filtrate flux rate over the transmembrane pressure for a given membrane.

$$\textbf{Water Permeabilty} = \frac{\textbf{Filtrate Flux (LMH)}}{\boldsymbol{TMP}}$$

Membrane Recovery: The percent ratio of the water NWP (normalized water permeability) after cleaning to the primary NWP measured before the membrane came into contact with a process fluid.

$$\boldsymbol{Membrane\ Recovery} = \frac{\boldsymbol{NWP\ (after\ cleaning)}}{\boldsymbol{NWP(primary)}} \times \mathbf{100}\%$$

Product Recovery: The amount of product (mass or activity) recovered after processing compared to the amount in the starting sample. Usually expressed as a percentage of starting material.

Retentate: The portion of the recirculation stream returning from the cassette back to the feed (does not pass through the membrane). Also known as the concentrate.

Tangential Flow Filtration (TFF) or Crossflow Filtration: A process where the feed stream flows parallel to the membrane face. Applied pressure causes one portion of the flow stream to pass through the membrane (filtrate) while the remainder (retentate) is recirculated back to the feed reservoir.

Transmembrane Pressure (TMP): It is the driving force for liquid transport through the ultrafiltration membrane. Calculated as the average pressure applied to the membrane minus any filtrate pressure. In most cases, pressure at filtrate port equals zero.

$$\boldsymbol{TMP} = \left(\frac{\boldsymbol{P\ feed + P\ retantate}}{\mathbf{2}}\right) - \boldsymbol{P\ filtrate}$$