# Delipidation Treatments for Large-Scale Protein Purification Processing

by

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# DELIPIDATION TREATMENTS FOR LARGE-SCALE PROTEIN PURIFICATION PROCESSING

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Triglycerides are the majority lipid component of most biochemical mixtures and are virtually water insoluble. Lipid removal is desired prior to protein purification processing to decrease nonspecific fouling of downstream chromatographic matrices. Transgenic pig milk was used as a model system to study delipidation from therapeutic protein sources. The majority of triglycerides was extracted from stable lipid micelles and removed with a method that can be incorporated in downstream protein purification processing without denaturing the target protein. An efficient delipidation treatment used TNBP, a non-polar solvent, to extract lipid micelles and then phase transfer milk lipids into a TNBP-swelled dextran particulate. A batch incubation of a whey/TNBP mixture with pre-swollen Sephadex LH-20 or hydroxyalkoxypropyl dextran (HAPD) beads at 4ºC for 24 hours removed 67 $\pm$ 2 % (0.645 mg triglycerides/ml Sephadex LH-20) and 71 $\pm$ 1 % (0.628 mg triglycerides/ml HAPD) of the triglycerides present in the skimmed transgenic whey, respectively. Fully swollen beads removed 20% more triglycerides than beads which were wetted but not swollen in TNBP, indicating that a larger phase volume and internal adsorption of the lipids onto the Sephadex matrix dominates over surface adsorption. Polyclonal ELISAs indicated that 89 $\pm$ 6% of the recombinant human Protein C was still present in the transgenic whey after this delipidation treatment, indicating this treatment did not denature or harm the target protein.

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# I. INTRODUCTION

## 1. Protein Purification

### 1.1 Protein Sources

While many important therapeutic proteins are purified from plasma, often these proteins cannot be easily purified because they are present at such low concentrations in plasma (such as Protein C). In addition, copurification of structurally similar proteins (i.e. Vitamin K Proteins) which have antagonistic activity can also complicate purification processing.

Biotechnology is creating new sources of many therapeutic proteins. Prokaryotic and eucaryotic systems can be genetically engineered to produce therapeutic proteins. Often, prokaryotic cells do not have the structural and biochemical framework needed to perform some important protein modifications. Eucaryotic systems can produce complex proteins that require post-translational modifications. Multiple disulfide bonds, proteolytic cleavage of the precursor protein, glycosylation, and carboxylation are examples of post-translational modifications which are often essential for biologically active proteins. Alternatively to eucaryotic cell culture systems and plasma as sources for therapeutic proteins are transgenic animals such as sheep, goats, and pigs which have been genetically engineered so as to use their mammary glands as bioreactors for the production of pharmaceutical proteins. The high protein productivity of the mammary gland is due to high secretory cell density. Furthermore, its ability to perform complex protein modifications make dairy livestock an excellent bioreactor for cloned gene products. The therapeutic proteins produced by transgenic mammals can then be purified from the large quantities of milk which contain high concentrations of the proteins.

Downstream purification processing of proteins from biological mixtures must preserve the protein's structure and biological activity. Irreversible folding of proteins is referred to as denaturation. Denaturation of proteins can be caused by physical agents such as heat, pressure, Ultra Violet irradiation, organic solvents, ionic detergents, or extreme pH values (1). Many proteins are folded into a globular structure which is dynamic in nature for that protein but has a time average conformation. Hydrogen bonding and salt linkages help to maintain a protein's

conformation. While the globular structure is dynamic, an event which causes extensive and simultaneous bond breakage will also cause the protein to unfold and fall into a more disorganized configuration. Generally, when the denaturing agent is removed, structural hysteresis occurs and the protein does not regain its original structure. The unfolding of the protein will likely expose hydrophobic groups (1) which are usually protected. These hydrophobic groups can then interact with lipid components of the mixture and thus the activation energy for proper refolding becomes high. The addition of nonpolar solvents can induce conformations which make refolding an unlikely event. In fact, the synthesis of protein occurs in an intracellular environment which minimizes undesired conformations which do not permit proper folding (2).

### 1.2 Presence of Lipids

Blood plasma, mammalian cell culture broth, and the milk of transgenic livestock are all potential sources of therapeutic proteins. However, because these sources are all from mammalian cells, their lipid content is quite high. In general, all living cells contain both hydrophobic and hydrophilic compounds which serve as nutrients or as transporters of nutrients and therefore these lipids will be present in biological fluids containing cells. For example, membrane-lipids are more prevalent in mammalian cells because lipids help provide intracellular compartmentalization, which is necessary for the diversity of complex chemical reactions that mammalian cells perform. In general, triglycerides and phospholipids are the major hydrophobic constituents of biological mixtures. Cholesterol and cholesterol esters are also important hydrophobic compounds which serve as base structures for steroidal hormones and also help to change the fluidity of membranes. Cell lyses is a contributor of these lipid species into culture broths. All of these hydrophobic compounds can complicate and foul adsorptive processes when purifying proteins from plasma, mammalian cell culture broth, or the milk from transgenic livestock.

Here, our goal is to gain an understanding of the lipid deposition process from biochemical mixtures onto protein adsorption matrices. In the context of the downstream processing, the lipid

is treated as a dispersed phase. The stability of the dispersed lipid phase can be characterized on the basis of its extractability into a second dispersed phase, created by the introduction of a solvent. The feasibility of using a solvent phase to extract lipids while permitting continued downstream processing of the feedstream without denaturation of the target protein is sought. The choice of solvent should be amendable to lipid solubilization without significant protein denaturation if it is also to be used as a delipidation step in the downstream processing. The whey of transgenic pig milk containing recombinant protein C is selected as a model system for a therapeutic protein source having a diversity of lipids present and containing recombinant protein C. The salient features of lipids which occur in plasma and milk are presented below. The chemical composition of milk is also presented.

### 1.3 Lipid Removal by Centrifugation

Lipids are commonly removed from a protein source by centrifugation. Unfortunately, the process of centrifugation is difficult to scale up for large volumetric throughput. For example, milk is skimmed on the industrial scale by use of an airtight bowl centrifuge at approximately 500g (3). This process cannot sediment and remove small lipid micelles of about 5 μm or smaller (4). For example, the largest tubular bowl centrifuges can handle a throughput of about 0.2-20 gal/minute but only reach a maximum centrifugal force of 15,900 g (5). Small lipid micelles can be removed with an ultracentrifuge, which can attain a centifugal force of 600,000g, but this is only practical for smaller volumes (4). Thus, residual lipid micelles amounting to about 0.25 mg/ml will occur and these residual lipids will be a potentenial source for fouling (lipid deposition).

### 1.4 Viral Inactivation

The transmission of infectious diseases has always been a problem with the use of blood and blood products. Today a single contaminated blood donation can infect many recipients due to the pooling and fractionated methods applied to blood donations. The lipid related structure is

central to the inactivation processing of many viral pathogens. Many infectious agents transmitted by plasma derivatives are viruses with a lipid envelope. The main viruses transmitted by blood products include, Hepatitis (B, non-A and non-B) and HIV. Two known exceptions to the lipid envelope viruses are the parvovirus, a non-enveloped, single stranded DNA particle which does not contain any lipid or glycoprotein, and the delta agent which is also a non-envelope particle but contains only RNA (6). Both physical and chemical procedures can be used to inactivate these viruses. However, it is difficult to assess effaciousness for viral inactivation because many logs of inactivation are sought as a part of any one step in downstream processing. Each step thus acts as a separate barrier through which infectious agents are successively diminished.

It is also challenging to inactivate the viruses without denaturing the important cellular components and proteins present in the blood plasma (7). Some organic solvents can disrupt lipid-enveloped viruses while not causing denaturation of plasma proteins (8, 9). The use of lipophilic solvents and a detergent such as TNBP and/or Triton X-100, (See Figures 1 and 2) inactivates all lipid-envelope viruses at levels greater than 4 logs in plasma products, but this procedure does not inactivate the non-envelope viruses such as the parvovirus or the delta agent (6). The New York Blood Center extracts all lipid-envelope viruses in plasma protein solutions such as AHF (Coagulation Factor VIII-SD) with a tri-N-butyl phosphate (TNBP) and cholate solution. Marker viruses indicate inactivation of all lipid-envelope viruses. This procedure does not affect pathogens which are not lipid-envelope, like the parvovirus and the delta agent. Horowitz (11) found that treatment of the plasma with TNBP and no detergent did inactivate viruses but detergent (Triton X-100) alone did not. The use of 0.3% TNBP plus Triton X-100 resulted in 90 percent or more recovery of AHF while achieving 4 logs of lipid coat inactivants of challenges with lipid envelope viruses. The lipid is thought to be extracted from the viral particle and sequestered into a separate micellular phase consisting of dispersed TNBP stabilized by surfactant.

## 1.5 Plasma Fractionation

In the plasma fractionation procedure, one of the first steps is the use of di-ethyl-aminoethyl (DEAE)-cellulose or DEAE -Sephadex resins for ion exchange chromatography of the cryopoor plasma to concentrate the factor IX complex (10). The factor IX complex is then purified further on a monoclonal antibody column (11). Presently, all of the remaining blood products except, the original CFIX (coagulation factor IX) concentrate and the CFIX-HT (heat treated coagulation factor IX), are treated with 0.3 percent tri-n-butyl phosphate (TNBP) and 1 percent Triton X-100 (11). A complication of the solvent detergent method is the removal of the TNBP and Triton X-100 during the manufacturing process (11). TNBP is currently removed from the protein by gel exclusion chromatography on Sephadex G-25 (9). But Tween 80, another commonly used detergent in viral inactivation can not be removed by dialysis or gel exclusion chromatography because it forms such large micelles (9). Using a solvent detergent treatment with the STREAMLINE™ expanded bed chromatography may allow the solvent and detergent to be removed in the same chromatographic processing step. Simultaneous adsorption of the target protein while the TNBP micelles pass through the chromatographic media may achieve removal of the solvent detergent. For example, a complication may be adsorption of TNBP onto the chromatographic media such as STREAMLINE™.

## 1.6 Lipid Fouling in Protein Purification Processing

Lipid removal is desired prior to protein purification processing to decrease non-specific fouling of downstream chromatographic matrices. In general, biopolymeric matrices such as agarose and cellulose are used to adsorb protein. They also provide both hydrophilic and hydrophobic environments where lipid adsorption can occur. Problems are often encountered with the chromatography of biological samples, especially milk and plasma (which have similar lipid content). "Untreated milk results in rapid degradation of the separation and eventual destruction of the chromatography column, which becomes evident with peak broadening, peak splitting, and increased back pressure" (12). Experiments with skimmed porcine milk (the milk fat

was removed by 3000g centrifugation for 1 hour at 4ºC) indicated chromatographic columns must be regenerated to remove lipids after as little as three cycles of processing of whey on DEAE-agarose (unpublished data, W.H. Velander). In addition, after several chromatographic cycles of skimmed whey with agarose immmunosorbents, lipid is clearly visible after centrifugation of the immunosorbent at 4ºC after batchwise equilibration in Tris/EDTA buffer. Past studies indicate that even well-skimmed milk contains about 1 percent of the milk fat (13) and one third of the phosphorus lipids content of whole milk (1), causing nonspecific binding and damage to the chromatographic column to occur. The exact mechanism by which lipid adsorption occurs onto the chromatographic matrixes has not been elucidated, but it likely can be treated as a phase transfer process from micellular phases to the surfaces of chromatographic supports.

## 2. Sources of Lipids in Biological Mixtures

Lipids are a ubiquitous biochemical species that serves as a highly concentrated energy store and the main components of cell membranes, to compartmentalize various sections of a cell (2). Depending on the derivatization of the lipid, it can occur as sparingly soluble solute species or as supramolecular colloidal assembles. Lipids generally account for two percent of the wet weight of cells and nine percent of the dry weight of cells (2). All cells employ lipid bilayer membranes to serve as selectively permeable barriers which act as a substrate and an information transport conduit between the cytoplasm and the outside of the cell. Eucaryotic cells in addition contain specialized internal membranes which surround structures called organelles, such as the mitochondria, chloroplasts, and lysosomes (2). These biological membranes are principally lipids, ranging in content from 80-25 percent of the membrane, depending on its function (2). The remaining membrane composition is mostly proteins having substantial hydrophobic character. Membrane lipids are generally small molecules with both hydrophobic and hydrophilic attributes causing them to have limited solubility in either nonpolar solvents or water. This thermodynamic property causes them to spontaneously form closed, stable bimolecular structures such as sheets or micelles (2) which are colloidal in nature.

While membranes can be disrupted and fragmented during purification processing, they will spontaneously reform complex, structured, colloidal assemblies. Biological membranes are held together by many reinforcing and cooperative noncovalent interactions (2). Hydrophobic interactions act chiefly through entropic mechanisms which seek to maximize the number of low energy water configurations. Thus, hydrophobic interactions are frequently referred to as a solvent effect (14). While hydrophobic interactions are the driving force for the lipid bilayers, they are stabilized by the full array of molecular forces which mediate molecular interactions. For example, there are the van der Walls attractive forces between proximolar nuclei of adjoining molecules and the electrostatic and hydrogen bonding attractive forces which occur between the polar head groups (such as phosphate moieties and the water at the outer surface of the lipid bilayer membrane) (2). The overall free energy of the system is lowered by the spontaneous formation of lipid bilayers which compartmentalize, creating an energetically unfavorable barrier that allows the least number of water molecules to be disrupted by hydrocarbon molecules (14).

The three main types of membrane lipids are: phospholipids, glycolipids, and cholesterol (2). The phospholipids are abundant in all biological membranes and will be discussed in more detail later. Glycolipids are sugar containing lipids, such as cerebrosides and gangliosides which are both prevalent in the brain. Cholesterol is generally found only in eucaryotes, in high concentration in the plasma membrane, modulating its flexibility (2).

### 2.1 Plasma Lipids

Colloidal structures containing lipids are prevalent in plasma. The total lipid concentration in human serum is about 644 mg/100 ml serum, while the triglyceride, phospholipid, and total sterol concentrations are 86, 220, and 188 mg / 100 ml of serum, respectively (15). Much of the lipids in blood are bound to albumin, which solubilizes them for transport and keeps the concentration of free fatty acids below 1 meq/L, where hemolysis (the destruction of red corpuscles) occurs (15). Cholesterol, triglycerides, and other lipids are also transported in the blood by colloidal structures created by lipoproteins. Lipoproteins and associated lipids form

hydrophobic core particles which are surrounded by a shell of polar lipids and apoproteins (2). Chylomicrons and Very Low Density Lipids (VLDL) are triglyceride rich particles that are secreted principally by the epithelial cells of the intestine and the liver, respectively. The chylomicrons are 98% lipid of which, 90% are triglycerides coming mostly from the diet. Chylomicrons are stabilized by a surface shell of phospholipids, cholesterol, and proteins (15) The VLDL has a composition similar to the chylomicrons particles, which makes both comparable in composition to the fat globules present in milk. Free fatty acids, not associated with lipoprotein particles are transported by the blood rapidly and are absorbed by all bodily tissues, like the skeletal muscles and the heart. In general, lipids are readily converted to triglycerides and stored in adipose cells (15). Thus, triglycerides are the majority component of lipid present in biological mixtures.

### 2.2 Lipid containing Structures in Milk

Fat membrane globules exist as an oil in water emulsion in milk and are the largest naturally occuring lipid micelles. There are approximately $1x10^{10}$ fat globules per 1 ml of milk with diameters 0.1-10 μm accounting for 0.042 of the volume fraction in milk (See Figure 3, (13)). The fat membrane globules have a membrane which consists mostly of polar lipids, proteins, and enzymes. The fat globule membrane stabilizes the emulsion, preventing the fat globules from coalescing (16), while allowing the insoluble, nonpolar lipids to exist in the aqueous solution. The fat globule membrane also provides a selectively permeable barrier to enzymes and is similar to cellular membranes in structure. Like cellular membranes, fat membrane globules can be disrupted into smaller micelles by mechanical force. Thus, skim milk can contain residual lipid consisting of small micelles formed from the disruption of much larger fat membrane globules.

Many different lipids are present in the fat globule such as cholesterol, digylcerides, free fatty acids, phospholipids, cholesterol esters, and triglycerides (see Figure 4). Lipids are defined as the esters of fatty acids which are not soluble in aqueous liquids. The main lipid type, triglycerides, accounts for 98% of the lipids in milk. Triglycerides have molecular weights around

750 (13), and are extremely nonpolar with no solubility in water, causing the triglycerides to be contained within the core of the fat globule. Triglycerides are not reactive and act as a solvent for other nonpolar substances within the fat globule. The triglycerides may become part of the lipoproteins which would account for traces of triglycerides in the milk plasma (13).

Compound lipids are lipids which also consist of nonlipid components such as phospho groups, organic bases, and glucides. Phospholipids are compound lipids composed mostly of neutrals but also contain two charged groups, which cause them to have a highly polar part of the molecule and an amphiphilic nature. For example, phosphotriglycerides have one phospho ester and two fatty acid esters on a glycerol backbone. Thus, phosphotriglycerides are amphiphilic and have both an ionic/polar and a nonpolar derivative of hydrocarbons. This directs the ionic/polar part of the molecule to the water side of the interface, because it is water soluble, while the remainder of the molecule is repelled by water. The interfacial properties of amphiphiles creates organized structures, such as micelles in nature (14). Thus, phospholipids do not dissolve well in polar water nor in nonpolar lipids but occur at, and stabilize the interfaces between these two phases.

The phospholipid molecules, with their amphiphilic nature, constitute a key structural component of the fat globule membrane by acting as an interfacial species that separates the hydrophobic lipids like triglycerides from the aqueous phase of milk. The phospholipids make up 0.8% of the lipids present in milk, and serve to solubilize hydrophobic core lipids. The phospholipids make up 33 % of the fat globule membrane with the two polar sections pointing to the outside and the inside of the membrane. Various proteins make up 48% of the fat globule membrane and water makes up 11% of the fat globule membrane (13). The proteins found in the fat globule membrane bind divalent metals which can decrease the hydrophilic nature of the phospholipid (1). There are essentially no phospholipids within the core of the fat globule because the amphiphilic quality of these molecules make them extremely surface active.

The 5 major phospholipids in milk are: sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl serine (17). The phospholipids bind

cations, stabilize the emulsion, and probably help orient enzymes onto the fat globule surface (16). When milk is skimmed by centrifugation, only the small fat globules remain. The ratio of membrane surface area to the volume of the fat globule increases with decreasing micelle sphere size. Therefore, the remaining small fat globules cause skimmed milk to have a higher percentage of phospholipids and other globule membrane components to the core components than the larger globules present in whole milk. The lipid globule surface is quite extensive, approximately 500 $cm^2$ in 1 ml of whole milk. Approximately 5 % of the total membrane surface (13) and 1/3 of the phospholipids remain after skimming (1).

The lipid globules in whole milk range in size from 0.1-10 μm in diameter. Thus, intact, native fat membrane globules would be excluded from intraparticle adsorption by cross-linked dextrans such as Sephadex G-25. The globules less than 1 μm in size account for about 80% of the globules but only 10% or less of the volume of the fat (16). The structural elements of milk are so small that they allow quick diffusion and rapid partition equilibrium between the fat globules and the milk plasma/whey (13). "The membrane surrounding lipid globules in milk closely resembles plasma membrane in ultrastructure, in that it has a typical bilayer appearance, with the space between bilayers being comparable to that of plasma membrane" (16).

Lipoproteins are particles with core hydrophobic lipids surrounded by a shell of polar lipids and apoproteins (2). Lipoproteins, also called milk microsomes, consist of cell membranes and microvilli from the mammary tissue. These are complexed with the lipids in the milk fat globule membrane, providing colloidal stability. Any lipids found in solution (0.4%) are usually associated with a lipoprotein.

Removal of the lipoproteins have been thought to destabilize the fat globule membrane. Often peripheral membrane proteins can be removed from the membrane by changing the pH or the salt concentration. To release integral membrane proteins from the membrane, usually a detergent such as Triton X-100 or sodium cholate in excess is required (2). About 25% of the proteins in the fat globule membrane are solubilized with Triton X-100 (18). Disruption of the milk lipid globule membrane (MLGM) can also occur with the help of freezing and thawing,

agitation, nonionic detergents, addition of conjugated bile salts such as those involved in digestion, or the addition of a polar, aprotic solvent. The addition of low molecular weight organic molecules which are soluble in oil and water can also release the membrane from the core (13). When the membrane proteins are to be studied, the membrane is usually collected by centrifugation of the samples (16). The proteins complexed with the phospholipids of the milk lipid globule membrane (MLGM) are disrupted by alcohols (1) and the mojohnier reagent also indicates ethyl alcohol dissociates the protein from the phospholipids (1), which is why we tried using this solvent (unsuccessfully) to disrupt the micelles and aid in lipid adsorption with the Sephadex beads.

### 2.3 Other Lipids in Milk

Cholesterols are the main unsaponifiable lipid present in milk. (Saponification is the irreversible base-promoted hydrolysis of an ester). These molecules are extremely nonpolar although they associate well with phospholipids. The cholesterols make up only 0.3% of the lipids present in whole milk, approximately 2% of the membrane components and 0.26% of the fat globule core (13).

There can also be trace amounts of free fatty acids, monoglycerides, diglycerides (usually from the hydrolysis of the triglycerides), and cholesterol esters in the milk fat globule.

## 3. Other Features of Milk

### 3.1 Other Chemical Features of Milk

Milk is necessarily complex as it must supply hydrophobic and hydrophilic nutrients and structural components for cellular membranes to the neonatant. It is a multiphase mixture of lipids, proteins, and low molecular weight electrolytes. There are four phases present in milk: an aqueous whey phase, a lipid emulsion phase (3.7wt%), a colloidal dispersion of caseins (2.8 wt %), and somatic cellular solids (See Figure 3).

Milk is 87% water (16) giving it mostly aqueous characteristics while bearing some

similarity in protein complexity to plasma. Milk is naturally proteolytic which aids in digestion by the neonatant. The aqueous soluble components of milk include: proteins (3.4 wt % in bovine milk), inorganic salts and vitamins (~0.7 wt% in bovine milk), phosphates,and carbohydrates. Some of the main proteins found in skim milk are casein (2.2-3.4 g/100ml), β-Lactoglobulin (0.2-0.4g/100ml), α Lactalbumin (0.07-0.15 g/100ml), blood serum albumin (0.02-0.05 g/ 100 ml), immune globulins (0.05-0.11 g/100 ml), and proteose-peptone (0.06-0.17 g/100 ml) (19,20). The aqueous phase also supplies many important vitamins to the young such as ascorbic acid, thiamin, riboflavin, pyridoxine, niacin, and biotin (16) which function in intermediate metabolism.

Of the carbohydrates found in the aqueous phase, lactose is the principal carbohydrate in milk, comprising 4.6 of the total weight percent in bovine milk (16). The concentration of lactose is a compromise between the high nutrient requirements and the constraints due to osmolarity (16). The lactose is a disaccharide causing it to have half the osmolarity as two equivalent monosaccharides such as glucose. The lower osmolarity decreases the possibility of postprandial osmotic stress in the infant who ingests large amounts of calories in the form of carbohydrates (16).

The salts found in the aqueous phase of milk serve three main purposes: provide nutrition (calcium and phosphate), help in the physical stability of the milk proteins (especially the caseins), and catalyze oxidation of milk lipids (certain metallic elements like copper and iron) (1). The monovalent ions, consisting mainly of $Na^+$, $K^+$, and $Cl^-$, exist largely in the ionized state in the aqueous phase of the milk ((16)--Table I p. 580 shows concentration of relevant ionic species in milk). Calcium is present in the aqueous fraction of milk and is also associated with the casein micelles. The zinc ion (4-6 µg/ml) is evenly distributed between the aqueous, lipid, and casein phases (16). Zinc is essential in over 200 enzymes which play catalytic or structural roles while the zinc metalloproteins help maintain the integrity of cell membranes. About half of the zinc was non dialyzable, indicating it is associated/bound to proteins (16). Copper, iron, and approximately 18 % of the magnesium is associated with the lipid globule or membrane (21). Iron (1-3 µg/ml in pig's milk) (16) is a constituent of the milk enzymes xanthine oxidase, peroxidase, and catalase

(1). Other trace minerals present in milk consist of manganese, molybdenum, iodine, fluoride, and selenium.

Salts are present free in the aqueous solution and in colloidal particles which contain casein, calcium, magnesium, phosphate, and citrate. The main dissolved salts consist of phosphate, citrate, chloride, sulfate, bicarbonate, sodium, potassium, magnesium, and calcium. About a third of the calcium and phosphate, 75 % of the magnesium, and 90% of the citrate are present in the dissolved state (1). Salts help buffer the pH and stabilize many of the proteins and the milk fat globule membrane.

Caseins represent about 80% of the total milk proteins. They are present as a fine dispersion of large aggregates composed of proteins and salts which assemble into micelles approximately 10-300 nm in diameter, 1/50th the size of the fat globules (16), and are held together by colloidal calcium phosphate and hydrophobic bonding (22,23). The calcium and phosphate associated with the casein in the casein micelles progressively dissolve at lower pHs until at the isoelectric point, pH 4.6-4.7, the casein is free of the linking salts (1) causing the caseins to precipitate out of solution. (16). In the procedures used in Dr. Velander's lab, casein micelles are solubilized by the addition of 100 mM EDTA, which releases these proteins and removes this phase (24). Milk plasma minus casein micelles is defined as milk serum (13).

Somatic cells are mainly leukocytes of various types, which contain all cytoplasmic components, most notably nucleic acids and enzymes, especially catalase. They can be considered extraneous particles usually 10 μm in diameter but are always present, numbering approximately 100,000 / ml, or about 0.005% of the milk's volume (13). Many of the proteolytic enzymes are associated with the somatic cells, so the higher the concentration of somatic cells, the higher the probability of faster proteolytic degradation of the proteins.

### 3.2 Enzyme Activity in Milk

Enzymes are proteins which catalyze reactions. The enzymes of milk are broadly proteolytic. Enzyme activity usually depends on pH and enzymes are often easily denatured at

elevated temperatures. Most enzymes present in milk are not there for nutrition and enter the milk unavoidably during the secretion process with somatic cells. Many classes of enzymes are endogenous to milk and some examples are: catalase, peroxidase, xanthine oxidase, alkaline phosphatase, acid phosphatase, amylases, protease, lipases, and aldolase. Many other enzymes present in milk come from bacterial origin which gain access sometime before or after the milk is drawn from the teat (1).

Lipases are specific esterases (esterases catalyze the hydrolysis of esters) that preferentially hydrolyze the triglycerides to free fatty acids and diglycerides and monoglycerides. Lipolytic activity produces undesirable flavors in milk. (Milk lipases and their action are reviewed in ref (25). Milk generally requires activation treatments for lipases to attack the fat globules. Some of these activation treatments include shaking and temperature manipulation such as cooling to 5$^{o}$C, warming to 30$^{o}$C, and cooling again to 5$^{o}$C, which are related to some treatments our transgenic milk receives. These treatments do not only activate the enzymes but also aid in the attack on the fat globule by helping the enzyme adsorb onto the globule or by changing the orientation on the adsorbed fat globule membrane. It appears that the enzymes are adsorbed to the fat globule because lower enzyme activity occurs in skimmed milk (1).

Nonspecific proteases can hydrolyze the peptide linkage of proteins, therefore degrading proteins. Significant proteolysis takes place in cow's milk at 37$^{o}$C even when antibiotics are present to inhibit any bacteria. These enzymes can be associated with caseins (1). The broad specificity of proteolytic milk enzymes can be expected to degrade recombinant proteins present in milk as well. These proteases are generally more active at higher temperatures, which is why it is preferable to keep transgenic milk at 2-4$^{o}$C at all times.

## 4. Adsorptive Matrices used for Protein and Lipid Purification

Sephadex is a bead formed, dextran gel with the dextran chains crosslinked to provide three dimensional structure. Sephadex LH-20 is Sephadex G beads (see Figure 5) which have been soaked in 4% aqueous sodium hydroxide and then suspended in propylene oxide to add β-

hydroxypropyl ether groups, which increases the carbon to hydroxyl ratio (26). Sephadex LH-20 has both hydrophilic and lipophilic characteristics. The Sephadex LH-20 will swell in water, polar organic solvents, and mixtures of each. The Sephadex LH-20 gel will preferentially absorb polar solvents over nonpolar solvents when exposed to a solution of both (27).

Sephadex LH-20 is supplied as a dry powder with dry bead diameters of 25-100 μm. The beads will swell to different volumes depending on the solvent. Sephadex LH-20 swells to 4.0-4.4 ml/g dry gel in water and to 3.9 ml/g dry gel in chloroform or TNBP (27). Solvents which cause swelling to 2.5 ml/ g dry gel or less are generally not useful because they do not provide enough interior phase volume to selectively partition molecules from other media, mostly in a chromatographic processing mode (28).

Sephadex matrices are generally used in gel filtration. Gel filtration separates molecules according to their size. The largest molecules have the shortest column residence time while the smallest molecules have longer residence times due to interparticle permeation, as long as molecular seiving is the only effect taking place. The upper molecular weight range depends upon how much the gel swells in its solvent. Sephadex G has a molecular weight exclusion limit of 5,000 with globular proteins while Sephadex LH-20's exclusion limit is slightly lower due to the hydroxyalkylation (28). A molecular weight of 5,000 Daltons corresponds to a globular protein with a hydrodynamic radius ($R_h$) of approximately $1.53 \pm 0.09$ nm (the hydrodynamic radius for the 5,000 Dalton globular protein was estimated from an empirical equation from ProteinSolutions Inc. [MW ($\pm$ 15%) = $(R_h * 1.2275)^{2.564} * 1000$] which was developed from globular proteins ranging in molecular weight from about 10,000-700,000 Daltons). As a comparison, the molecular weight of Protein C, the target protein in our model transgenic milk is about 71,100 Daltons with a hydrodynamic radius of $4.3 \pm 0.5$ nm and the radius size of the lipid micelles range from 50-5,000 nm. The low molecular weight size exclusion limit of these Sephadex beads excludes most whey proteins and lipid micelles from the dextran interior phase volume.

Hydroxyalkoxypropyl Sephadex, a more hydrophobic matrix with long chain alkyl ether

chains added to Sephadex LH-20 which can be used for both straight and reversed-phase chromatography for the separation of lipids and steroids of a wide range of polarity (37).

There are many references which have used Sephadex LH-20 for the adsorption of lipids and lipid purification (29-36) but all of these references utilized the use of a nonpolar (usually immiscible) solvent which already contains the extracted lipids.

Previous studies have removed lipids from small quantities of aqueous protein, mainly albumin. Albumin is a well characterized protein which serves as a depot and transporter of lipid in blood plasma. These delipidation methods utilized changing the pH to 3.0 or 12.5 to partially unfold the protein, allowing the charcoal or the hydroxyalkoxypropyl derivatives of dextran to partition lipid from the protein. About 97% of the protein was recovered with this procedure using the hydroxyalkoxypropyl derivatives of dextran while removing 90% or more of the lipids. Their treatments at pH's of 5-10 showed removal of only 10% of the fatty acids. There was no significant difference of lipid removal at 2 or 37° C. Increasing amounts of dextran present increased the lipid removal till a maximum was reached at 10-15 times more dextran than protein present. These previous studies only looked at solutions containing 1% or 0.2% protein concentration with 1-2 moles of endogenous fatty acids per mole of protein and small quantities of protein, 2-10 mg protein in a 1% aqueous solution with 10 times the hydroxyalkoxypropyl derivatives of dextran (38) or 10 w/w % albumin in water with 0.2-0.4 mg of charcoal/mg of protein (30). Nonaliphatic, hydrophobic ligands attached to agaroses proved to be poor defatters at high or low pH's. Results with L-lysine bound to sepharose by an α-amino group suggest that electrostatic forces are not important for fat removal. The Sepharose was ineffective at removing fatty acids and independent of the charges on the fatty acid and the acceptor ligand. Lipid removal with charcoal generally showed the same results except for a lower protein recovery, of less than 80%. (30, 38)

## 5. Summary of Objectives

The main objective of this work is to develop a method which removes or prevents lipids from fouling chromatographic matrices. Scientific emphasis will be placed on the rate limitations of phase transfer of triglycerides from lipid micelles to a dextran particulate. The delipidation method should permit continued downstream processing of the large feedstream volumes without denaturation of the target protein. The diversity and complexity of the lipids present in transgenic milk, the model therapeutic protein source for this study, are of the same composition and type as those found in the other common therapeutic protein sources. The recombinant Protein C present in the transgenic milk allows this study to monitor the effect of the delipidation process on an active therapeutic protein. The success of delipidation with milk should be applicable to the other protein sources, such as plasma and eucaryotic cell culture systems.

## II. EXPERIMENTAL METHODS SECTION

### 1. Materials

Tributyl phosphate and Tween 80 was from Sigma, Triton X-100 was from Roman Haas. HPLC grade methanol, HPLC grade chloroform, HCl, were used for the extractions. Thin Layer Chromatography utilized: reagent grade diethyl ether, HPLC grade benzene, 100% ethanol, reagent grade glacial acetic acid, HPLC grade hexane, disposable Preval power unit sprayers, and Whatman LH-K 20 x 10 cm HPTLC plates with preadsorbent stripwere used for the TLC procedure. Cholesterol, Tristearin, and phospholipid standards were from Sigma.

### 2. Experimental Procedures

#### 2.1 Whey Preparation

Non-transgenic or transgenic whole pig milk pools were diluted 1:1 with 200 mM EDTA pH 7.0 to solubilize the casein micelles and reduce interactions between rhPC and the micelles (24). The milk/EDTA solution was centrifuged at 3000g for 45 minutes at 3°C. The solidified fat layer was skimmed off and the liquid phase filtered though Whatman #4 filter paper. The aqueous 200 mM EDTA-treated skim milk is denoted as "whey" for the remainder of this paper. After the transgenic whey was skimmed it was dialyzed to remove any traces of the EDTA, so it would not interfere with the $ZnCl_2$ or $MgCl_2$ studies.

#### 2.2 Batch Incubation of Whey and Beads

Dry Sephadex G-25 coarse, or Sephadex LH-20 beads were weighed into 50 ml conical bottomed polypropylene centrifuge tubes. Duplicates or triplicates of each sample were made. 20 ml of whey was added to all the tubes and placed on a rotator at the conditions for incubation (i.e. 4°C for 24 hours or 23°C for 1 hour).

Tributyl phosphate, Triton X-100, and Tween 80 or just tributyl phosphate were added to the whey and these components were added and allowed to mix with the whey at 4°C for 30-45 minutes before exposure to the Sephadex beads.

## 2.3 Lipid Milk Extraction

The whey was extracted with organic solvent , concentrated by evaporation, and then the organic solvent concentrate was assayed for lipid using Thin Layer Chromatography (TLC). The solvent extraction procedure was adapted from Gentner et al. (1981) (40).

All whey samples (16-20 ml total volume; after delipidation treatment with Sephadex), were filtered through Whatman #4 filter paper into a 50 ml conical bottomed centrifuge tube and mixed with 10 ml of methanol. Then 5 ml of chloroform was added to the filtered whey samples and the vials were thoroughly mixed by shaking for 1 minute. The samples were incubated for 15 minutes at 37°C and then cooled to room temperature. An additional 5 ml of chloroform was added to each sample and shaken well for 1 minute. The pH of each sample was then adjusted to 4.5-5.0 with HCl. The samples were then placed in 4°C water in a 4°C cold room for 15 minutes with shaking every 3-5 minutes. The whey/organic solvent samples were phase separated by centrifugation at 2°C for 15 minutes at 2500g. The bottom/organic layer was removed and placed in a 100 ml round bottom flask. The solvent was evaporated almost to dryness on a rotary evaporator at 40°C. The lipids were then redissolved and quantitatively transferred in 2 or 4 ml of 4:1 chloroform:methanol and stored at -50°C.

## 2.4 Thin Layer Chromatography

The thin layer chromatography (TLC) procedure is adapted from the procedure of Freeman and West (1966): a double TLC development procedure of a sequence of polar and then a nonpolar solvent system was used (41).

Whatman LH-K 20 x 10 cm HPTLC plates with preadsorbent strip, and disposable Preval power unit sprayers were used for the TLC procedure. Each plate was cleaned in pure chloroform or solvent system 1 by complete migration of the solvent to the top of the Thin Layer Chromatography plate and then heated at 125°C for 1 hour. All standards and samples were made up in 4-1 chloroform -methanol. One cm lanes were used with 3 µl samples applied 1 cm from the bottom of the plate and allowed to dry completely. Solvent mixtures were prepared

fresh and allowed to equilibrate in the developing tanks 1 hour prior to use. The plates were first allowed to migrate 6 cm above the application line in solvent system 1, diethyl ether - benzene - ethanol - acetic acid (40 - 50 - 2 - 0.2). The plates were air dried for 5 minutes and then heated at 60 $^{o}$C for 5 minutes to remove trace acetic acid. The plates were subsequently allowed to migrate 8 cm from the application point in solvent 2, diethyl ether - hexane (6 - 94). The plates were air dried and then heated at 60$^{o}$C for 30 minutes. The plates were detected by spraying with 4 : 1, water : sulfuric acid and heated at 125$^{o}$C for 30 minutes. The char-spots on the plates were scanned in the reflectance mode on a Shimadzu 9000 Flying Spot scanner; a beam wavelength of 240 nm and beam width of 10 mm provided integration of signal for the entire area of each sample lane.

### 2.5 TLC Milk Lipid Standards

Cholesterol, Tristearin, and phospholipid standards from Sigma were used. Standard references for pig whey lipids were obtained from an extraction of 20 ml of control whey; the weight of lipids obtained from direct extraction of control whey was used as a lipid reference mixture standard. The lipids were diluted in 4:1 chloroform -methanol, ranging in concentration from 0.391 to 12.5 mg/ml. This standard curve was linear for the majority triglyceride components. Phospholipids, cholesterols, and cholesterol esters are also seen on the developed TLC plates.

### 2.6 Densitometry/Scanning of TLC plate

The HPTLC plates were scanned in the reflectance mode at a wavelength of 240 nm. The plates were scanned in the linear mode with a beam size of 0.4 x 10 mm down the whole length of the lane. The minimum peak area was set at a total reflectance signal of 250.

## 2.7 Determination of Protein C by Polyclonal ELISA

Immulon II plates were coated overnight at 4°C with 100 µl/well of 5 µl/ml of rabbit anti-human Protein C in 0.1 M $NaHCO_3$, pH 9.3. Wells were washed with TBS-Tween. TBS solution is 25 mM Tris Hydrochloride with 50 mM NaCl. The TBS-Tween solution is 2 ml Tween 20 / 4L TBS solution. 100 µl of standard and samples in the dilution buffer (TBS-0.1% Bovine Serum Albumin (BSA), pH 7.0) was added to all the wells and incubated at 37°C for 20 minutes. Wells were washed four times and the bound Protein C was detected by a sandwich of goat anti-human protein C and HRP-conjugated sheep anti-goat IgG. The plates were read at 490 nm (42).

## 2.8 Experimental Delipidation Treatment of Whey using Sephadex LH-20 and STREAMLINE™ Expanded Bed Chromatography

Sephadex LH-20 was pre-swelled in TNBP for 24 hours, then sedimented and the excess TNBP was removed by decantation. The TNBP-swollen Sephadex LH-20 beads were mixed with pig whey containing 29.3 TNBP g/kg whey. The "free-TNBP" treated whey was mixed by rotary agitation for 30 minutes at 4°C prior to addition of swollen Sephadex. This mixture was agitated at 4°C for 24 hours and then loaded at 5 cm/min at 4°C onto STREAMLINE™ DEAE from Pharmacia in a packed bed mode. The fall through and initial wash were collected, then the STREAMLINE™ DEAE was expanded at 5 cm/min to remove the TNBP and Sephadex LH-20. The STREAMLINE™ DEAE was repacked at 5 cm/min and proteins eluted with 125 mM NaCl, 250 mM NaCl, and 500 mM NaCl. The fall through, Sephadex LH-20 and NaCl fractions were analyzed for protein content using a spectrophotometer set at a wavelength of 280 nm, rhPC by ELISA, 12% PAGE stained with silver and by western. The fall through and Sephadex LH-20 fractions were extracted with chloroform and analyzed for lipid content. The liquid fraction containing Sephadex LH-20 was filtered through a 8 µm pore size Nylon filter membrane. The filtrate was collected and stored for future use. The filtered material contained the Sephadex LH-20. Both the nylon filter and the Sephadex LH-20 beads were put in a large container with 50 ml

of chloroform for 3 days to extract the lipids from the beads and membrane. The chloroform was concentrated down to 2 ml on a Rotovap. The extracted phase was then analyzed on HPTLC plates and quantified with densitometry.

## III. RESULTS

### 1. Time Course Studies of Triglyceride Adsorption from whey in Treatment Type G (with 29.3g TNBP/kg whey, Triton X-100, and Tween 80 by Sephadex LH-20 at 4ºC)

The rate of adsorption of triglycerides was studied by incubating samples with 0.8 g dry Sephadex LH-20 beads and whey which had been mixed with TNBP, Triton X-100, and Tween 80 for various times from 1-21 hours at 4℃. The results from this experiment are displayed in Table I. This table shows a trend of gradual removal of triglycerides from the whey at incubation times to 21 hours. The most triglyceride removed was 46% after incubation for 21 hours at 4ºC. The standard error for the samples ranged from 1-7%.

The time course of adsorption of triglycerides from whey samples with 29.3 g free TNBP/kg whey (no Triton X-100 nor Tween 80) when added to 0.8 g dry Sephadex LH-20 (eventually 20 % swelled bead volume) indicated similar results to those reported above which had TNBP, Triton X-100, and Tween 80 added to the whey. The values reported in Table II just present the results at 1, 20 and 24 hours. The 44% of the triglycerides in the whey were adsorbed after 24 hours, which is comparable to the amount removed with TNBP, Triton X-100, and Tween 80 present in the whey, 46%.

Tables III and IV presents the results of dextran/solvent treatments investigative of transport barriers which could prevent nonpolar lipids and solvent from entering the Sephadex LH-20 beads. Sephadex beads were either wetted or fully swollen in solvent. Wetting occured only for solvent incubation of 1 hour or less. Samples of 0.8 g of dry Sephadex LH-20 were wetted in the presence of 5 ml of TNBP or water for 1 hour at room temperature. The excess TNBP liquid was removed from the TNBP-swelled dextran beads. These TNBP wet beads were incubated in whey which had been mixed with 3% free TNBP for 1 hour at room temperature.

Table III shows the lipid content of extracts from treatments having prewetted (not fully swelled dextran beads**)** Sephadex LH-20 beads in water or TNBP before adding the whey solution to the samples. The whey solution used in the samples reported in Table III were mixed with (the NYBC-viral inactivation with 10 times the level of TNBP, but the same concentration of Triton

X-100, and Tween 80 = 10NYBC) 29.3 g/ kg whey, 11.0 g Triton X-100/ kg whey, and 3.24 g Tween 80/kg whey. This table indicates that not much triglyceride adsorption occurred in beads pre-wetted in water, only about 13% was removed. The samples pre-wetted in TNBP did not adsorb any more lipids than those pre-wetted in water. The samples had standard errors less than 10%.

**2. Bead Swelling Experiments**

Bead swelling experiments were performed to see how quickly swelling would occur and how much effect temperature and swelling solvent (aqueous versus organic) had on the fully swelled bead volume. The results in Table V shows that equal weights of Sephadex G-25 coarse and Sephadex LH-20 swelled to approximately the same volume, 4.7-4.8 ml in water at both room temperature and 4°C. The size of the Sephadex LH-20 beads are approximately half the size of the Sephadex G-25 coarse when dry and when swollen in water. The beads swelled to almost full swollen volume in water within an hour.

The effect of temperature on bead swelling is shown in Table VI. The Sephadex G-25 coarse beads did not swell at either temperature in the TNBP. The Sephadex LH-20 beads did not swell in TNBP at 4°C after 48 hours. The Sephadex LH-20 beads incubated with TNBP at room temperature for 24 hours swelled to more than three times the size of the dry and 4°C wetted beads. Hydroxyalkoxypropyl dextran type X beads swelled 60% in volume at room temperature as compared to that at 4°C.

**3. HPTLC Resolution of Lipid Mixtures**

The different lipid components present in milk were resolved on the HPTLC plates and were detected by $H_2SO_4$ charring; a typical HPTLC detection of milk lipids is shown in Figure 7. The phospholipids remain at the origin, the cholesterols are about half way up the plate to an $R_f$ = 0.46, the triglycerides migrate two-thirds up the plate with an $R_f$= 0.69 and the cholesterol esters nearly comigrate with the solvent front to the top of the plate with an $R_f$= 0.84. This TLC

method is a technique which allows multiple samples to be simultaneously resolved and thus facilitates statistical analysis of component concentrations. A linear relationship between peak area measured by densitometry and concentration was established for only the triglycerides. While the solvent system used here was optimal for triglyceride quantification, it enables only semiquantitative estimation of the other lipid components due to spot spreading by diffusion. However, differences in the relative signal intensities of cholesterol, cholesterol esters, free fatty acids, and phospholipids to that of the triglyceride signal could be detected.

## 4. Batch Contacting Delipidation Experiments

Batch experiments were performed with skimmed transgenic whey and Sephadex beads to test if lipids can be removed from the whey to decrease the amount of lipids fouling chromatographic columns. The general procedure used to investigate lipid removal from skimmed whey is illustrated in Figure 6a while Figure 6b presents specifically the different batch contacting experiments done with whey. Twenty-three different contacting experiments were tried and are labeled with a letter, A-W. The results from each experiment are presented below. These results assume the lipid adsorbed by the dextran supports to be the difference between the lipid content of extracts from starting whey and various treatment wheys

### 4.1 Treatment Type A

Tables VII and VIII present the concentration and mass of triglycerides from 20 ml whey samples added to 0, 0.2 g, 0.4 g, and 0.8 g dry beads of Sephadex G-25 or LH-20 bead after incubation at 4°C for 26 hours. Triplicates of chloroform extracts of each whey-dextran sample were used to calculate average values and standard error; and each chloroform sample was assayed in triplicate by HPTLC. For all treatment groups and within the precision of the data, there is no significant difference in the detected amount of triglycerides between the samples with and without the Sephadex present during incubation at 4°C. No significant triglyceride concentration effects were seen for the 4°C treatment due to volume reduction of the whey phase

as a result of bead swelling. However, a slight decrease in the relative signal intensities of cholesterol, cholesterol esters, free fatty acids, and phospholipids to that of the triglyceride signal was seen as a result of the whey treatments at the 20 % swelled volume of Sephadex LH-20 level relative to that with no Sephadex beads (See Figure 7: triplicate whey extract applications are given in lanes 17-19 for the 20% swelled volume Sephadex LH-20 treatments; compare to triplicate whey extract applications given in Lanes 9-11). The average standard error of the 4°C treatment for the Sephadex G-25 coarse bead group was about 6%, while the LH-20 group was about 13%.

### 4.2 Treatment Type B

Tables IX and X show the amount of triglycerides in whey after 0.2g, 0.4g, and 0.8g dry Sephadex G-25 coarse and Sephadex LH-20 beads were incubated with 20 ml of whey (no preswelling) at 23°C for 1 hour. These values show that approximately 30% of the triglycerides present in the original starting whey were not detectable by HPTLC of extracts from whey samples that had been incubated with the Sephadex G-25 coarse beads. Approximately 15% of the triglycerides present in the extracts of the starting whey were not detectable in extracts from whey contacted with the Sephadex LH-20 beads at room temperature for 1 hour. In general, the samples of treatment type B resulted in increased concentration in the whey proteins. This increased concentration is likely due to the reduction in the whey volume caused by bead swelling. The beads swelled slightly more at room temperature than at 4°C (See Table V). The average standard error of the Sephadex G-25 coarse group was about 8%, while the LH-20 group was about 5%.

### 4.3 Treatment Type C (1 M NaCl, Room Temperature, 1 hour)

Tables XI and XII show the amount of triglycerides extracted from 20 ml of whey incubated with 1 M NaCl at 23° for 1 hour with 0, 0.2 g, 0.4 g, and 0.8 g dry Sephadex G-25 coarse and Sephadex LH-20 beads (no preswelling of the beads). Within the standard error, no

triglycerides were adsorbed by the Sephadexes in the presence of 1 M NaCl. However, extensive precipitation of lipid (as evidenced by low density floating white precipitate) and other components occurred as a result of the 1 M NaCl treatment. This precipitation phenomena is likely responsible for the much higher standard errors obtained for the treatment type C group of about 20% to 50%.

### 4.4 Treatment Type D (4 mM $MgCl_2$, RT 1 hour)

Tables XIII and XIV show the effect of 25 mM $MgCl_2$ in 20 ml of whey on detected triglyceride mass and concentration when incubated with 0.8 g dry Sephadex G-25 coarse or LH-20 (no preswelling of the beads) for 1 hour at room temperature. The samples with magnesium chloride added in Table XIII indicate the triglyceride concentration went up significantly, approximately 30%, when compared to the samples of just whey. Table XIV shows no significant effect on triglycerides with the magnesium and Sephadex G-25 coarse beads present.

### 4.5 Treatment Type E
### (2.93 g TNBP/kg whey, 11 g Triton X-100/kg whey, 3.24 g Tween 80/kg whey, RT, 1hour)

Tables XV and XVI present the detected amount of triglycerides in whey after incubation of 20 ml of whey with the components used in viral inactivation and 0.8 g dry Sephadex G-25 coarse or LH-20 beads (no preswelling) at room temperature for 1 hour. Within the standard error, no triglycerides were adsorbed by the samples incubated with Sephadex G-25 coarse under these conditions. The average standard error for the Sephadex G-25 coarse bead samples was 13%.

The samples which were incubated with TNBP, Triton X-100, and Tween 80 present in the whey at the concentrations recommended by the New York Blood Center solvent/detergent viral inactivation procedure (NYBC) showed about 30 % removal of the triglycerides with or without the 0.8g dry Sephadex LH-20 beads (swelled bead volume = 20%) added to the mixture. The samples without Sephadex LH-20 also showed the same triglyceride removal as the samples

with the Sephadex LH-20 beads present during the incubation. The standard error for these LH-20 samples was low, only 4-10%.

As soon as the TNBP was mixed with the whey, an emulsion was visible with the whey becoming apparently whiter, much less translucent. When the concentration of TNBP was increased to 29.3 g/kg whey, an oily film of TNBP was visible on top of the whey. The 29.3 g TNBP/kg whey is well above the solubility limit of TNBP in water, 1 ml TNBP/164 ml water. The samples which contained TNBP in them took much longer to filter when trying to remove the beads. A small amount of oily, translucent phase remained behind on top of the filter paper when filtered.

### 4.6 Treatment Type F

**(29.3 g TNBP/kg whey, 11 g Triton X-100/kg whey, 3.24 g Tween 80/kg whey, RT, 1 hour)**

Tables XVII and XVIII show the mass and concentration of triglycerides present in organic extract of whey after batch contacting of 20 ml of whey with 10 times the concentration of TNBP recommended by the NYBC viral inactivation procedure with the suggested viral inactivation concentration of Triton X-100 and Tween 80 and 0.8 g dry beads of Sephadex G-25 coarse or LH-20 at room temperature for 1 hour. Once again the extract samples from Sephadex G-25 coarse contacted whey showed similar triglyceride levels to the extract samples which were not contacted with Sephadex G-25 coarse beads. The extracted samples did not indicate a lower triglyceride detection under these conditions. The samples incubated with the Sephadex LH-20 beads showed a 13-26% decrease in triglyceride mass than extracts from whey samples which were not contacted with beads (Tables XVIII and XXI). Based upon adjustment in whey volume due to the absorption of liquid from the whey into the Sephadex, the concentration of lipids in the samples were comparable with the concentration of triglycerides in the original whey.

### 4.7 Treatment Type G

**(29.3 g TNBP/kg whey, 11g Triton X-100/kg whey, 3.24g Tween 80/kg whey, 4ºC, 24 hours)**

Tables XIX and XX present HPTLC extract data taken on sample treatments consisting of 20 ml of whey plus 29.3 g TNBP/ kg whey, 11 g Triton X-100/kg whey, and 3.24 g Tween 80/kg whey (10NYBC). These samples are different from Treatment F as the contacting was made at 4ºC for 24 hours instead of 23ºC for 1 hour. As with the Sephadex G-25 samples incubated at room temperature (Treatments E and F), there was no significant triglyceride adsorption by treatment type G. The standard error for the HPTLC analysis of the treatment type G, Sephadex G-25 sample extracts ranged from 1-10%.

Similar to the results at room temperature for an hour, the corresponding Sephadex LH-20 samples incubated at 4ºC for 24 hours showed detection of 26-44% less of the triglycerides.

### 4.8 Comparison of Treatment Type F and L

**(Treatment F: 29.3 g TNBP/kg whey with 0.8 g Sephadex at 23ºC, 1 hour; Treatment L: 29.3 g TNBP/kg whey, 11 g Triton X-100/kg whey, 3.24 g Tween 80/kg whey with 0.8 g Sephadex at 23ºC, 1 hour)**

Table XXI presents the HPTLC results of extracted samples of 20 ml of whey, 29.3 g TNBP/kg whey, 11 g Triton X-100/kg whey, 3.24 g Tween 80/kg whey, contacted with 0.8 g dry Sephadex LH-20 beads at room temperature for 1 hour. This table also shows the results of samples which only had 29.3 TNBP /kg whey (no Triton X-100 nor Tween 80 added to the whey) so the effect of Triton X-100 and Tween can be seen. By difference of original and dextran contacted extracted whey samples, Table XVII shows that approximately 56% of the triglycerides were adsorbed at room temperature from samples which had only TNBP mixed with the whey and incubated with Sephadex LH-20. The samples which had TNBP, Triton X-100, and Tween mixed with the whey only adsorbed 26% of the triglycerides. The standard error for all these

samples was below 6%. The results indicate that the lipids are as well or more greatly removed from the whey with only TNBP, and no Triton X-100 and Tween 80, present in solution.

## 5. Effect of 29.3 g TNBP/kg whey,11 g Triton X-100/kg whey, 3.24 g Tween 80/kg whey on Protein C ELISA Detection

These particular experiments utilized a TNBP concentration 10 times higher than that normally used in NYBC viral inactivation procedures. The polyclonal ELISA's indicated the high concentration of TNBP in the transgenic whey caused a loss, or loss of recognition, of 10% -15% of the rhPC at 4°C for 1-24 hours. The samples with TNBP, Triton X-100 and Tween 80 caused a loss of ELISA signal corresponding to 10-20% of the rhPC when incubated at 4°C for 1-20 hours (see Table XXII). The error in the ELISA samples ranged from 4-16%.

Table XXIII shows that at high concentration of TNBP in the transgenic whey, a 33% to 37% lower rhPC content is detected by Polyclonal ELISA when TNBP is incubated at 23°C for 1 hour. The samples with TNBP, Triton X-100, and Tween 80 had 38 to 41% less rhPC detectable by ELISA when incubated at 23°C for 1 hour. These results indicate almost twice as much rhPC signal was lost from samples incubated at room temperature for 1 hour with TNBP, Triton X-100, and Tween 80, than the samples incubated at 4°C for 24 hours with these solvents.

## 6. Transport Studies

### 6.1 Effect of Sephadex LH-20 Prewetted in Water and TNBP upon the adsorption of Triglycerides from Whey with 3% TNBP at 23°C, 1 hour; No Surfactant.

Table IV presents the results from an experiment designed to investigate transport barriers which could prevent nonpolar lipids and solvent from entering the Sephadex LH-20 beads. Samples of 0.8 g of dry Sephadex LH-20 were allowed to wet in the presence of 5 ml of TNBP or water for 1 hour at room temperature. The excess TNBP liquid was removed from the TNBP dextran swelled beads. These TNBP preswelled beads were incubated in whey which had been mixed with 29.3 g free TNBP/kg whey for 1 hour at room temperature having no surfactant. The

results show that the Sephadex LH-20 samples swelled in TNBP adsorbed substantially more triglycerides (56% versus 27%) than the samples swelled in water when in the presence of free/dispersed TNBP in whey. The amount of triglyceride removed (39%) by dry Sephadex LH-20 which had free TNBP in the whey solution is more than that of water-pre-swelled beads which had free TNBP in the whey solution (27%). The standard error for these samples was 8% or lower.

### 6.2 The Effect of Partially Swollen Sephadex LH-20 Wet with Water and TNBP at 4ºC for 1 hour upon the adsorption of Triglycerides from Whey with 3% TNBP at 4ºC for 24 hours; No Surfactant.

Table XXIV shows the results of the above Transport Study (discussed above) when conducted at 4ºC for 24 hours; no surfactants (Treatments M,N,R) . This table presents similar levels of adsorbed lipid to those found at room temperature. The treatment having Sephadex LH-20 wetted in TNBP at 4ºC for 1 hour with free/ dispersed TNBP added to the whey, removed the most triglycerides, 47%, while the samples wetted in TNBP but did not have free TNBP added to the whey and the samples wetted in water only adsorbed about 38% of the triglycerides in whey. A bead swelling experiment showed that Sephadex LH-20 only swelled substantially in TNBP at room temperature and not at 4ºC after 24-48 hours. The standard error for these samples was below 7%.

### 6.3 Delipidation in the Presence of Fully Preswelled Sephadex LH-20 at Room Temperature for 24 hours

Table XXV shows delipidation results after incubating whey with TNBP plus Sephadex LH-20 after the Sephadex LH-20 beads have been fully pre-swollen at room temperature for 24 hours in water or TNBP. This table shows the highest triglyceride adsorption at 67% from the samples which went through Treatment Type W (whey with 29.3 g free TNBP/kg whey incubated with Sephadex LH-20 beads which had been pre-swollen in TNBP at room temperature for 24

hours). The standard error for these samples were less than or equal to 5%. These results are similar to the delipidation studies described in Tables III, IV, and XXIV but with a higher triglyceride removal. The 24 hour TNBP-swollen Sephadex LH-20 beads appeared more fully swollen relative to 1 hour pre-wetted/pre-swollen dextran beads when studied by light microscopy.

### 6.4 The Effects of Preswelling Hydroxyalkoxypropyl Dextran Type X at Room Temperature

Table XXVI presents the results from incubating the whey with dispersed TNBP mixture with Hydroxyalkoxypropyl Dextran type X (HAPD) beads for 24 hours at 4°C. The HAPD beads had been preswollen in TNBP for 24 hours at room temperature. When the transgenic whey was added to the dry HAPD beads, the beads did not swell, nor even completely wet. HAPD beads preswollen in TNBP and then incubated with whey which had free TNBP dispersed in it, adsorbed approximately 70% of the triglycerides present in whey. This is a significant reduction, removing about the same amount as the Sephadex LH-20 preswollen in TNBP at room temperature. The standard of error for these HPTLC analyses of delipidation was less than 7% for these samples.

## 7. Lipids Extracted From Sephadex LH-20 Beads Exposed to Treatment Type W and a STREAMLINE™ Column

A chloroform extraction and HPTLC analysis of Sephadex LH-20 beads which had been exposed to treatment type W (excess TNBP was removed after the beads were swelled in TNBP for 24 hours at room temperature and the pre-swollen beads were then incubated with whey and 29.3 g free TNBP/kg whey for 24 hours at 4°C) and then run through a STREAMLINE column, showed that lipids were being adsorbed from the whey onto/into the Sephadex LH-20 beads as seen with the results of Table XXXI. Table XXXI presents the mass of triglycerides extracted from 20 ml of Sephadex LH-20 beads after exposure to treatment type W with 100 ml of pig whey and then loaded as feed (whey plus beads with TNBP) and run through a STREAMLINE™

column at 4°C.

**8. Zinc Precipitation Effects**

From the results in Tables XXVII and XXVIII, it was evident that zinc chloride was having a significant effect on delipidation. Table XXVII shows that the incubation of zinc chloride with Sephadex LH-20 beads essentially removed all lipids present in the whey. A light floating precipitate was visible in the zinc treated mixtures. This low density precipitate was filterable with Whatman #4 filter paper.

Table XXVIII shows the results of incubation of 4 mM $ZnCl_2$ for various times from 1-24 hours. The samples with or without Sephadex LH-20 beads gave similar results. Zinc treatments resulted in soluble triglyceride removal ranging from 56 to 100 % for all incubation times. The standard error for the Zinc treatments was less than 9% .

Table XXIX shows the effect of various (0.5-3 mM) zinc chloride concentrations on triglyceride detection levels. The results indicate a linear relationship
(mg Triglyceride detected = 6.10 mg TRG -1.81*mM $ZnCl_2$, $r^2$= 0.98))
between zinc chloride concentration and residual triglyceride (see Figure 13). About 77% less of the triglycerides were detected at 3 mM $ZnCl_2$ and no detectable triglycerides were present after treatment at 4 mM $ZnCl_2$. The standard error for these samples was about 20% or less. The nondisperse lipid precipitate may have contributed variable residual soluble lipid after filtration which resulted in high standard error.

**9. Effect of TNBP on Lipid Adsorption by Sephadex LH-20**

Whey mixed with 2.93 wt% TNBP was used in samples with and without preswollen Sephadex LH-20 beads to investigate the effect of the 10 fold additional TNBP to that of NYBC-solvent detergent formula. Filtration was done with the samples prior to solvent extraction. An oily residue was found on the filtration paper. Table XXX shows that 79% delipidation occured

with Sephadex LH-20 preswollen in TNBP and having 10 fold excess free TNBP present in the whey. Extracts from whey contacted with 10 fold higher TNBP but no Sephadex LH-20 removed 53% of the triglycerides.

## IV. DISCUSSION

### 1. Milk as a Model Mixture for Delipidation Treatment

### 1.1 Introduction

Figure 7 presents the lipid components of whey on a High Performance Thin Layer Chromatography Plate. Using $H_2SO_4$ charring, TLC analyses show that triglycerides are the majority lipid component in the whey. Triglycerides typically represent over 98% of the total lipid mass of raw milk (1). Submicron phospholipid micelles can form after disruption of the much larger fat-(phospholipid) membrane globules which naturally occur at 0.1 to 10 μm in diameter (13), to encapsulate the triglycerides found in milk. Due to the triglycerides limited solubility in an aqueous environment, the triglycerides are likely sequestered by micelle formation in the presence of free phospholipids provided by membrane fragmentation and soluble phospholipids. Thus, the phospholipid micelles are too large for intraparticle transport in cross-linked dextran such as Sephadex. Hence, the phospholipid encasement of the triglycerides in small micelles in an aqueous environment would provide kinetic and transport resistance to subsequent partitioning, even under conditions which would thermodynamically favor the residence of triglycerides in the Sephadex phase. (Figure 8 shows schematically two possible routes of lipid transport into the Sephadex phase.) TLC of the lipid extract from whey suggests free phospholipids are present in the whey which could form micelles after the fat-lipid membrane globule has been disrupted and skimmed away (see Figure 7). In general, phospholipid micelle formation is a likely occurrence in biochemical mixtures derived from higher eukaryotes due to the high content of triglycerides and phospholipid membranes. For example, chylomicron is a colloidal form composed of 98% triglycerides stabilized by a surface shell of phospholipids, cholesterol, and proteins (10) found in blood plasma. Thus, these experiments with pig whey provide a good example case for the study of biochemical mixtures derived from eukaryotes.

Our studies initially sought optimized conditions for the delipidation by Sephadex beads from aqueous whey without introduction of a separate nonpolar solvent phase. Removal of the lipoproteins in the fat globule membrane have been thought to destabilize the fat globule

membrane. Often peripheral membrane proteins can be removed from the membrane by changing the pH, salt concentration, or adding a detergent such as Triton X-100 in excess (2).

The disruption of micelles can occur by raising the ionic strength which can create charge shielding within the whey solution. By increasing the ionic strength, the relative strength of the hydrophobic interaction of the Sephadexes with the lipid increased by decreasing the ionic, polar, and hydrogen bonding which normally help to stabilize the phospholipid structure of the micelle. Thus, NaCl salt was added to the whey to try and remove the peripheral membrane proteins and destabilize the fat globule membrane (2). We thought this would decrease the lipid solubility and possibly cause surface adsorption onto the Sephadex beads. Some lowered solubility of the whey lipids was evident from precipitation and this caused a wide range of error in the amount of lipid present in the organic solvent extracts of the whey. Thus, it could not be determined if significant amount of lipids were adsorbed onto Sephadex beads in the presence of 1 M NaCl.

Another factor to consider is that hydrophobic interactions increase in strength as temperature increases, due to kinetic disruption of the other lower energy forces such as ionic or hydrogen bonding decrease in strength (14). Thus, incubation of whey and Sephadex LH-20 or G-25 beads at room temperature for one hour should result in more significant adsorption than at 4$^{o}$C. The higher percentage of triglyceride removal was evident in these experiments with about 15% and 30% of the total triglycerides adsorbed at room temperature (Tables VII and VIII) and only 0-20% removed at 4$^{o}$C (Tables IX and X).

The range and duration of the extraction conditions at warm conditions are limited due to the labile nature of protein in mixtures such as whey and plasma. Residence times at room temperature should be minimized to prevent activation of the endogenous milk protease cascades and subsequent proteolysis of the target protein. A polyclonal ELISA for human Protein C was used to monitor the effect of various treatments on the target protein. The polyclonal ELISA uses antibodies to recognize the native conformation of human Protein C. If there is a dramatic change of the native Protein C's structure, then it will not be detectable by the ELISA. If the detected concentration of Protein C did not change, it is assumed that there is no gross

denaturation of the Protein C, although an activity assay should be done to definitively show that the target protein is still fully active.

Increased degradation or denaturation of rhPC may have occurred as the concentration of the rhPC detected by ELISA was lowered by almost 35% when the whey and TNBP were kept at room temperature for 1 hour (Table XXIII). Only 10-15% lower rhPC concentration was detected for whey held at 4ºC for 24 hours with TNBP mixed with the whey. The TNBP may be a main determinant in hPC stability because all whey samples exposed to TNBP or TNBP, Trition X-100, and Tween 80 showed lower rhPC signal by ELISA. A possible mechanism is that the presence of TNBP at higher temperatures more rapidly denatures rhPC so that it is not recognizable by ELISA.

The New York Blood Center found that TNBP, Triton X-100, and Tween 80 can effectively disrupt and extract lipid enveloped viruses without denaturing the important cellular components and proteins present in plasma (6). However, the introduction of a nonpolar solvent alone, is an efficient method to disrupt the phospholipid micelle and extract lipid. The solvent extraction used to isolate lipids for TLC analyses is itself an example of the great efficiency that a separate, nonpolar solvent phase has in both shifting equilibrium partitioning of lipid from the aqueous whey phase and the speed at which the equilibrium can be approached in the presence of solvent. In contrast, a separate aqueous hydrogel phase which has both internal hydrophilic and hydrophobic surfaces (i.e., Sephadex LH-20 beads) would necessarily be less effective than a nonpolar, fluid, solvent phase, such as chloroform. Most previous studies utilizing Sephadex LH-20 for lipid removal employed a nonpolar (usually immiscible) solvent to initially extract lipids from the aqueous solvent phase (29-36).

### 1.2 Lipid Phase Transfer

Figure 8 shows schematically two possible routes of lipid transport into the Sephadex phase. The aqueous wet dextran phase also serves as a model surface onto which fouling by lipid deposition may occur. However, here we use it as a pretreatment that acts as a sink into which

delipidation can occur prior to chromatographic processing of the whey. The phospholipid encased lipid micelles are naturally stable in the aqueous whey phase (see phospholipids in Figure 7) and enable the insoluble triglycerides to be present in whey. Hence, no significant lipid adsorption was observed when dry Sephadex LH-20 beads were added to and incubated with whey. A solvent is used to create a phase where the intermediate lipid would have a lower free energy than when residing in the naturally occuring fat globules. Mechanical contacting can disrupt the micelles and encourage phase transfer of the lipids from the fat membrane globule. The addition of a nonpolar solvent, such as, but not necessarily limited to TNBP, destabilizes the fat globules and serves to extract the nonpolar lipids. During the process of separating the Sephadex beads from the whey, the excess TNBP was partially removed by gravity filtration. The extracted lipids in the TNBP phase did not transfer to the Sephadex, since the presence of the aqueous wet Sephadex beads did not increase the delipidation in the presence of free TNBP. The exposure of the Sephadex or HAPD beads to a nonpolar solvent before addition of the whey helped to create a nonpolar environment with which free TNBP could exchange lipid. In summary, whey plus free TNBP was needed in addition to the solvent pre-wetted beads.

From the aqueous bead swelling experiments, it was observed that both Sephadex LH-20 and Sephadex G-25 coarse swelled to its full size almost immediately in water at room temperature or 4℃. However, the Sephadex LH-20 beads swelled much more slowly in TNBP, requiring about 24 hours at room temperature to reach full swollen volume. Thus, both Sephadex G-25 and Sephadex LH-20 beads will preferentially adsorb polar rather than nonpolar solvents (27, 28). Since very little delipidation occured with dry Sephadexes, it is likely that the polar character of the aqueous solvated dextran beads was not a favorable environment for the triglycerides.

**2. Lipid Removal with Free TNBP**

The gravity filtration of whey with free TNBP tended to remove some of the TNBP as an oily residue which appeared on the filter surface. We noted that vaccum filtration tended to

create a TNBP emulsion in the filtrate. However, the gravity filtered aqueous sample filtrate extracted with chloroform contained about 40% less triglyercerides than extracts from control whey. Direct extraction of the oily filter residue was not done. Although the TNBP is difficult to fully separate from the aqueous whey, these experiments show that the separate TNBP phase which remained in the filter media probably contained most of the triglycerides from the whey.

While the presence of free TNBP may have extracted lipids from the fat membrane globule, these lipids do not transfer well into the aqueous swelled Sephadex LH-20 beads added to the solution. In summary, no difference in delipidation occured for treatments having free TNBP with and without dry dextran.

**3. Most Efficient Delipidation Process**

The most significant extraction of triglycerides, 67%, from the stable phospholipid micelle to the Sephadex LH-20 matrix occurred when TNBP was present in both the whey phase and the beads which were fully pre-swollen in TNBP (see Table XXV). From TNBP only treated whey, the aqueous (dispersed) TNBP served as an intermediate vehicle into which lipid could be extracted from naturally occurring micelles (See Figure 8). This TNBP may have been dispersed to a high surface area/volume ratio due to micelle formation from the presence of natural surfactants such as phospholipids. However, it was easily filtered into an oily phase which indicates the TNBP micelles may have been unstable. Further extraction from the dispersed TNBP phase would occur due to hydrophobic partitioning into the nonpolar TNBP/Sephadex LH-20 phase where lipids can then be adsorbed into the low dielectric constant, lipophilic sites contained within the TNBP-swollen dextran matrix. Thus, the TNBP/Sephadex provides phase partitioning for lipid, but also offers the advantage of a structured internal environment where adsorption can occur. In terms of a free energy cascade: the free energy of the adsorbed lipid within the triglyceride/TNBP/Sephadex LH-20 phase and the dispersed fluid TNBP/extracted/triglyceride phase, are both lower than the naturally occurring phospholipid/triglyceride micelles occurring in the whey.

The Sephadex LH-20 beads fully swollen in water before incubation with whey and free TNBP did not adsorb triglycerides. Conversely, the dielectric constant of the matrix phase can be significantly lowered due to the presence of TNBP instead of water, within the bead. In the case of TNBP swelled beads, the TNBP acts as a barrier against the transport of aqueous soluble species such as simple electrolytes and most hydrophilic proteins into the dextran matrix. This barrier to hydrophilic species can be maintained when the whey is already saturated with TNBP.

Delipidation treatments using only free TNBP and no Sephadex LH-20 beads adsorbed 26% less triglycerides than treatments with the same total amount of TNBP contributed by free and TNBP-preswollen beads (Table XXX). Hence, TNBP residing in the swollen matrix apparently acted as a more efficient sink than simply adding the same phase volume of free TNBP to the whey. In summary, the need for both free TNBP and TNBP swelled Sephadex LH-20, suggests a transport from free TNBP to TNBP swelled/saturated Sephadex LH-20 beads.

**4. Bead Swelling Experiments**

We chose to investigate TNBP wet, but not fully swollen beads and compare them to fully swollen beads. Wetted beads which are not fully swollen have more external surface area available for the lipid adsorption. In contrast, fully swelled beads have less surface area but access to the bead interior may yield increased lipid adsorption and removal. After incubation of the beads with the solvent, the beads were examined with light microscopy to help detail the extent of swelling. A time course study for bead swelling was performed which indicated (Table VI) that the Sephadex G-25 coarse beads would become wet but did not swell or increase in size in the nonpolar TNBP solvent when contacted with the TNBP at 4$^{o}$C or room temperature. This is probably due to a minimal content of hydrophobic or lipophilic sites and is consistent with the lack of lipid adsorption onto the Sephadex G-25 beads.

The LH-20 Sephadex beads were found to be fully wettable by TNBP. The relative importance of surface adsorption (deposition of lipids onto the Sephadex bead surface) versus internal adsorption of lipids onto the matrix is seen from experiments using equivalent dry masses

of Sephadex LH-20 with fully TNBP-swollen versus partially swollen (but thoroughly TNBP wet beads as seen by microscopic analysis) using long batch contacting at 4°C with whey (compare Table XXIV with Table XXV). In general, the surface area to volume ratio of the TNBP/Sephadex LH-20 phase is small relative to the TNBP and phospholipid micelle phases and thus likely has rate limited mass transport. However, using a large phase volume of dextran solids for adsorption of lipid provides for significant adsorption capacity in a batch processing mode where contacting can occur over many hours at 4°C while limiting target protein degradation. More fully swollen Sephadex LH-20 were obtained by batch contacting at room temperature for 24 hours in TNBP. This gave a swelling of 3.9 ml/g (Table VI) which corresponds to three times the phase volume obtained by swelling at 4°C for an hour (1.3 ml/g, Table VI). Relative to the matrix swollen for 24 hours, the Sephadex LH-20 beads were fully wetted by solvent but not fully swollen after 1 hour contacting with TNBP at room temperature. Thus, at constant dry mass Sephadex LH-20, a smaller phase volume of TNBP but higher external matrix surface area is provided at 1.3 mg/ml TNBP/Sephadex than at 3.9 ml/g TNBP/Sephadex. Conversely, the more fully swollen TNBP Sephadex LH-20 beads had less surface area but a larger phase volume of TNBP.

The higher phase volume of the fully-TNBP-swollen Sephadex LH-20 removed 67% of the triglycerides present in the whey after incubation with the whey at 4°C for 24 hours (Table XXV). This is a significantly greater capacity than the lesser swelled Sephadex LH-20 beads which removed only 47% of the triglycerides in the whey (Table XXIV). This is also significantly greater than treatments with only (dispersed) TNBP (no Sephadex beads) which removed 40% of the triglycerides. The removal of the 40% of the triglycerides probably resided in the TNBP/oily phase which tended to become trapped in the filter surface. By contrast, lipid adsorption by TNBP pre-wetted (not fully swollen) beads at room temperature for 1 hour removed 56% of the triglycerides (Treatment Type S, Table IV). Thus, the better lipid extraction by the larger internal phase volume of TNBP at constant dry Sephadex LH-20 mass suggests that batch contacting capacity is not solely by a surface area dependent adsorption.

**5. Hydroxyalkoxypropyl Dextran Beads**

The greater hydrophobicity of the TNBP-swollen Sephadex LH-20 relative to the Sephadex G matrix resulted in increased delipidation. Therefore, hydroxyalkoxypropyl dextran (HAPD) beads were also tried to see if these even more highly hydrophobic beads would remove more of the triglycerides in whey. HAPD is made from Sephadex LH-20 by further derivatization with boron trifluoride ethyletherate and alkyl olefin oxide to add the long chain alkyl ethers onto the bead structure (26). In comparison to Sephadex LH-20, the HAPD matrix would not wet with aqueous whey. This is consistent with previous studies which found that even moderately substituted hydroxyalkyl derivatives are not wetted by water (26). Table XXVI presents the results from contacting whey with TNBP-swelled-HAPD beads for 24 hours at 4$^{o}$C (the HAPD beads, type X had been pre-swelled at room temperature for 24 hours in TNBP). The HAPD swelled to 3.4 ml/g (Table VI) in TNBP, which is slightly smaller but comparable to the swelling of the Sephadex LH-20 in TNBP (3.9 ml/g). The TNBP/HAPD adsorbed approximately 70% of the triglycerides from the whey which is also similar to the (67%) lipid adsorption of the TNBP/Sephadex LH-20 beads. Thus, the main difference between delipidation treatments using TNBP/Sephadex LH-20 and TNBP/HAPD treatments was that the TNBP/HADP beads were not efficiently wetted with the whey as compared to the TNBP/Sephadex LH-20 beads. This may have advantages under conditions where protein precipitates are necessarily present. For example, in the presence of protein precipitate, the more easily wetted TNBP/Sephadex LH-20 treatment system became entrained and agglomerated in protein precipitation (data not shown).

**6. Extracted Lipid from Sephadex LH-20 Beads**

The chloroform extraction of the Sephadex LH-20 beads show that lipids were transferred into the TNBP-swelled dextran matrix from the free TNBP-whey mixture (see Figure 9). These beads were exposed to treatment type W but with an additional step of loading the whey/TNBP/Sephadex LH-20 mixture onto a STREAMLINE$^{TM}$ column. This resulted in the filtration of the TNBP swelled Sephadex LH-20 by the STREAMLINE$^{TM}$ bed. It is also likely

that the free TNBP accumulated in the filtered TNBP-swelled Sephadex LH-20 and that some phase transfer occured during that step between free TNBP and TNBP swelled Sephadex LH-20. The lipid material balances for the fractions from the STREAMLINE™ runs are shown in Tables XXXII-XXXV. The extraction of the Sephadex LH-20 beads collected from various runs of the STREAMLINE™ column, indicate a wide range of 0.6-5.3 mg of triglyceride adsorbed / 20 ml of swelled Sephadex LH-20 beads (Table XXXI), which corresponds to 0.03- 0.265 mg trigylceride removed / ml of swelled Sephadex LH-20 beads. The wide range of lipids extracted from the beads could be caused by the handling errors involving the large volume of the fractions from the STREAMLINE™ runs. In comparison to the studies where the Sephadex LH-20 was removed by gravity filtration of the most effective batch delipidation treatment, (treatment type W for Sephadex LH-20 or HAPD; delipidation was measured by difference of lipid content between starting and final treated wheys) about 2.5 mg of triglyceride removed / 4 ml of swelled Sephadex LH-20 beads which corresponds to 0.625 mg / ml of Sephadex LH-20 beads.

## 7. Summary

In summary, about 70% of the triglycerides can be extracted from lipid micelles and removed to prevent lipid fouling of chromatographic matrices with a method that can be incorporated in downstream protein purification processing without denaturing the target protein. Figure 14 shows an overview of the important lipid removal treatments. This figure shows that the most efficient lipid removal treatment required TNBP dispersed in the aqueous whey phase and Sephadex LH-20 or HAPD which was fully pre-swollen in the non-polar TNBP. The TNBP is a non-polar solvent which extracts the lipids from the lipid micelles without affecting the therapeutic protein and acts as a vehicle to carry the lipids into the Sephadex beads. The Sephadex LH-20 or HAPD must be swollen in a non-polar solvent to provide a non-polar environment for the free TNBP (which contains the extracted lipids) to be attracted to so the lipids in the free TNBP phase can be deposited onto the lipophilic sites of the dextran beads. The fact that the beads fully swollen remove 20% more triglycerides than the beads which were just

wetted in TNBP indicate that internal adsorption of the lipids onto the Sephadex matrix is more important than surface adsorption. The Sephadex fully swollen in TNBP has less surface are but three times the phase volume available for lipid deposition/adsorption.

## 8. Future Studies

This work shows that the majority of triglycerides can be extracted from micelles and subsequently adsorbed with a combination of free TNBP and TNBP-swelled-Sephadex LH-20 or HAPD matrixes while not significantly affecting the target protein. TNBP is an expensive solvent and the amount of TNBP used here would represent $2.10 / kg feed stream (Sigma). Future studies should include the gradual addition of 0.1 to 4 mM Zn2+ or Mg2+ which causes phospholipid micelle instability during the adsorption step in combination with lesser amounts of TNBP. The use of less expensive solvents to swell the beads such as ethanol, propanol, or an immiscible solvent such as hexane should also be investigated. In addition, the TNBP/HADP system will be tried in the presence of protein precipitate to see if agglomeration occurs.

The batch lipid adsorption pretreatment can be combined with downstream processing on STREAMLINE™ DEAE. Initial probe experiments show that the batch Sephadex LH-20 /whey mixture can be processed on the STREAMLINE™ DEAE. The Sephadex and excess TNBP can be filtered by columnwise, packed bed (top-down) loading without significant pressure drop at 20% swelled volume Sephadex/volume of whey. The Sephadex particulates are efficiently removed from the top of the STREAMLINE™ DEAE in expanded mode due to the great Stokes drag differential between STREAMLINE™ DEAE and Sephadex LH-20. Centrifugation could also be used to separate the Sephadex pretreatment for delipidation. In summary, it is likely that delipidation by TNBP/Sephadex LH-20 or TNBP/HAPD can be combined with downstream processing steps to decrease the probability of lipid fouling of chromatographic matrices.

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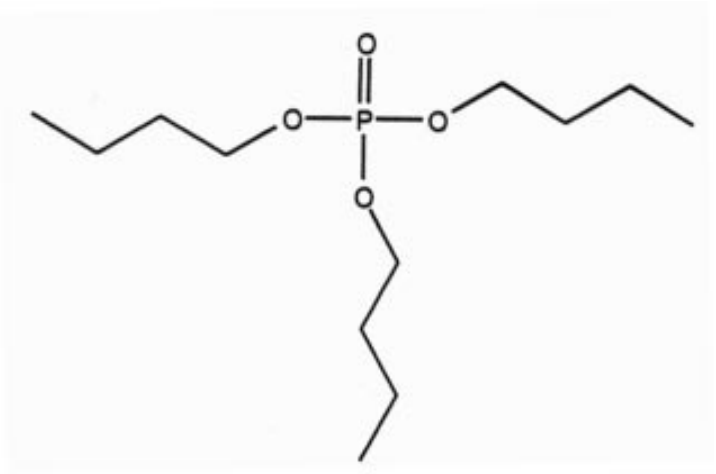
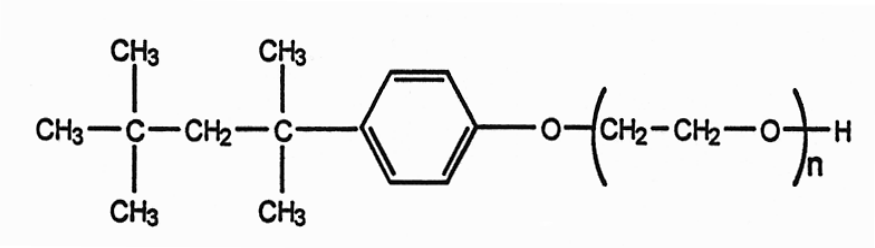


Tri-n-Butyl Phosphate

**Figure 1. The Chemical Structure of Tri-n-Butyl Phosphate (TNBP)**




Polyoxyethylene-p-t-octylphenol
(Triton X - series)

**Figure 2. The Partial Chemical Stucture of Polyoxyethylene-p-t-octylphenol (Triton X)**

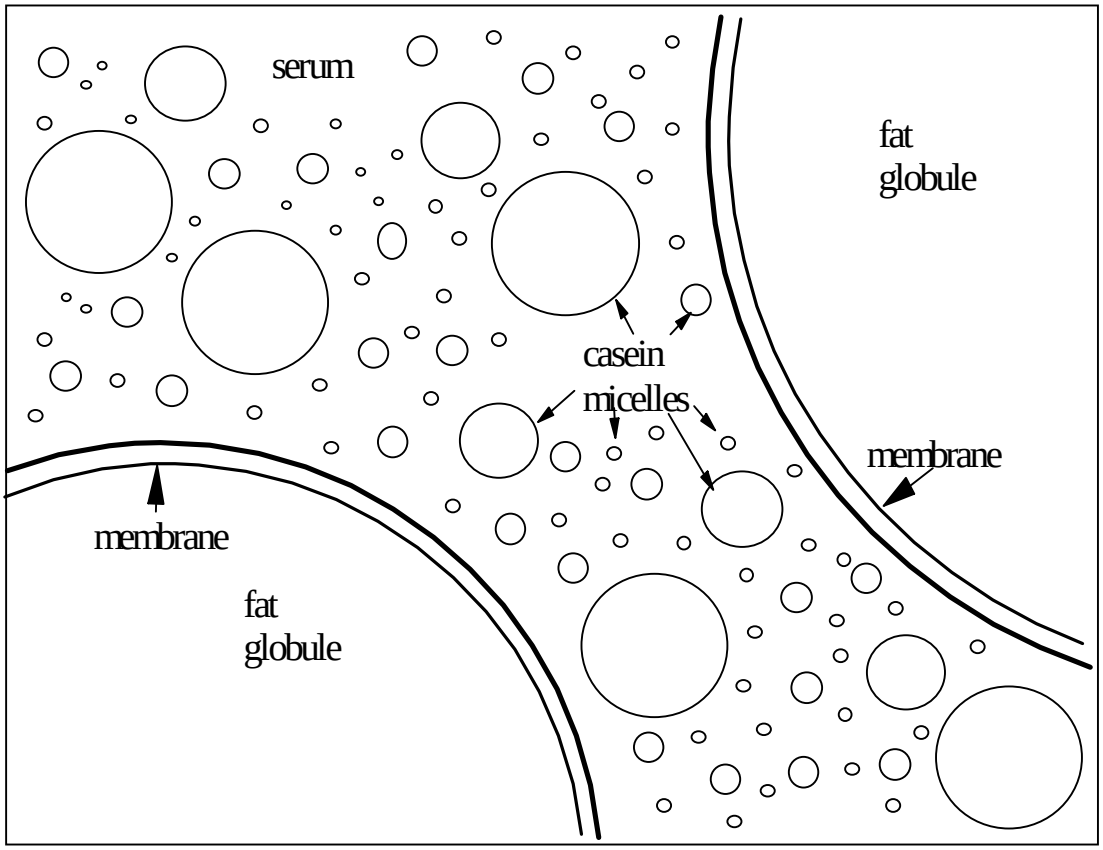



**Figure 3. The Main Structural Components of Milk viewed at approximately 50,000X magnification.** This figure shows the relative size of the main structural elements in milk: fat globules, casein micelles, and serum or whey. The serum or whey contains various proteins, vitamins, and enzymes. The serum is still opalescent so it contains even smaller particles.

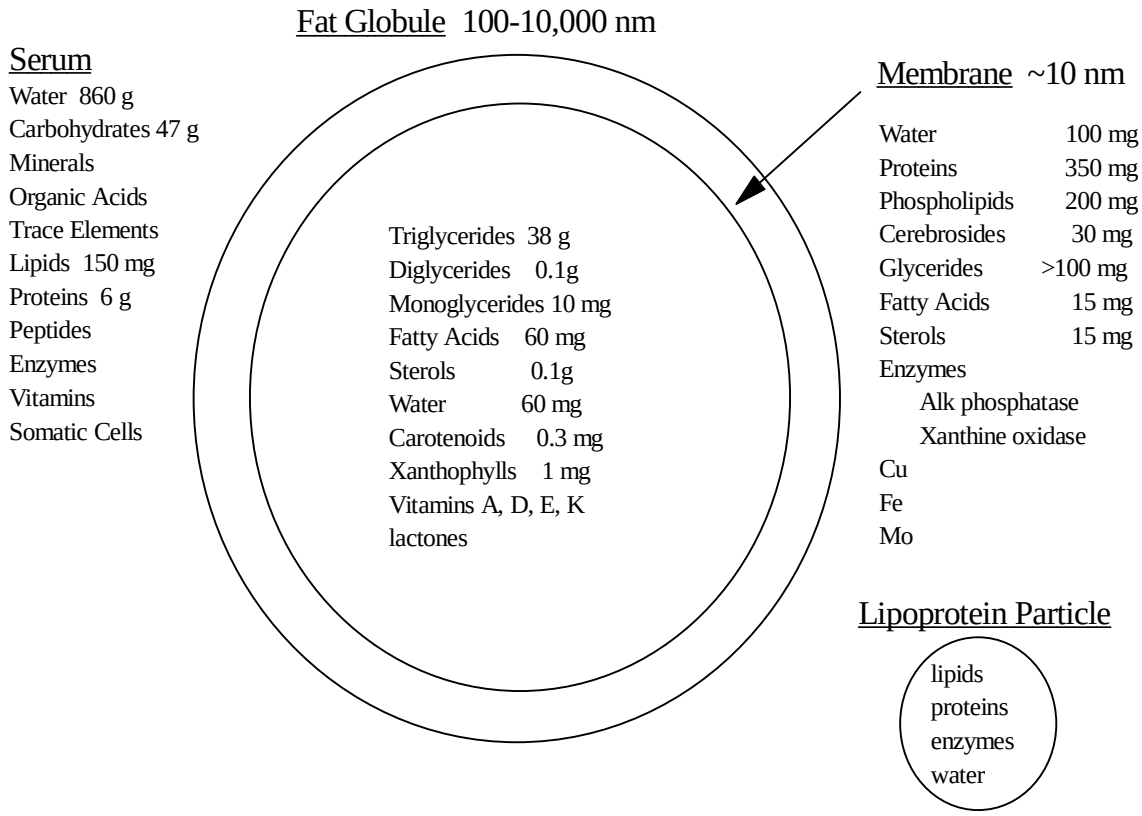



**Figure 4. Composition of the Milk Fat Globule and the Milk Fat Globule Membrane.** The amounts refer to 1 kg of milk (from The Milk Fat Globule, by Mulder and Walstra).

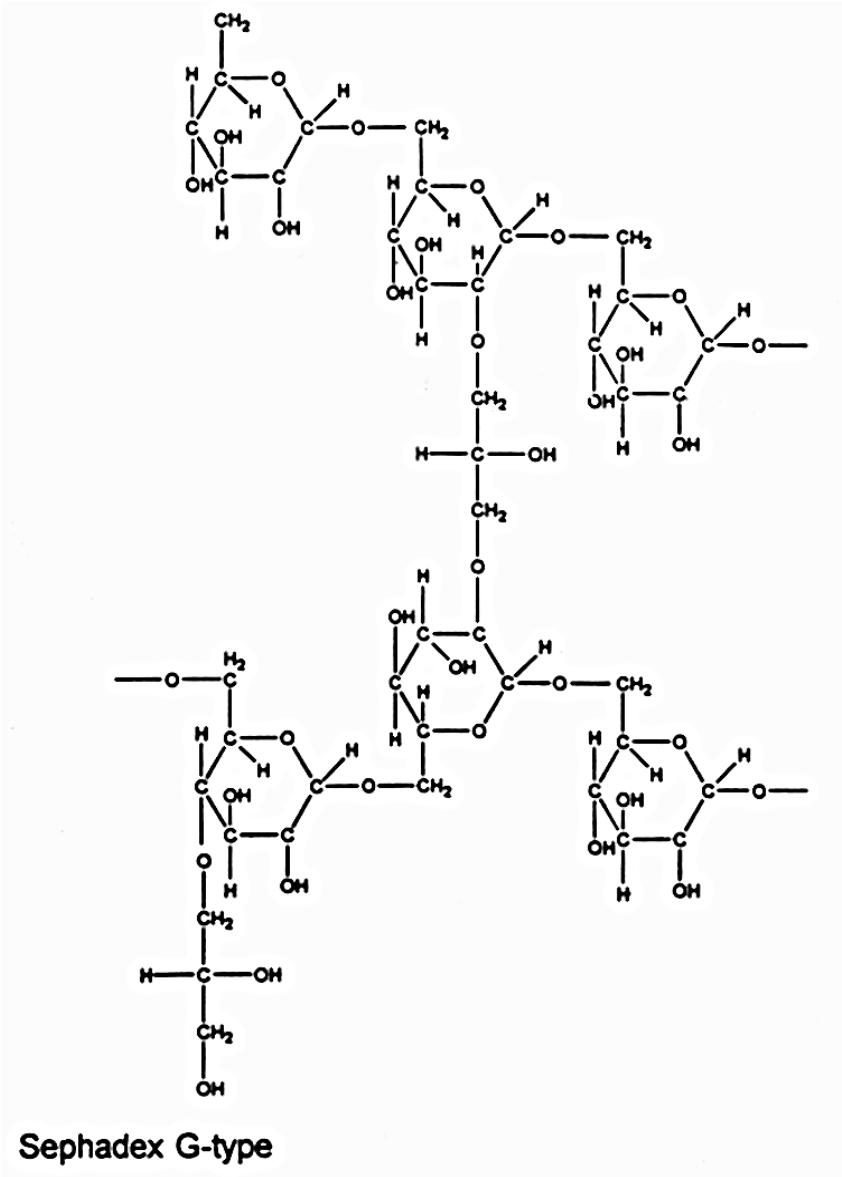



**Figure 5. The Partial Chemical Structure of Sephadex G**

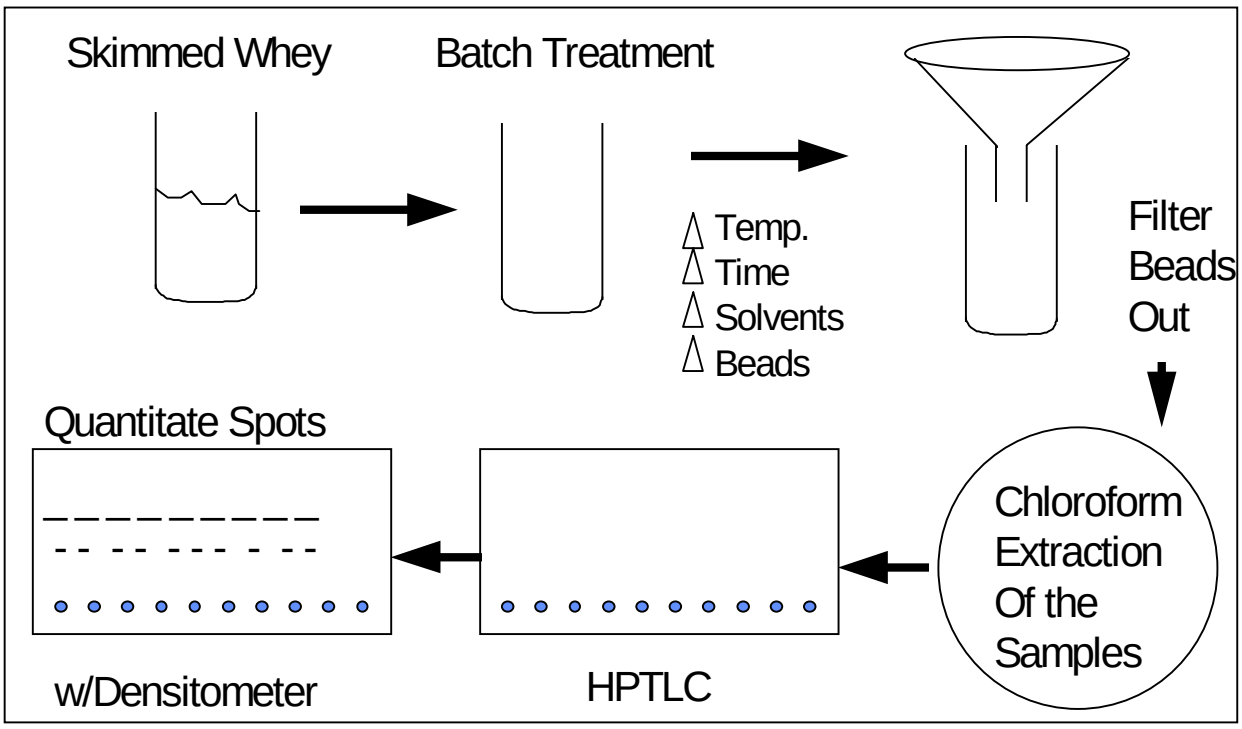



**Figure 6a. General Experimental Procedure for all Treatment Types**

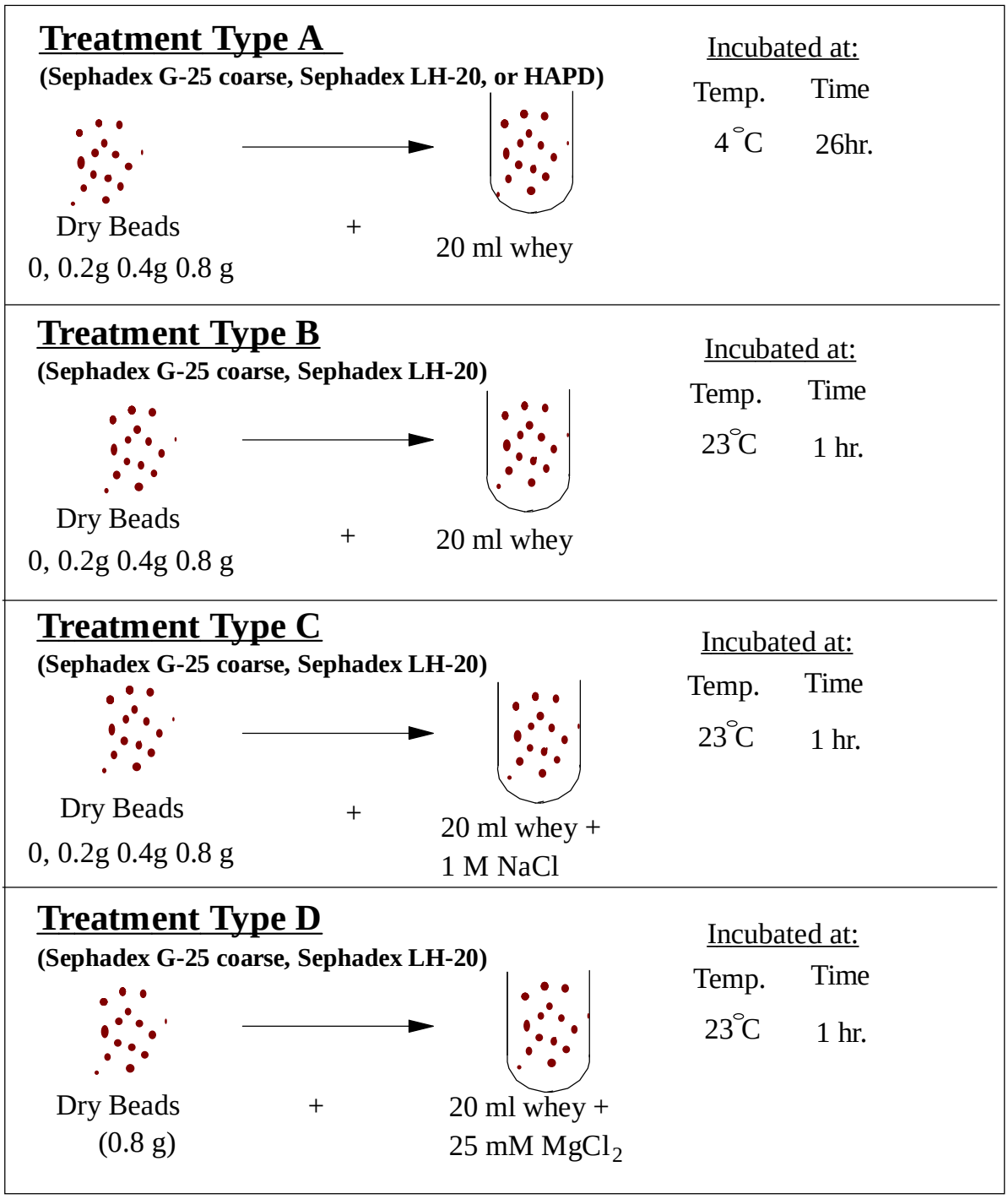



**Figure 6b. Descriptions of each Type of Sample Treatments used to Try and Remove Lipids from Whey.**

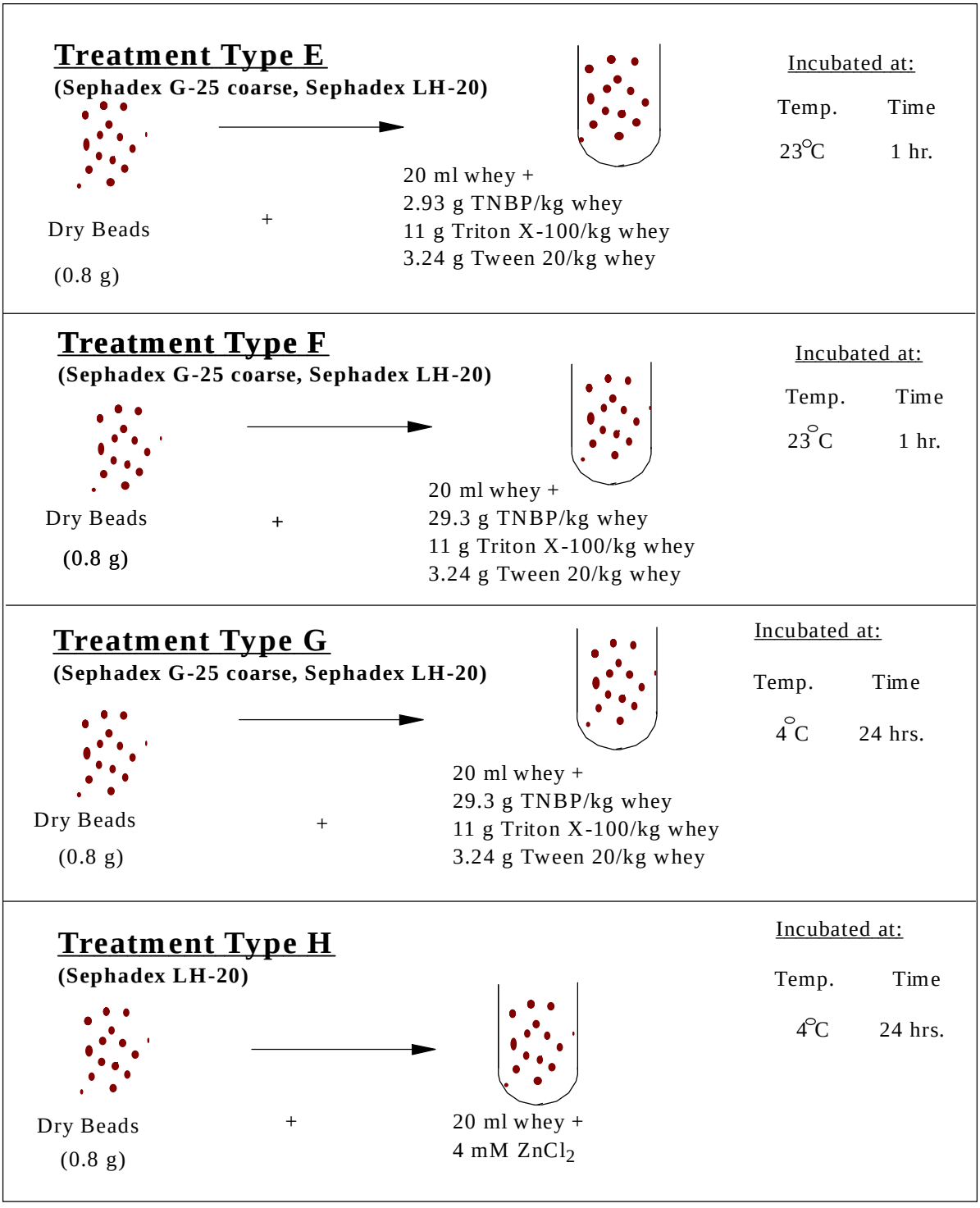



**Figure 6b. (Continued)**

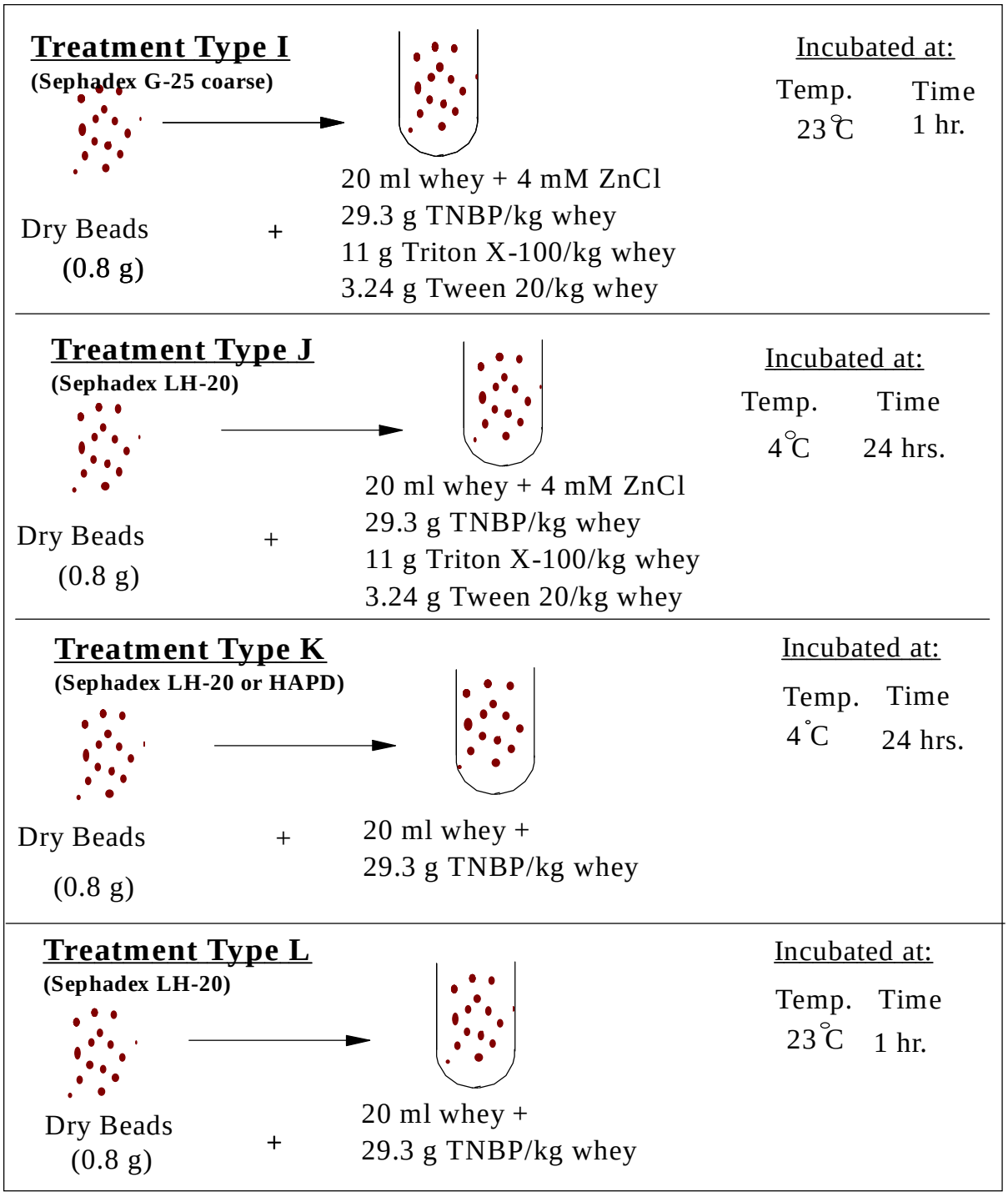



**Figure 6b. (Continued)**

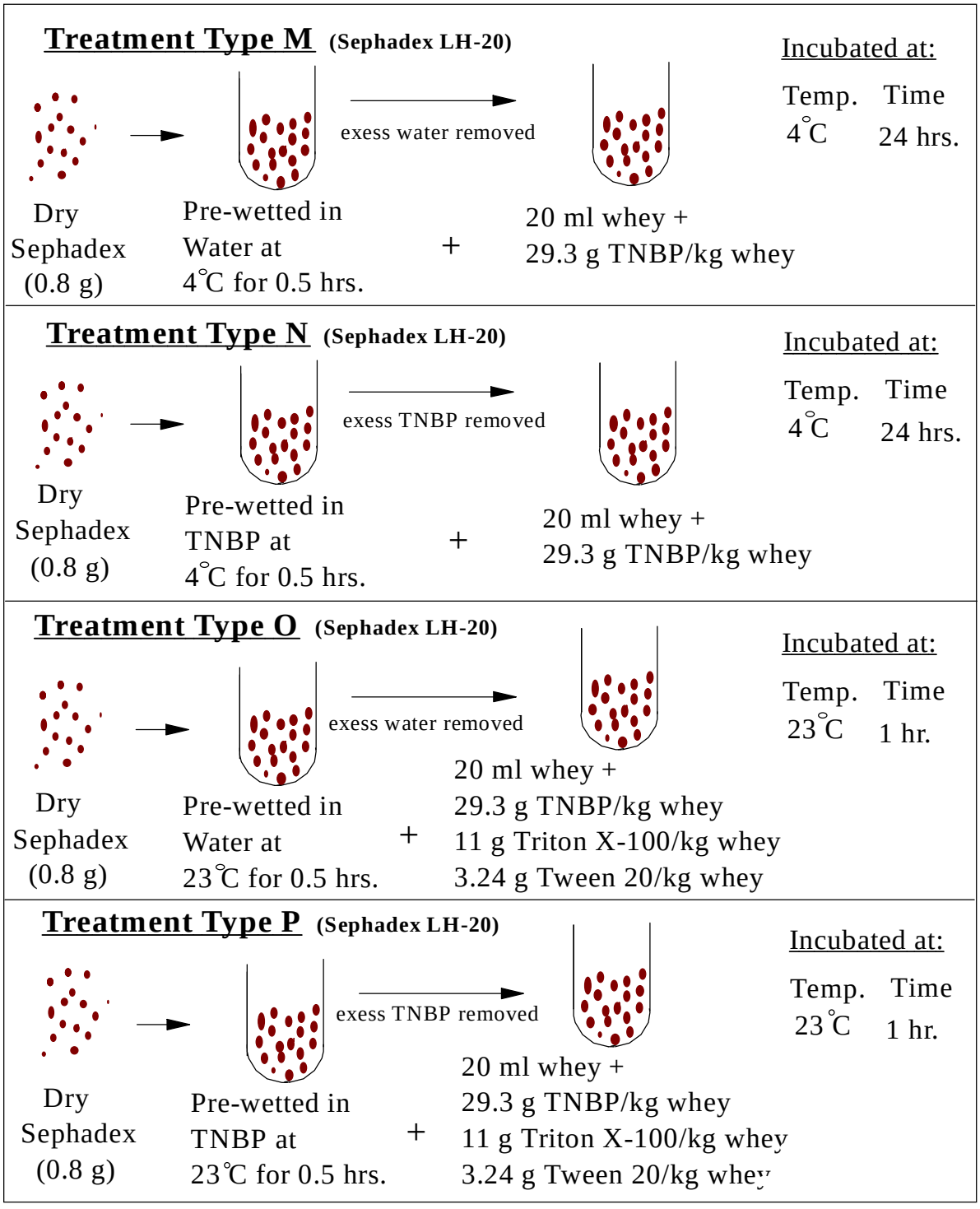



**Figure 6b. (Continued)**

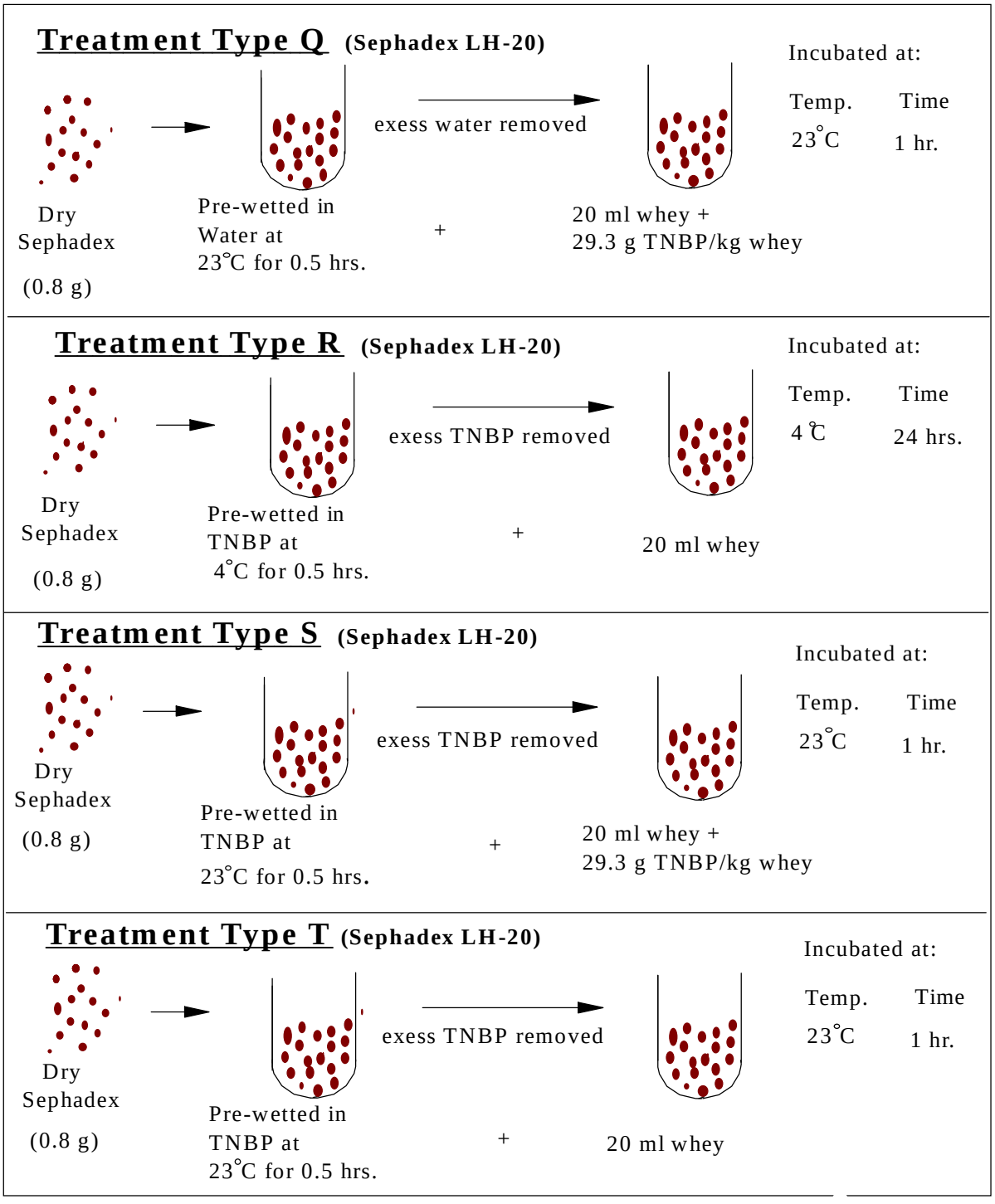



**Figure 6b. (Continued)**

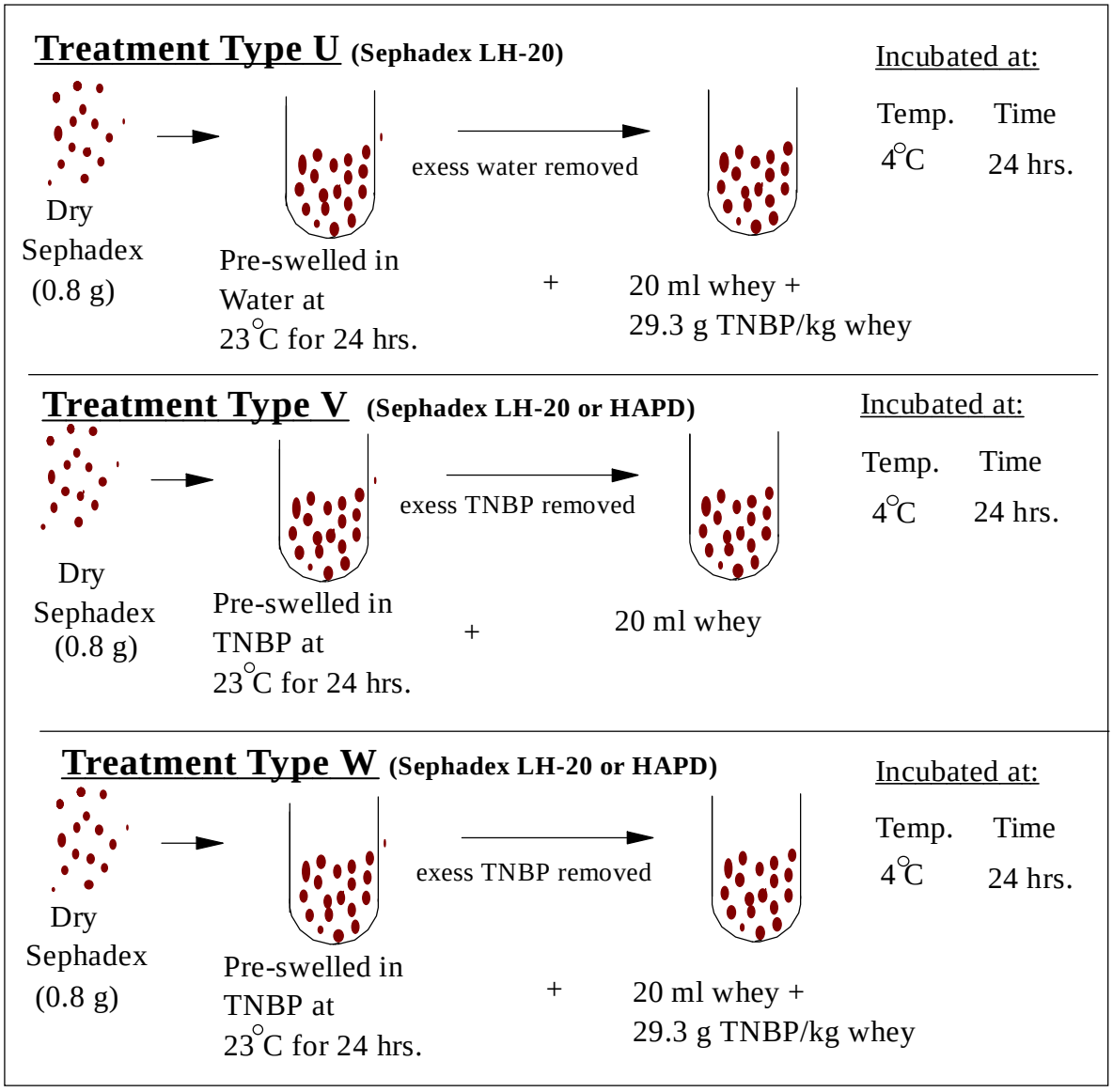
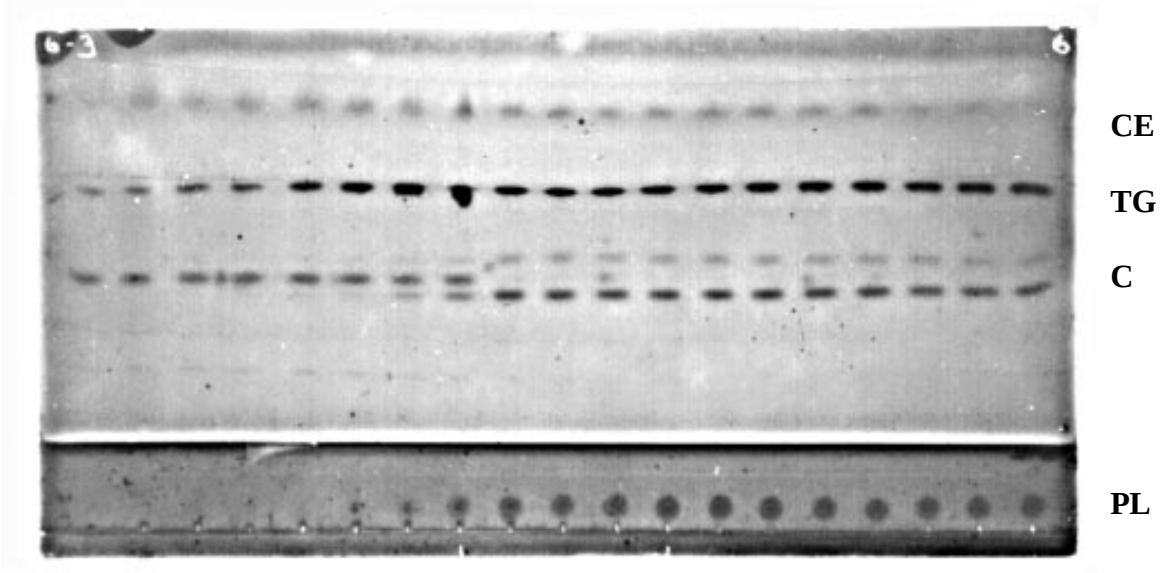



**Figure 6b. (Continued**




**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19**

**CE = Cholesterol Esters TG = Triglycerides C = Cholesterols PL = Phospholipids O = Origin**

**Figure 7. Thin Layer Chromatography Plate of Lipids Extracted from Whey.** This TLC plate shows a standard curve ranging from 1.17 µg to 37.5 µg in lanes 1-8. Lanes 9-11 are 3 different lipid extraction samples of 20 ml whey exposed to 23°C for 1 hour without any beads present. Lanes 12-19 show 3 lipid extraction samples (spotted multiple times) of 20 ml of whey exposed to 20 % swelled bead volume of Sephadex LH-20 at 23°C for 1 hour.

| Lane | Sample |
|---|---|
| 1 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 2 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 3 | 2.35 µg of lipid from whey (3 µl application of 0.781 µg /µl ) |
| 4 | 2.35 µg of lipid from whey (3 µl application of 0.781 µg /µl ) |
| 5 | 4.68 µg of lipid from whey (3 µl application of 1.56 µg /µl ) |
| 6 | 9.37 µg of lipid from whey (3 µl application of 3.125 µg /µl ) |
| 7 | 18.75 µg of lipid from whey (3 µl application of 6.25 µg /µl ) |
| 8 | 37.50 µg of lipid from whey (3 µl application of 12.5 µg /µl ) |
| 9 | 3 µl of sample 1) 20 ml of whey without any beads |
| 10 | 3 µl of sample 2) 20 ml of whey without any beads |
| 11 | 3 µl of sample 3) 20 ml of whey without any beads |
| 12 | 3 µl of sample 1) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 13 | 3 µl of sample 1) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 14 | 3 µl of sample 2) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 15 | 3 µl of sample 2) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 16 | 3 µl of sample 2) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 17 | 3 µl of sample 3) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 18 | 3 µl of sample 3) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |
| 19 | 3 µl of sample 3) 20 ml of whey with 20% swelled bead volume of Sephadex LH-20 |

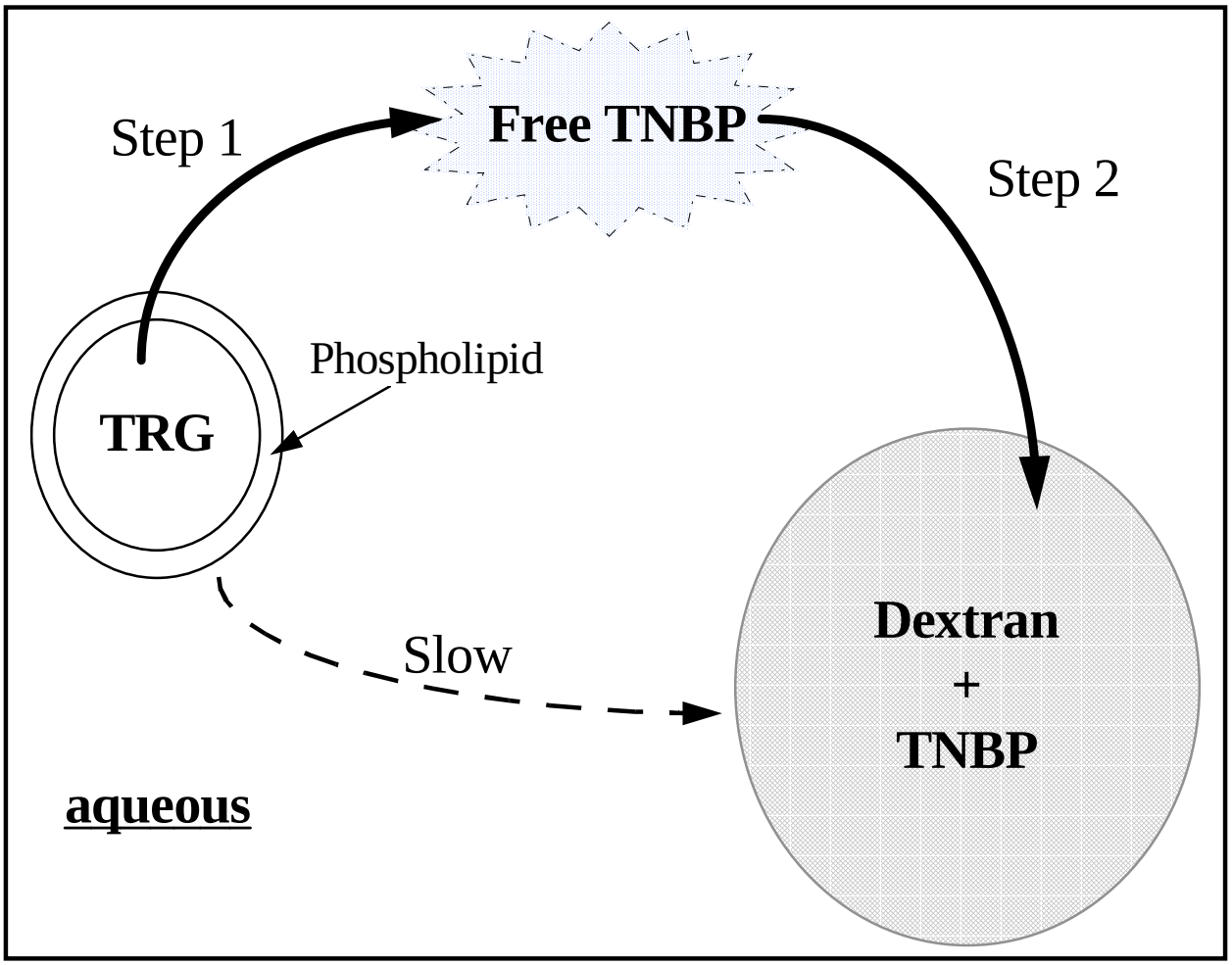



**Figure 8. Phase Transfer Diagram for Free TNBP /TNBP-Swelled Sephadex / Whey Solution**

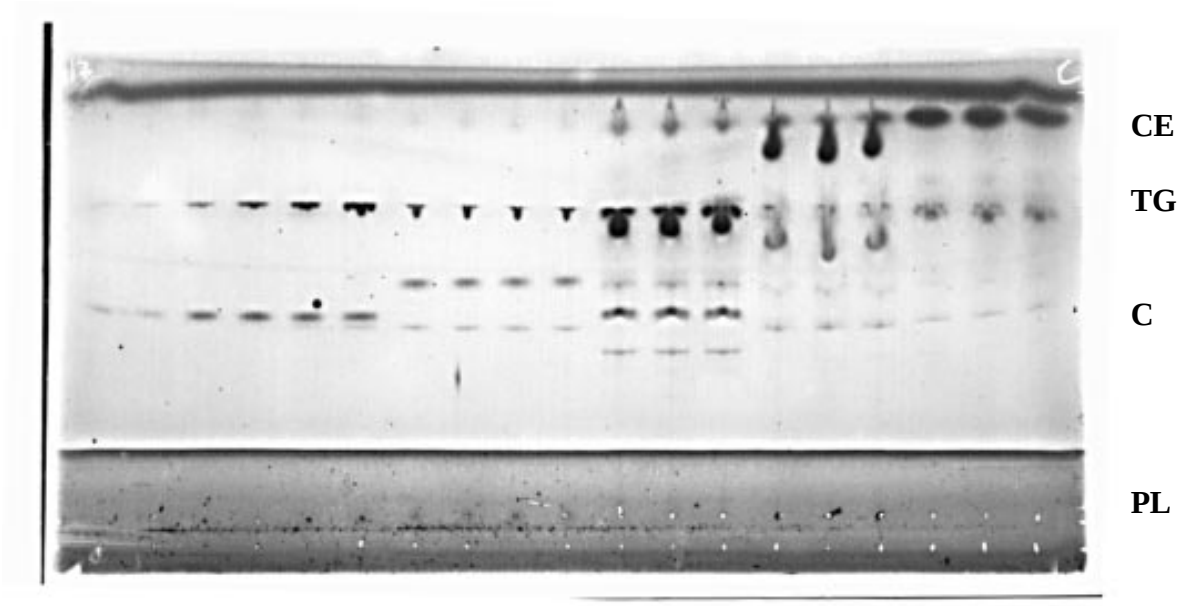
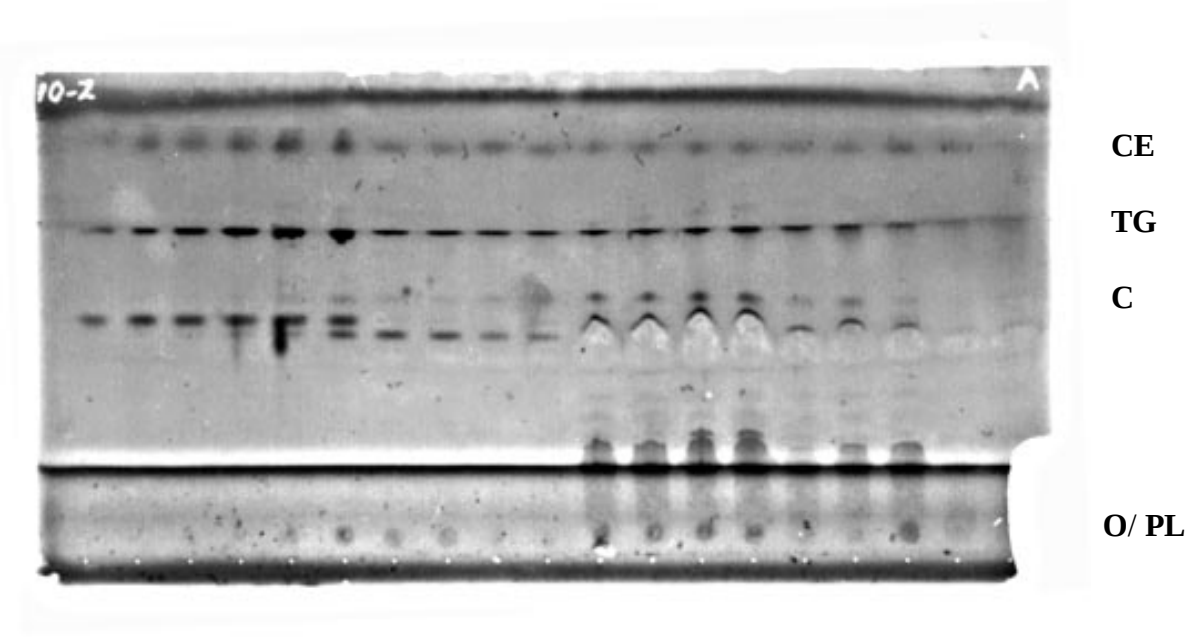



**Figure 9. Thin Layer Chromatography Plate of Lipids Extracted from 20 ml of Sephadex LH-20 Beads which had been exposed to Treatment Type W and run through a STREAMLINE column.**

| Lane | Sample |
|---|---|
| 1 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 2 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 3 | 2.35 µg of lipid from whey (3 µl application of 0.781 µg /µl ) |
| 4 | 4.68 µg of lipid from whey (3 µl application of 1.56 µg /µl ) |
| 5 | 9.37 µg of lipid from whey (3 µl application of 3.125 µg /µl ) |
| 6 | 18.75 µg of lipid from whey (3 µl application of 6.25 µg /µl ) |
| 7 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 8 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 9 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 10 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 11 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #7 |
| 12 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #7 |
| 13 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #7 |
| 14 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #9 |
| 15 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #9 |
| 16 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #9 |
| 17 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #10 |
| 18 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #10 |
| 19 | 6 µl sample from extracting 20 ml of swelled Sephadex LH-20 from run #10 |




**CE = Cholesterol Esters TG = Triglycerides C = Cholesterol PL = Phospholipids O = Origin**

**Figure 10. Thin Layer Chromatography Plate of Lipids Extracted from Samples Exposed to Treatment Types F, L, S, and Q** (figure caption continued on next page).

[A] Densitometer Scan of this Lane is Shown in (A) of Figure 11
[B] Densitometer Scan of this Lane is Shown in (B) of Figure 11
[C] Densitometer Scan of this Lane is Shown in (C) of Figure 11
[D] Densitometer Scan of this Lane is Shown in (D) of Figure 11

**Figure 10. Thin Layer Chromatography Plate of Lipids Extracted from Samples Exposed to Treatment Types F, L, S, and Q**. This TLC plate shows a standard curve ranging from 1.17 µg to 18.75 µg in lanes 1-5. Lanes 6 and 7[A] show the lipids extracted from duplicate samples of 20 ml of pig whey after incubation at 23ºC for 1 hr. (Treatment type B without beads). Lanes 8 and 9 show the extracted lipids from duplicate samples of 20 ml of whey with 29.3g TNBP/kg whey after incubation at 23ºC for 1 hour (Treatment Type L w/o Beads). Lanes 10 and 11 are the extracted lipids from duplicate samples of 20 ml of whey with 29.3g TNBP/kg whey, 11.0g Triton X-100/kg whey, and 3.24g Tween 80/kg whey after incubation at 23ºC for 1 hour (Treatment Type F w/o Beads). Lanes 12 and 13[B] show the extracted lipids from duplicate samples of 20 ml of whey with 29.3g TNBP/kg whey with 0.8g dry Sephadex LH-20 incubated at 23ºC for 1 hr. (Treatment Type L). Lanes 14 and 15 show the extracted lipids from duplicate samples of 20 ml of whey with 29.3g TNBP/kg whey, 11.0g Triton X-100/kg whey, and 3.24g Tween 80/kg whey with 0.8g dry Sephadex LH-20 after incubation at 23ºC for 1 hr. (Treatment Type F). Lanes 16[C] and 17 show duplicate samples of 0.8g dry Sephadex LH-20 swelled in 6 ml of water for 0.5 hrs at 23ºC, then excess liquid was removed, and 20 ml of whey with 29.3g/kg whey was added and incubated with the swelled beads for 1 hour at 23ºC (Treatment Type Q). Lanes 18 and 19[D] show the lipids extracted from duplicate samples of 0.8g of dry Sephadex LH-20 swelled in 6 ml TNBP for 0.5 hrs at 23ºC, then excess liquid was removed and 20 ml of whey with 29.3g TNBP/kg whey was added and incubated with the swelled beads for 1 hour at 23ºC (Treatment Type S).

**CE = Cholesterol Esters TG = Triglycerides C = Cholesterols PL = Phospholipids O = Origin**

| Lane | Sample |
|---|---|
| 1 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 2 | 2.35 µg of lipid from whey (3 µl application of 0.781 µg /µl ) |
| 3 | 4.68 µg of lipid from whey (3 µl application of 1.56 µg /µl ) |
| 4 | 9.37 µg of lipid from whey (3 µl application of 3.125 µg /µl ) |
| 5 | 18.75 µg of lipid from whey (3 µl application of 6.25 µg /µl ) |
| 6 | 3 µl of sample 1) 20 ml of whey without any beads |
| 7 | 3 µl of sample 2) 20 ml of whey without any beads[A] |
| 8 | 3 µl of sample 1) 20 ml of whey exposed to Treatment Type L without Beads |
| 9 | 3 µl of sample 2) 20 ml of whey exposed to Treatment Type L without Beads |
| 10 | 3 µl of sample 1) 20 ml of whey exposed to Treatment Type F without Beads |
| 11 | 3 µl of sample 2) 20 ml of whey exposed to Treatment Type L without Beads |
| 12 | 3 µl of sample 1) 20 ml of whey exposed to Treatment Type L |
| 13 | 3 µl of sample 2) 20 ml of whey exposed to Treatment Type L[B] |
| 14 | 3 µl of sample 1) 20 ml of whey exposed to Treatment Type F |
| 15 | 3 µl of sample 2) 20 ml of whey exposed to Treatment Type F |
| 16 | 3 µl of sample 1) 20 ml of whey exposed to Treatment Type Q[C] |
| 17 | 3 µl of sample 2) 20 ml of whey exposed to Treatment Type Q |
| 18 | 3 µl of sample 1) 20 ml of whey exposed to Treatment Type S |
| 19 | 3 µl of sample 2) 20 ml of whey exposed to Treatment Type S[D] |

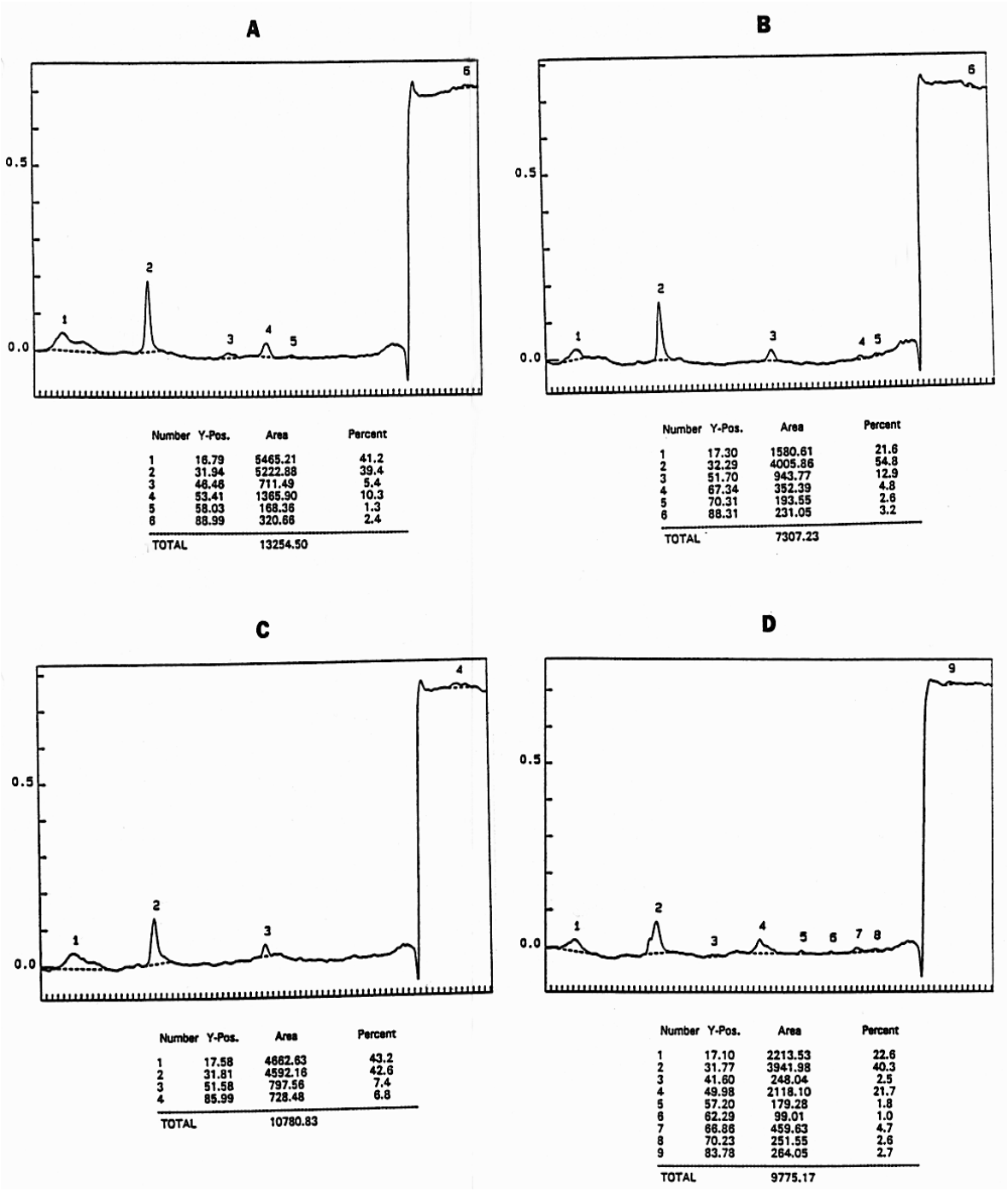



**Figure 11. Densitometer Scans of Lanes 7, 13, 16, and 19 of the TLC plate shown in Figure 10** (Caption Continued on next page).

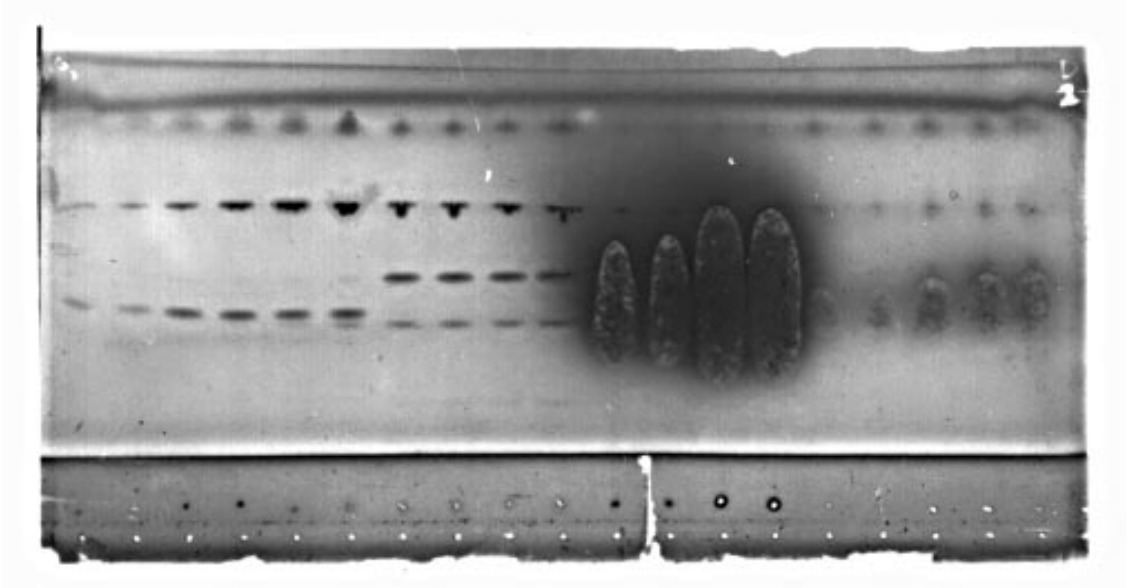
**Figure 11. Densitometer Scans of Lanes 7, 13, 16, and 19 of the TLC plate shown in Figure 10**. The TLC plates are scanned on a Shimadzu Dual-Wavelength Flying Spot Scanner CS-9000 at a wavelength of 240 nm to quantitate the spots with peak areas. Peak 2 (at y position ~32) represents the Triglyceride spot on the scanned TLC plate. Peak 1 (y postition ~17) is the Cholesterol Esters, and the spots at y positions around 53 are Cholesterols.

(A) A scan of lane 7 in Figure 7 which shows the densitometer readings of the lipids extracted from 20 ml of whey incubated for 1 hour at 23°C (Treatment Type B without beads).

(B) A scan of lane 13 in Figure 7 which shows the densitometer readings of the lipids extracted from 20 ml of whey with TNBP added and incubated with the Sephadex LH-20 beads for 1 hour at 23°C (Treatment L).

(C) A scan of lane 16 in Figure 7 which shows the densitometer readings of the lipids extracted from a sample of 0.8 g dry Sephadex LH-20 swelled in 6 ml of water for 0.5 hours at room temperature, then excess liquid was removed, and 20 ml of whey with 29.3 g TNBP/ kg whey was added and incubated with the swelled beads for 1 hour at 23°C (Treatment Type Q).

(D) A scan of lane 19 in Figure 7 which shows the densitometer readings of the lipids extracted from a sample of 0.8 g of dry Sephadex LH-20 swelled in 6 ml TNBP for 0.5 hours at 23°C, then excess liquid was removed and 20 ml of whey with 29.3 g TNBP / kg whey was added and incubated with the swelled beads for 1 hour at 23°C (Treatment Type S).




**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19**

**CE = Cholesterol Esters TG = Triglycerides C = Cholesterols PL = Phospholipids O = Origin**

**Figure 12. Thin Layer Chromatography Plate of Lipids Extracted from Various Fractions of a STREAMLINE column which used Sephadex LH-20 exposed to Treatment Type W (preswelled in TNBP at room temperature for 24 hours and then incubated with 100 ml of whey with 29.3 g TNBP/ kg whey at 4ºC for 24 hours) as feed.**

| Lane | Sample |
|---|---|
| 1 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 2 | 1.17 µg of lipid from whey (3 µl application of 0.391 µg /µl ) |
| 3 | 2.35 µg of lipid from whey (3 µl application of 0.781 µg /µl ) |
| 4 | 4.68 µg of lipid from whey (3 µl application of 1.56 µg /µl ) |
| 5 | 9.37 µg of lipid from whey (3 µl application of 3.125 µg /µl ) |
| 6 | 18.75 µg of lipid from whey (3 µl application of 6.25 µg /µl ) |
| 7 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 8 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 9 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 10 | 3 µl sample of extracted 25 ml of dialyzed whey |
| 11 | 3 µl sample from extracting 350 ml of the Fall Through fraction of run #10 |
| 12 | 3 µl sample from extracting 350 ml of the Fall Through fraction of run #10 |
| 13 | 6 µl sample from extracting 350 ml of the Fall Through fraction of run #10 |
| 14 | 6 µl sample from extracting 350 ml of the Fall Through fraction of run #10 |
| 15 | 3 µl sample from extracting 350 ml of fraction run #10 containing TNBP & Beads |
| 16 | 3 µl sample from extracting 350 ml of fraction run #10 containing TNBP & Beads |
| 17 | 6 µl sample from extracting 350 ml of fraction run #10 containing TNBP & Beads |
| 18 | 6 µl sample from extracting 350 ml of fraction run #10 containing TNBP & Beads |
| 19 | 6 µl sample from extracting 350 ml of fraction run #10 containing TNBP & Beads |

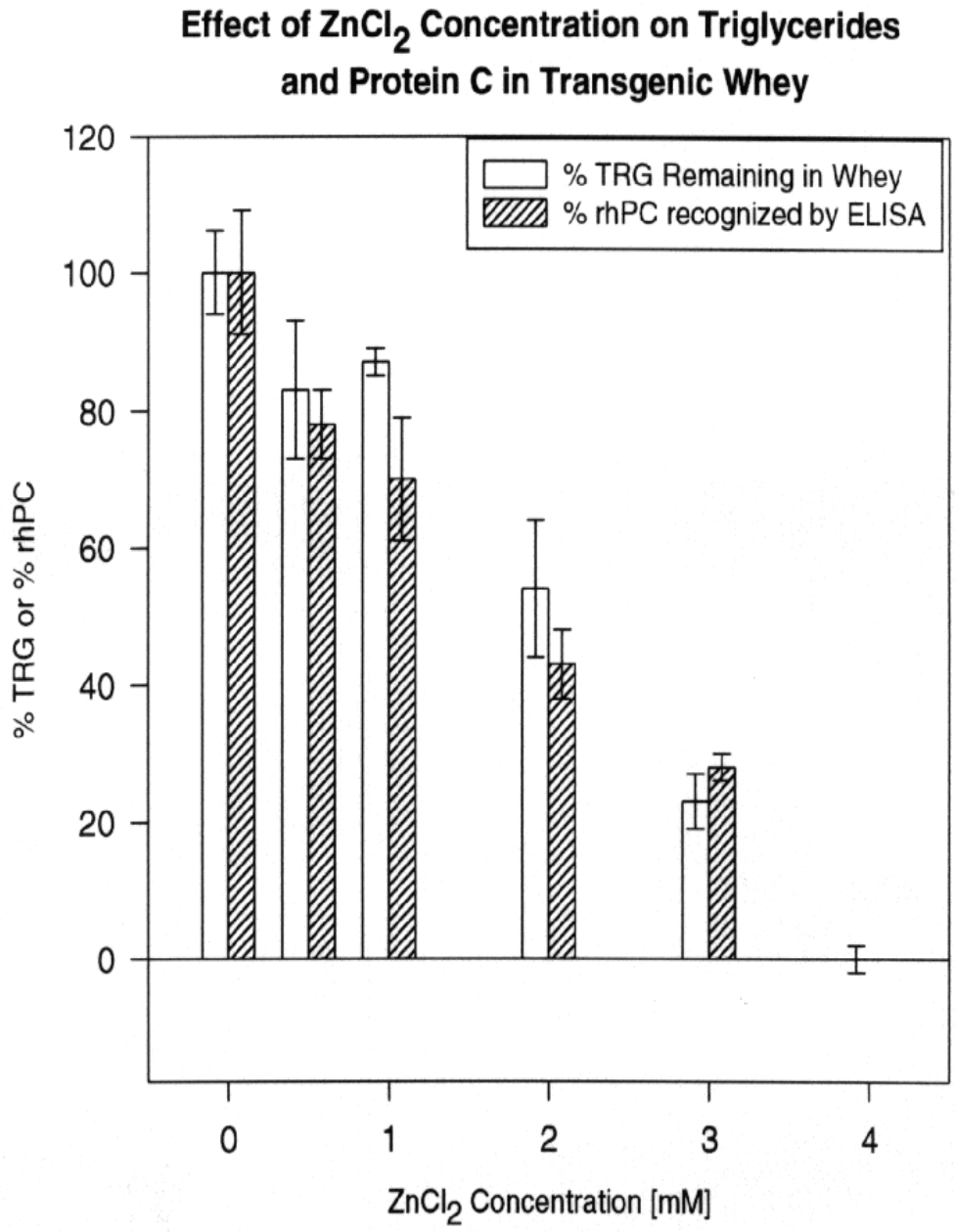



**Figure 13. The Effect of 0-3 mM Zinc Chloride on Triglycerides and Protein C Remaining in Transgenic Whey.**

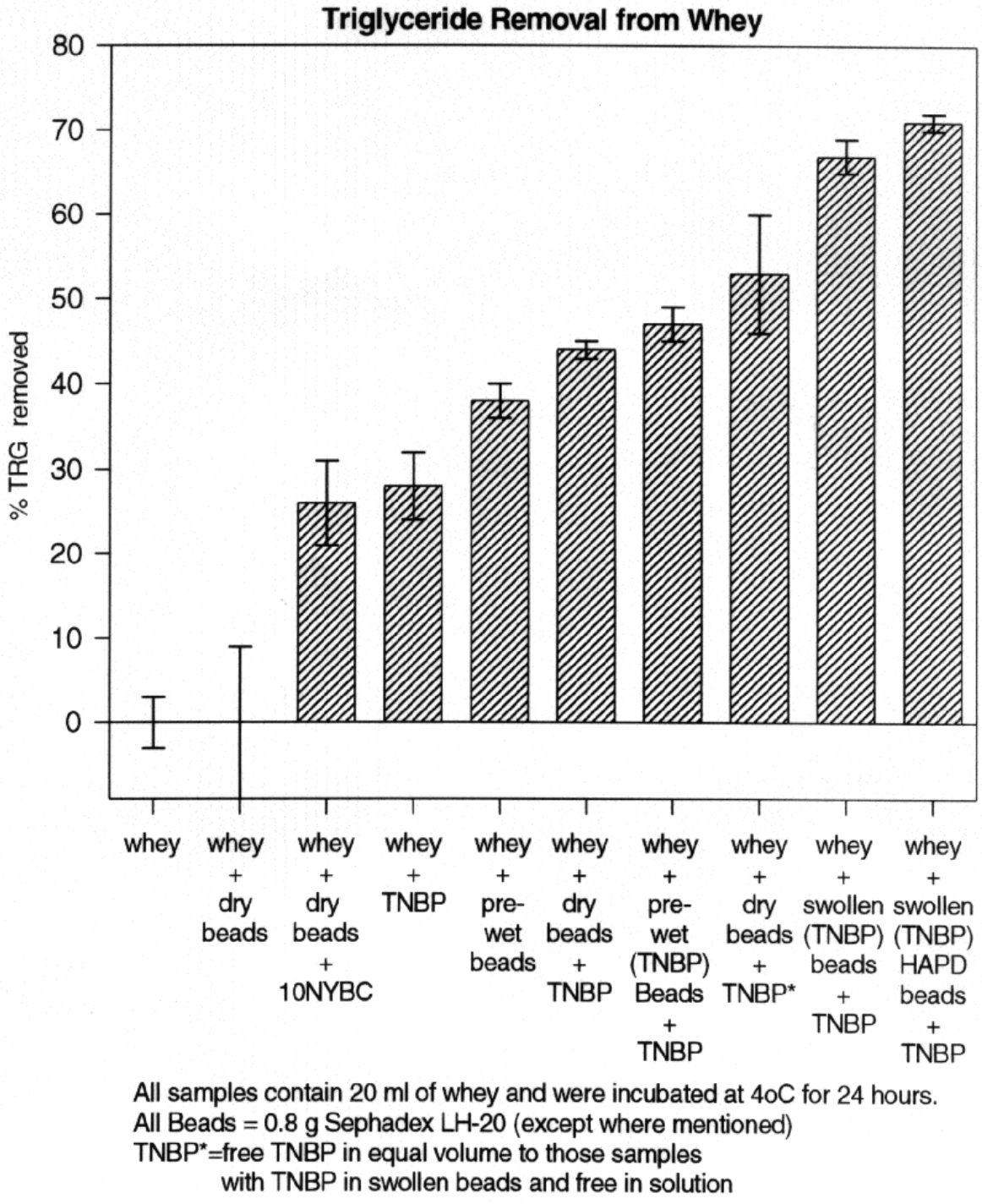



**Figure 14. Results of Significant Delipidation Treatments**

**Table I. Treatment Type G for Various Times between 1-20.5 hours** (Triglyceride detection from whey with 29.3g TNBP/kg whey, 11g Triton X-100, and 3.24g Tween-80/kg whey incubated with 0.8g dry Sephadex LH-20 for various times between 1-20.5 hours at 4°C).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (4°C,1 h) | 5.36 ± 0.01 | 0.282 + 0.001 | 0 ± 1 |
| Treatment G w/o Beads for 1 h | 4.55 ± 0.15 | 0.228 ± 0.007 | 15 ± 3 |
| Treatment G for 1h | 4.24 ± 0.06 | 0.257 ± 0.004 | 21 ± 1 |
| Treatment G for 2 h | 5.35 ± 0.15 | 0.324 ± 0.009 | 0 ± 3 |
| Treatment G for 4 h | 5.79 ± 0.22 | 0.362 ± 0.014 | 0 ± 4 |
| Treatment G for 9 h | 3.89 ± 0.28 | 0.236 ± 0.017 | 27 ± 5 |
| Treatment G for 20.5 h | 2.91 ± 0.13 | 0.176 ± 0.008 | 46 ± 2 |

**Table II. Treatment Type K for various times between 1-20.5 hours** (triglyceride detection from whey with 29.6 g TNBP / kg whey + 0.8 g dry Sephadex LH-20 for various times between 1-20.5 hours at 4°C).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (4°C,1 h) | 6.07 ± -.-- | 0.303 + -.--- | 0 ± -.- |
| Whey(4°C, 24 h) | 8.13 ± 0.46 | 0.407 ± 0.023 | 0 ± 8 |
| Treatment K w/o Beads for 1h | 4.99 ± 0.09 | 0.250 ± 0.005 | 18 ± 2 |
| Treatment K w/o Beads for 24 h | 5.25 ± 0.49 | 0.266 ± 0.025 | 13 ± 8 |
| Treatment K for 1h | 4.84 ± 0.26 | 0.289 ± 0.016 | 20 ± 4 |
| Treatment K for20h | 3.70 ± 0.24 | 0.218 ± 0.014 | 39 ± 4 |
| Treatment K for24h | 3.43 ± 0.01 | 0.205 ± 0.001 | 44 ± 1 |

**Table III. Transport Study with Sephadex LH-20 Beads which compares the Treatment Types O, T, and P** (Effect of Pre-wetting Sephadex LH-20 in water and TNBP upon the detection of triglycerides from whey with 29.3 g TNBP/kg whey, 11 g Triton X-100 /kg whey, and 3.24 g Tween 80 /kg whey after incubation at room temperature for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 4.36 ± 0.08 | 0.218 ± 0.005 | 0 ± 2 |
| Treatment F w/o Beads | 3.94 ± 0.07 | 0.197 ± 0.004 | 0 ± 5 |
| Treatment O | 3.76 ± 0.03 | 0.188 ± 0.002 | 14 ± 4 |
| Treatment T | 3.78 ± 0.13 | 0.229 ± 0.008 | 13 ± 5 |
| Treatment P | 3.83 ± 0.24 | 0.216 ± 0.014 | 12 ± 5 |

**Table IV. Transport Study with Sephadex LH-20 Beads which compares the Treatment Types Q, S, T, and L** (effect of pre-wetting Sephadex LH-20 in Water and TNBP at 23°C for 0.5 hours upon the detection of triglycerides from whey with TNBP after incubation with the pre-wetted beads at 23°C for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 3.76 ± 0.03 | 0.188 ± 0.001 | 0 ± 1 |
| Treatment L w/o Beads | 2.82 ± 0.13 | 0.141 ± 0.007 | 25 ± 3 |
| Treatment Q | 2.76 ± 0.02 | 0.138 ± 0.001 | 27 ± 1 |
| Treatment S | 1.65 ± 0.07 | 0.100 ± 0.004 | 56 ± 2 |
| Treatment T | 3.60 ± 0.31 | 0.221 ± 0.019 | 4 ± 8 |
| Treatment L | 2.28 ± 0.12 | 0.130 ± 0.007 | 39 ± 3 |

**Table V. Size of Sephadex G-25 and LH-20 swollen in 6 ml of water for 48 hours at 23ºC or 4ºC.**

| | Dry weight (g) | Dry Volume (ml) | Wet Volume (ml) | Avg Wet Size (µm) |
|---|---|---|---|---|
| G-25c @ RT | 1.0037 | 1.2 | 4.8 | 263 ± 60 |
| G-25c @ 4ºC | 1.0005 | 1.2 | 4.7 | 171 ± 35 |
| LH-20 @ RT | 1.0010 | 1.2 | 4.3 | 111 ± 23 |
| LH-20 @ 4ºC | 1.0019 | 1.2 | 4.7 | 90 ± 15 |

**Table VI. Size of Sephadex G-25, Sephadex LH-20, and Hydroxyalkoxypropyl Dextran (HAPD) swollen in 5 ml of TNBP for 48 hours at 23ºC or 4ºC.**

| | Dry weight (g) | Dry Volume (ml) | Wet Volume (ml) | Avg Wet Size (μm) |
|---|---|---|---|---|
| G-25c @ RT | 1.0024 | 1.2 | 1.1 | 126 ± 40 |
| G-25c @ 4ºC | 1.0026 | 1.2 | 1.1 | 114 ± 37 |
| LH-20 @ RT | 1.0015 | 1.2 | 3.9 | 103 ± 24 |
| LH-20 @ 4ºC | 1.0026 | 1.2 | 1.3 | 57 ± 17 |
| HAPD @ RT | 1.0075 | 1.5 | 3.4 | ----- |
| HAPD @ 4ºC | 1.0065 | 1.6 | 2.1 | ----- |

**Table VII. Treatment Type A with Sephadex G-25 coarse Beads.** The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey with 0, 0.2g 0.4g, and 0.8 g dry Sephadex G-25 coarse beads at 4℃ for 26 hours. Triglycerides (TRG) quantified by densitometric analyses of TLC.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Treatment A w/o Beads | 16.4 ± 1.0 | 0.82 ± 0.05 | 0 ± 6 |
| Treatment A w/ 0.2 g of Beads | 16.6 ± 0.8 | 0.87 ± 0.04 | 0 ± 5 |
| Treatment A w/ 0.4 g of Beads | 18.5 ± 1.3 | 1.03 ± 0.07 | 0 ± 8 |
| Treatment A w/ 0.8 g Beads | 16.9 ± 1.0 | 1.05 ± 0.06 | 0 ± 6 |

**Table VIII. Treatment Type A with Sephadex LH-20 Beads.** The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey with 0, 0.2g, 0.4g, and 0.8 g dry Sephadex LH-20 beads at 4°C for 26 hours. Triglycerides (TRG) quantified by densitometric analyses of TLC.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Treatment A w/o Beads | 18.3 ± 2.3 | 0.91 ± 0.12 | 0 + 13 |
| Treatment A w/ 0.2 g of Beads | 11.8 ± 1.8 | 0.62 ± 0.10 | 36 ± 10 |
| Treatment A w/ 0.4 g of Beads | 15.8 ± 2.8 | 0.88 ± 0.16 | 14 ± 15 |
| Treatment A w/ 0.8 g Beads | 21.8 ± 1.7 | 1.36 ± 0.11 | 0 ± 9 |

**Table IX. Treatment Type B with Sephadex G-25 coarse Beads.** The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey with 0, 0.2 g 0.4 g, and 0.8 g dry Sephadex G-25 coarse beads at 23℃ for 1 hour. Triglycerides (TRG) quantified by densitometric analyses of TLC.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Treatment B w/o Beads | 17.0 ± 1.1 | 0.85 ± 0.06 | 0 ± 6 |
| Treatment B w/ 0.2 g of Beads | 11.0 ± 1.0 | 0.58 + 0.05 | 36 ± 6 |
| Treatment B w/ 0.4 g of Beads | 11.8 ± 0.9 | 0.65 ± 0.05 | 31 ± 5 |
| Treatment B w/ 0.8 g Beads | 13.6 ± 1.2 | 0.85 ± 0.08 | 20 ± 7 |

**Table X. Treatment Type B with Sephadex LH-20 Beads**. The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey with 0, 0.2g 0.4g, and 0.8 g dry Sephadex LH-20 beads at 23ºC for 1 hour. Triglycerides (TRG) quantified by densitometric analyses of TLC.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Treatment B w/o Beads | 15.7 ± 0.6 | 0.78 ± 0.03 | 0 ± 4 |
| Treatment B w/ 0.2 g of Beads | 12.6 ± 0.2 | 0.66 ± 0.01 | 20 ± 1 |
| Treatment B w/ 0.4 g of Beads | 13.2 ± 0.7 | 0.73 ± 0.04 | 16 ± 4 |
| Treatment B w/ 0.8 g Beads | 14.8 ± 1.3 | 0.93 ± 0.08 | 6 ± 8 |

**TableXI. Treatment Type C with Sephadex G-25 coarse Beads.** The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey with 1 M NaCl and 0, 0.2 g, 0.4 g, and 0.8 g dry Sephadex G-25 coarse beads at 23℃ for 1 hour. Triglycerides (TRG) quantified by densitometric analyses of TLC.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Treatment C w/o Beads | ***** | ***** | ***** |
| Treatment C w/ 0.2 g of Beads | 5.2 ± 0.4 | 0.28 ± 0.02 | -- ± 8 |
| Treatment C w/ 0.4 g of Beads | 5.6 ± 0.3 | 0.31 ± 0.02 | -- ± 5 |
| Treatment C w/ 0.8 g Beads | 5.4 ± 1.9 | 0.34 + 0.12 | -- ± 35 |

***** Below Standard Curve Range

**Table XII. Treatment Type C with Sephadex LH-20 Beads.** The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey with 1 M NaCl and 0, 0.2 g 0.4 g, and 0.8 g dry Sephadex LH-20 beads at 23℃ for 1 hour. Triglycerides (TRG) quantified by densitometric analyses of TLC.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Treatment C w/o Beads | ***** | ***** | ***** |
| Treatment C w/ 0.2 g of Beads | 3.8 ± 0.7 | 0.20 ± 0.04 | -- ± 18 |
| Treatment C w/ 0.4 g of Beads | 7.8 ± 4.1 | 0.43 ± 0.23 | -- ± 53 |
| Treatment C w/ 0.8 g Beads | 3.4 + 1.0 | 0.21 ± 0.06 | -- ± 29 |

***** Below Standard Curve Range

**Table XIII. Treatment Type D with Sephadex LH-20 Beads.** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 25 mM $MgCl_2$ with 0.8g dry Sephadex LH-20 beads at 23°C for 1 hour).

| | Total mg TRG | TRG Conc [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (RT 1 hr) | 6.11 ± -.-- | 0.32 ± -.-- | 0 ± -.- |
| Treatment D w/o Beads | 7.96 ± 0.76 | 0.42 ± 0.04 | 0 ± 12 |
| Treatment D | 8.03 ± 0.80 | 0.45 ± 0.04 | 0 ± 13 |

**Table XIV. Treatment Type D with Sephadex G-25 coarse Beads** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 25 mM $MgCl_2$ with 0.8g dry Sephadex G-25 coarse beads at 23ºC for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Control Whey (4ºC 1 hr) | 5.52 ± -.-- | 0.30 ± -.-- | 0 ± --.- |
| Whey (RT 1 hr) | 2.09 ± -.-- | 0.11 ± -.-- | 62 ± -.-- |
| Treatment D w/o Beads | 4.78 ± 0.10 | 0.24 ± 0.01 | 13 ± 2 |
| Treatment D | 5.58 ± 0.42 | 0.37 ± 0.03 | 0 ± 8 |

**Table XV. Treatment Type E with Sephadex G-25 coarse Beads.** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 2.93g TNBP/kg whey, 11 g Triton-X 100/kg whey, and 3.24 g Tween 20/kg whey with 0.8 g dry Sephadex G-25 coarse beads at 23°C for 1 hour).

| | Total mg TRG | TRG Conc [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (RT 1 hr) | 2.87 ± 0.28 | 0.144 ± 0.014 | 0 ± 10 |
| Treatment E w/o Beads | 2.76 ± 0.12 | 0.138 ± 0.006 | 4 ± 4 |
| Treatment E | 3.64 ± 0.65 | 0.212 ± 0.038 | 0 ± 23 |

**Table XVI. Treatment Type E with Sephadex LH-20 Beads.** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 2.93g TNBP/kg whey, 11 g Triton-X 100/kg whey, and 3.24 g Tween 20/kg whey with 0.8 g dry Sephadex LH-20 beads at 23°C for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (RT 1 hr) | 5.13 ± 0.76 | 0.257 ± 0.038 | 0 ± 15 |
| Treatment E w/o Beads | 3.51 ± 0.17 | 0.175 ± 0.008 | 32 ± 3 |
| Treatment E | 3.58 ± 0.13 | 0.205 ± 0.007 | 30 ± 3 |

**Table XVII. Treatment Type F and I with Sephadex G-25 coarse Beads.** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 29.3g TNBP/kg whey, 11 g Triton-X 100/kg whey, and 3.24 g Tween 20/kg whey with 0.8g dry Sephadex G-25 coarse beads at 23ºC for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (RT 1 hr) | 5.69 ± 0.04 | 0.28 ± 0.01 | 0 ± 1 |
| Treatment F w/o Beads | 6.46 ± 0.60 | 0.31 ± 0.03 | 0 ± 10 |
| Treatment F | 7.79 ± 0.24 | 0.45 + 0.01 | 0 ± 4 |
| Treatment I | 3.15 ± 0.84 | 0.21 ± 0.06 | 45 ± 15 |

**Table XVIII. Treatment Type F with Sephadex LH-20 Beads** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 29.3g TNBP/kg whey, 11 g Triton-X 100/kg whey, and 3.24 g Tween 20/kg whey with 0.8g dry Sephadex LH-20 beads at 23ºC for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (RT 1 hr) | 3.45 ± 0.37 | 0.17 ± 0.02 | 0 ± 11 |
| Treatment F w/o Beads | 3.71 ± 0.68 | 0.18 ± 0.03 | 0 ± 17 |
| Treatment F | 3.00 ± 0.03 | 0.17 ± 0.01 | 13 ± 1 |

**Table XIX. Treatment Type G with Sephadex G-25 coarse Beads.** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 29.3g TNBP/kg whey, 11 g Triton-X 100/kg whey, and 3.24 g Tween 20/kg whey with 0.8 g dry Sephadex G-25 coarse beads at 4°C for 24 hours).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (4°C 24 hrs) | 4.01 ± 0.04 | 0.201 ± 0.002 | 0 ± 1 |
| Treatment G w/o Beads | 3.88 ± 0.01 | 0.187 ± 0.001 | 3 ± 1 |
| Treatment G | 3.63 ± 0.28 | 0.204 ± 0.016 | 10 ± 7 |

**Table XX. Treament Type G and V with Sephadex LH-20 Beads.** (The mass and concentration of triglycerides remaining in whey after incubating 20 ml whey plus 29.3g TNBP/kg whey, 11 g Triton-X 100/kg whey, and 3.24 g Tween 20/kg whey with 0.8 g dry Sephadex LH-20 beads at 4°C for 24 hours).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (4°C 24 hrs) | 5.74 ± 0.13 | 0.287 ± 0.006 | 0 ± 2 |
| Treatment G w/o Beads | 6.65 ± 0.94 | 0.321 ± 0.045 | 0 ± 16 |
| Treatment G | 4.27 ± 0.26 | 0.251 ± 0.015 | 26 ± 5 |
| Treatment J | 3.52 ± 0.29 | 0.204 ± 0.017 | 39 ± 5 |

**Table XXI. Comparison of Treatment Types J and F with Sephadex LH-20 Beads.** (The effect of triglyceride detection from whey containing TNBP (29.3 mg/kg whey) and Whey containing TNBP(29.3 g/kg whey), Triton X-100 (11.0 g/kg whey), and Tween (3.24 g/kg whey) after incubation with 0.8 g dry Sephadex LH-20 at room temperature for 1 hour).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (RT 1hr) | 4.26 ± 0.03 | 0.213 ± 0.002 | 0 ± 1 |
| Treatment L w/o Beads | 2.74 ± 0.13 | 0.137 ± 0.006 | 36 ± 3 |
| Treatment F w/o Beads | 4.54 ± 0.17 | 0.227 ± 0.008 | 0 ± 4 |
| Treatment L | 1.87 ± 0.10 | 0.107 ± 0.006 | 56 ± 2 |
| Treatment F | 3.13 ± 0.14 | 0.181 ± 0.008 | 26 ± 3 |

**Table XXII. Effect of 29.3 g TNBP /kg whey and 29.3 g TNBP/ kg whey + 11 g Triton X-100/kg whey + 3.24 g Tween 80/kg whey (TTT) on rhPC in transgenic whey at 4ºC for 24 hours.**

| | µg/ml Protein C | % in Whey |
|---|---|---|
| Whey | 198 ± 31 | 100 ± 15 |
| Whey+TNBP(1 h) | 168 ± 9 | 85 ± 4 |
| Whey+(TTT)(1 h) | 181 ± 29 | 92 ± 15 |
| Whey+TNBP+LH20(24 h) | 176 ± 12 | 89 ± 6 |
| Whey+(TTT)+LH20(20 h) | 161 ± 8 | 81 ± 4 |

**Table XXIII. Effect of TNBP and TNBP, Triton X-100, and Tween 80 (TTT) on rhPC in transgenic whey at 23ºC for 1hour.**

| | µg/ml Protein C | % in Whey |
|---|---|---|
| Whey (4ºC, 1h) | 151 ± 16 | 100 ± 10 |
| Whey (RT, 1h) | 149 ± 8 | 98 ± 5 |
| Whey+TNBP(1 h) | 95 ± 9 | 63 ± 6 |
| Whey+(TTT)(1 h) | 89 ± 4 | 59 ± 3 |
| Whey+TNBP+LH20(1 h) | 102 ± 12 | 67 ± 8 |
| Whey+(TTT)+LH20(1 h) | 93 ± 3 | 62 ± 2 |

**Table XXIV. Transport Study with Sephadex LH-20 which compares Treatment types K, M, R, and N.** (Triglyceride detection from whey with 29.6 g TNBP / kg whey + pre-wetted Sephadex LH-20 incubated for 24 hours at 4°C).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey(4°C, 24 h) | 5.01 ± 0.14 | 0.250 ± 0.007 | 0 ± 3 |
| Treatment K w/o Beads | 2.90 ± 0.21 | 0.147 ± 0.010 | 42 ± 4 |
| Treatment K | 2.88 ± 0.08 | 0.172 ± 0.005 | 42 ± 1 |
| Treatment M | 3.08 ± 0.12 | 0.154 ± 0.006 | 38 ± 2 |
| Treatment R | 3.12 ± 0.08 | 0.201 ± 0.005 | 38 ± 2 |
| Treatment N | 2.67 ± 0.10 | 0.167 ± 0.006 | 47 ± 2 |

**Table XXV. Comparison of Treatment Types K, U, V, and W with Sephadex LH-20 Beads** (The effect of pre-swelling Sephadex LH-20 beads in water and TNBP at room temperature for 24 hours upon the detection of triglycerides after incubation of the whey with TNBP and the pre-swollen beads at 4°C for 24 hours).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 3.86 ± 0.20 | 0.218 ± 0.005 | 0 ± 5 |
| Treatment K w/o Beads | 3.15 ± 0.50 | 0.197 ± 0.004 | 18 ± 13 |
| Treatment K | 2.43 ± 0.08 | 0.149 ± 0.005 | 37 ± 2 |
| Treatment U | 2.40 ± 0.02 | 0.123 ± 0.001 | 38 ± 1 |
| Treatment V | 1.89 ± 0.10 | 0.111 ± 0.006 | 51 ± 3 |
| Treatment W | 1.28 ± 0.07 | 0.071 ± 0.004 | 67 ± 2 |

**Table XXVI. Comparison of Treatment Types K, A, V, and W with Hydroxyalkoxypropyl Dextran (HAPD) Beads Type X.** (The effect of pre-swelling Hydroxyalkoxypropyl Dextran (HAPD) in TNBP for 24 hours at 23°C upon the detection of triglycerides from whey with or without TNBP after incubation with the pre-swollen HAPD at 4°C for 24 hours.).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 3.56 ± 0.07 | 0.178 ± 0.003 | 0 ± 2 |
| Treatement K w/o Beads | 2.44 ± 0.08 | 0.122 ± 0.004 | 31 ± 2 |
| Treatment K | 3.43 ± 0.13 | 0.211 ± 0.008 | 4 ± 4 |
| Treatment A | 2.45 ± 0.03 | 0.126 ± 0.002 | 31 ± 1 |
| Treatment V | 1.33 ± 0.09 | 0.078 ± 0.005 | 63 ± 2 |
| Treatment W | 1.05 ± 0.03 | 0.058 ± 0.001 | 71 ± 1 |

**Table XXVII. Comparison of Treatment Types G, H, and J while using Sephadex LH-20 Beads.** ($ZnCl_2$ Study of samples with 29.3 g TNBP / kg whey + 11.0 g Triton X-100 /kg whey + 3.24 g Tween 80/ kg whey and / or 4 mM $ZnCl_2$ incubated with 0.8 g dry Sephadex LH-20 beads at 4°C for 24 hours).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 4.42 ± 0.33 | 0.22 ± 0.02 | 0 ± 7 |
| Treatment G w/o Beads | 3.92 ± 0.13 | 0.20 ± 0.01 | 11 ± 3 |
| Treatment G | 2.48 ± 0.07 | 0.12 ± 0.01 | 44 ± 2 |
| Treatment H* w/o Beads | 5.14 ± 0.35 | 0.26 ± 0.02 | 0 ± 8 |
| Treatment J w/o Beads | 4.70 ± 0.28 | 0.23 ± 0.02 | 0 ± 6 |
| Treatment H | 0.00 ± -.-- | 0.00 ± 0.01 | 100 ± 0 |

*was not filtered

**Table XXVIII. Treatment Type H for Various Times Between 0.5 and 24 hours**
(Triglyceride detection from whey with 4 mM $ZnCl_2$ + 0.8 g dry Sephadex LH-20 beads incubated for various times between 0.5-24 hours at 4°C.

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey (4°C,0.5 h) | 4.89 ± 0.11 | 0.257 + 0.006 | 0 ± 2 |
| Whey (4°C,24 h) | 4.56 ± 0.34 | 0.240 ± 0.018 | 7 ± 7 |
| Treatment H w/o Beads for 0.5h | 0.92 ± 0.11 | 0.046 ± 0.005 | 81 ± 2 |
| Treatment H w/o Beads for 24h | 2.14 ± 0.39 | 0.130 ± 0.024 | 56 ± 8 |
| Treatment H for 0.5h | 0.85 ± 0.14 | 0.051 ± 0.009 | 83 ± 3 |
| Treatment H for 1h | 1.49 ± 0.05 | 0.093 ± 0.003 | 70 ± 1 |
| Treatment H for 2 h | 0.42 ± 0.07 | 0.025 ± 0.004 | 91 ± 1 |
| Treatment H for 4 h | 0.00 ± -.-- | 0.000 ± -.--- | 100 |
| Treatment H for 8 h | 0.00 ± -.-- | 0.000 ± -.--- | 100 |
| Treatment H for 20 h | 2.11 ± 0.03 | 0.128 ± 0.002 | 57 ± 1 |
| Treatment H for 24 h | 1.48 ± 0.12 | 0.090 ± 0.007 | 70 ± 2 |

**Table XXIX. Effect of $ZnCl_2$ Concentration on Triglyceride Detection from Whey at 4°C for 30 minutes.**

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 4.92 ± 0.28 | 0.246 ± 0.014 | 0 ± 6 |
| Whey + 0.5 mM $ZnCl_2$ | 4.06 ± 0.48 | 0.203 ± 0.024 | 17 ± 10 |
| Whey + 1 mM $ZnCl_2$ | 4.26 ± 0.09 | 0.213 ± 0.005 | 13 ± 2 |
| Whey + 2 mM $ZnCl_2$ | 2.64 ± 0.51 | 0.132 ± 0.026 | 46 ± 10 |
| Whey + 3 mM $ZnCl_2$ | 1.15 ± 0.21 | 0.058 ± 0.010 | 77 ± 4 |

**Table XXX. Comparison of Treatment Types K and W while holding the TNBP Concentration in the samples constant.** (Triglyceride detection from transgenic pig whey after incubation at 4ºC for 24 hours with or without pre-swollen (at 23ºC for 24 hours) Sephadex LH-20 while holding the concentration of TNBP constant).

| | Total mg TRG | TRG Conc. [mg/ml] | % Removal of TRG in Whey |
|---|---|---|---|
| Whey | 3.55 ± 0.14 | 0.177 ± 0.007 | 0 ± 4 |
| Treatment K w/o Beads | 2.55 ± 0.13 | 0.133 ± 0.007 | 28 ± 4 |
| Treatment W* | 0.76 ± 0.03 | 0.041 ± 0.002 | 79 ± 1 |
| Treatment K* | 1.68 ± 0.24 | 0.086 ± 0.012 | 53 ± 7 |
| Treatment K | 1.98 ± 0.03 | 0.124 ± 0.002 | 44 ± 1 |

*Same Total volume of TNBP in these samples
Treatment K* = Whey + 29.3 g TNBP/kg whey + amount of TNBP in the preswollen Sephadex LH-20 beads of Treatment W

**Table XXXI. Mass of Triglycerides Extracted from 20 ml of Sephadex LH-20 beads after exposure to Treatment Type W with 100 ml of pig whey and then loaded as feed (whey + beads + TNBP) and run through a STREAMLINE column at 4ºC.**

| | mg Triglyceride | mg TRG / mL beads | % Error |
|---|---|---|---|
| Run 7 | 5.3 | 0.265 | 1.0 |
| Run 8 | 4.3 | 0.215 | 2.2 |
| Run 9 | 0.6 | 0.030 | 17.1 |
| Run 10 | 2.1 | 0.105 | 8.3 |

**Table XXXII. STREAMLINE Run #7 Triglyceride Material Balance.** Triglyceride (TRG) Material Balance from extracted fractions of Runs 7 on a STREAMLINE column at 4°C. The feed for the column consisted of Sephadex LH-20 beads exposed to Treatment Type W, with the swelled volume of Sephadex equaling 20 ml and the volume of whey incubated with the swollen beads being 100 ml.

| | mg Triglyceride | % Error |
|---|---|---|
| Feed = 100 ml whey | 12.5 | 3.7 |
| Fall Through | 5.8 | 20.9 |
| Sephadex LH-20 Beads (20 ml swelled beads) | 5.3 | 1.0 |
| TNBP Blow Out | 0.6 | 2.0 |
| Missing Lipid | 0.8 | 6.4 |

**Table XXXIII. STREAMLINE Run #8 Triglyceride Material Balance.** Triglyceride (TRG) Material Balance from extracted fractions of Run 8 on a STREAMLINE column at 4ºC. The feed for the column consisted of Sephadex LH-20 beads exposed to Treatment Type W, with the swelled volume of Sephadex equaling 20 ml and the volume of whey incubated with the swollen beads being 100 ml.

| | mg Triglyceride | % Error |
|---|---|---|
| Feed = 100 ml whey | 21.7 | 2.3 |
| Fall Through | 15.6 | 10.2 |
| Sephadex LH-20 Beads (20 ml swelled beads) | 4.3 | 2.2 |
| TNBP Blow Out | 4.4 | 4.9 |
| Missing Lipid | 0.0 | 12.0 |

**Table XXXIV. STREAMLINE Run #9 Triglyceride Material Balance.** Triglyceride (TRG) Material Balance from extracted fractions of Run 9 on a STREAMLINE column at 4ºC. The feed for the column consisted of Sephadex LH-20 beads exposed to Treatment Type W, with the swelled volume of Sephadex equaling 20 ml and the volume of whey incubated with the swollen beads being 100 ml.

| | mg Triglyceride | % Error |
|---|---|---|
| Feed = 100 ml whey | 18.5 | 1.0 |
| Fall Through | 3.3 | 1.0 |
| Sephadex LH-20 Beads (20 ml swelled beads) | 0.6 | 17.1 |
| TNBP Blow Out | 4.4 | 12.7 |
| Missing Lipid | 10.2 | 55.1 |

**Table XXXV. STREAMLINE Run #10 Triglyceride Material Balance.** Triglyceride (TRG) Material Balance from extracted fractions of Run 10 on a STREAMLINE column at 4ºC. The feed for the column consisted of Sephadex LH-20 beads exposed to Treatment Type W, with the swelled volume of Sephadex equaling 20 ml and the volume of whey incubated with the swollen beads being 100 ml.

| | mg Triglyceride | % Error |
|---|---|---|
| Feed = 100 ml whey | 18.9 | 4.4 |
| Fall Through | 9.1 | 7.0 |
| Sephadex LH-20 Beads (20 ml swelled beads) | 2.1 | 8.3 |
| TNBP Blow Out | 5.6 | 1.3 |
| Missing Lipid | 2.1 | 11.1 |

**Table XXXVI. Effect of $ZnCl_2$ concentration on rhPC in transgenic whey at 4ºC for 30 minutes.**

| | µg/ml Protein C | % in Whey |
|---|---|---|
| Whey | 123 ± 11 | 100 ± 9 |
| Whey + 0.5 mM $ZnCl_2$ | 97 ± 6 | 78 ± 5 |
| Whey + 1 mM $ZnCl_2$ | 87 ± 11 | 70 ± 9 |
| Whey + 2 mM $ZnCl_2$ | 54 ± 6 | 43 ± 5 |
| Whey + 3 mM $ZnCl_2$ | 35 ± 3 | 28 ± 2 |

## Appendix

### 1. Discussion of Other Treatments

#### 1.1 Magnesium and Ethanol

We tried to optimize the shift in whey/lipid/Sephadex equilibrium and also provide fast lipid partitioning by using magnesium and 20 % ethanol. These treatments were thought to have the potential to disrupt the phospholipid layer of the micelles because the proteins in the fat globule membrane bind divalent metals, which decrease the hydrophilic nature of the phospholipids and destabilize the fat globule membrane (1). This would allow the lipids to be adsorbed by the Sephadex beads without inactivation or precipitation of the recombinant protein, rhPC. These treatments with 1 hour residence times at room temperature were tried. The results in Table XIII, which show the triglyceride concentration increased, indicate that other phenomena may be occurring between the Sephadex G-25 coarse beads or the magnesium whey. The beads did not swell notably more in the presence of $MgCl_2$. Possibly, less of the lipid micelles are adhering to the extraction tube walls with the magnesium chloride present when compared to normal whey without magnesium present. This phenomena should be studied further to understand what is happening. This treatment may have possibilities to keep the lipids and triglycerides from adhering to the chromatography medium if it is helping to stabilize the lipid micelles and preventing them from coming out of solution and adhering to the walls of the tubes. The 25 mM $MgCl_2$ and the 20% ethanol added to the whey did not disrupt the micelle as hoped. The micelles remaining after skimming are extremely small and stable, since these added components did not aid in lipid removal at room temperature they were not investigated any further at 4$^{o}$C.

#### 1.2 Solvent and Detergent Treatment (NYBC)

After trying to disrupt the micelles with two different salts and ethanol, it was decided to try adding a more nonpolar solvent which may help perform a mini extraction of the remaining

lipids in the whey. Most previous work with Sephadex LH-20 used a nonpolar, usually immiscible, solvent like chloroform to separate and then purify the lipids (29-36). Many nonpolar solvents can precipitate the target recombinant protein and would also pose a problem in validation for Good Manufacturing Practice (GMP). However, a viral inactivation procedure for plasma, extracts lipids from viral packaging using TNBP, Triton X-100, and Tween 80 (11). The viral inactivation procedure has been used with plasma for years and has not shown any significant negative effects on the many important proteins and enzymes present in plasma. This procedure extracts lipids without affecting most product proteins significantly, including human protein C and has already been incorporated into GMP processes.

The initial studies with the solvent/detergent viral inactivation procedure used the recommended concentrations of TNBP, Triton X-100, and Tween 80 at room temperature for 1 hour (See Tables XV and XVI). These results show no significant lipid extraction occurred at this concentration of solvent/detergent at room temperature. Since there was no significant lipid extraction at this higher temperature, it was not tried at lower temperatures, where the transfer of lipids would be less likely to occur.

We thought the results of incubating the whey with 2.93 g TNBP/kg whey with 11 g Triton X-100, and 3.24 g Tween 80 (Tables XV and XVI) were caused by too small an amount of nonpolar phase with which to extract lipid from the stable phospholipid micelle. So a ten fold higher concentration of TNBP solvent than that used in the viral inactivation procedure was tried. This experiment did show a more significant decrease in triglyceride mass (27-50%) with the Sephadex LH-20 beads but not much triglyceride was removed with the Sephadex G-25 coarse beads (0-13%). The Sephadex G-25 does not have the lipophilic sites inside the bead like the Sephadex LH-20 beads which would account for the better removal of lipids with the LH-20. There was significant triglyceride adsorption at both 4$^{o}$C and 23$^{o}$C with the Sephadex LH-20 and 29.6 g TNBP/kg whey plus 11 g Triton X-100/kg whey and 3.24 g Tween 80/kg whey mixed in with the whey. The results of most experiments, showed a lower triglyceride level when the whey was mixed with TNBP, even without the Sephadex LH-20, which indicates that this level of

TNBP disrupts the lipid micelles or extracts the lipids into the nonpolar phase. At 29.3 g TNBP/kg whey, or approximately 3% TNBP, you are well over the solubility limit of TNBP in water (1ml TNBP dissolves in 164 ml of water). The excess TNBP which has extracted the lipids could be significant because it may prefer (have a lower free energy) and be slightly more soluble in the lipophilic Sephadex LH-20 beads.

It is likely that the presence of Triton X-100 and Tween 80 created the potential to form micelles consisting of these added surfactants and the triglycerides. These micelles would have likely been highly stable and probably presented a barrier to extraction /adsorption into the Sephadex (9). Past studies have used Triton X-100 or Tween 80 to destabilize the fat globule membrane (2). The experiments performed with free TNBP, Triton X-100, and Tween 80 mixed with the whey generally adsorbed about 25% of the triglycerides in the whey, which left a higher quantity of lipids remaining in the whey than those samples treated the same but with only free TNBP (no Triton X-100 or Tween 80). These results could be caused by the Tween 80 forming micelles which cannot get into the Sephadex LH-20 therefore not allowing the lipids to be adsorbed and removed. Therefore, the Tween 80 may have hindered the removal of lipids which were associated with the Tween 80.

When the extracted samples that had been mixed with TNBP, Triton X-100, and Tween 80 were spotted on a HPTLC plate, five more peaks, which almost appear as one long smear from the phospholipid spot to the cholesterol spot (sometimes masking the cholesterol spot), covered the bottom third of the TLC plate (See Figure 12). This smear sometimes affected the TLC assay, depending on how much of these solvents filtered through and were extracted and remained in the spotted samples.

### 1.3 Zinc Precipitation

Zinc has been known to precipitate protein and in this study it was found that the zinc also effectively broke up the lipid micelles and precipitated the lipids more effectively than any other method tried. The fat membrane globules contain integral membrane proteins. The precipitation

of these proteins, which make up 48% of the fat globule membrane may also cause disruption of the micelle (13). The precipitated lipids were evident on the walls and the lids of the sample containers after treatment with zinc. TLC analysis of filtered, zinc treated whey showed less than 5% detection of all types of lipids present (triglycerides, phospholipids, cholesterol, etc.).

The precipitation appears to occur immediately and the time study supports this by showing no relationship between incubation time and lipid removal. The various numbers for triglyceride removal in Table XXVIII and the relatively high standard errors, close to 20% for the 2 and 3 mM $ZnCl_2$ samples in Table XXIX, could be due to how well the samples were mixed when the zinc chloride solution was added, affecting the precipitates size, and therefore how well the precipitates were filtered out. The relationship between lipid and protein C precipitation (see Table XXXVI and Figure 13) negates the value of this method of lipid removal.

Several conditions were used to induce partitioning of the lipids into the Sephadex without affecting levels of the target protein residing in the whey. The lipids appeared stable in whey at 4°C for 26 hours and did not spontaneously partition into the Sephadex, as no reduction in lipid concentration was detected when the whey was added to dry Sephadex LH-20 and Sephadex G-25 coarse beads and then incubated at 4°C for 26 hours. The control treatment groups which were incubated under the same conditions, also showed no reduction of triglycerides. The swelling of dry dextran beads by the whey did not concentrate but appeared to decrease the amount of some lipids remaining in the whey by decreasing the whey volume at 4°C. Some selective adsorption was seen for certain lipid components in dry Sephadex LH-20 bead treatments at either 4°C or room temperature. The TLC of the samples treated with the dry Sephadex LH-20 beads detected less cholesterol esters, free fatty acids, and phospholipids in the whey in proportion to the triglycerides. However, because triglycerides are the major lipid component, removal of the triglycerides was still the primary goal.

## 2. MANUAL FOR USING THE SCAN COMPUTER PROGRAM TO INTERFACE A COMPUTER WITH THE SHIMADZU DENSITOMETER

Please consult the Shimadzu 9000 manual for instructions on how to correctly use the Shimadzu Scanner to obtain your desired information.

### DESCRIPTION OF ALL THE FUNCTIONS IN THE SCAN PROGRAM

**F1 - Load**

This command will load a certain file. After you press F1," Enter file name" will appear on the screen. Just enter the file's directory, backslash, and then the file name.

For example:

Enter file name **scanner\ file1**

**F2 - Save**

This function will save the file where the cursor arrow is.

After you press F2, "Enter file name" will appear on the screen. And the computer will default and put the file name which appears where the cursor is. If you hit [enter], the computer will save that lane onto the **c** drive as its own file with the name which the computer displayed. If you want to change the filename, just erase the file name and change it. If you want to save the file to the **a** drive, you must backspace over the name the computer showed and type **a:filename**. You want the red cursor in front of the lane/file you are saving.

**F3 - Directory**

This function allows you to see and load any files within a directory.

After you press F3, "Enter Directory" will appear on the screen. If you hit [enter] now, the computer will show you all the directories on the c drive. You can select the **scanner** directory and see what files are saved there and choose a file if you want to load it. To choose a file, use

the arrow keys to highlight a file and press [enter]. To look at the files on a disk, type **a:** after the "Enter Directory" command and select the file you want to load.

**F4 - View**

This function allows you to view a loaded file but it will not let you manipulate the file or its peaks.

**F5 - Print**

This function allows you to print the loaded file or the data table showing the peak areas for the various peaks in that file. When you press F5, a menu page will appear. Select your desired choice. This function will only allow you to print one file at a time, the loaded file. If you choose the F1 function in the print menu, "Start Printing on an HP3", the scan will print with the peak heights and area % in a table below.

The program is set up so it can ONLY print on a Hewlett Packard Lasar Printer III. After it is done printing, the computer will return to the main screen.

**F6 - Peak Edit**

This function will display the scan file and allow you to manipulate it with the functions at the bottom of the screen.

| | | | | |
|---|---|---|---|---|
| **F1-Move Start** | **F3-Insert Peak** | **F5-Zoom X** | **F7-Unzoom** | **F9-Peak dat** |
| **F2-Move End** | **F4-Delete Peak** | **F6-Zoom Y** | **F8-Recalc** | **Esc-Quit** |

Move the cursor with the arrow keys. To move in larger jumps, hold the control button down while pressing the arrow key(s).

**F1-Move Start**

To move the start of a peak, place the cursor where you want the peak to start and then press F1. The closest beginning of a peak will where the cursor is.

**F2-Move End**

To move the end of a peak, place the cursor wher you want the new peak ending to be and then press F2. This command will affect the closest peak.

**F3-Insert Peak**

This command will insert a peak where the cursor is. The new peak begins where the cursor is placed and does not "recognize" the size of the peak. It marks the peak's end, 2 mm from the peaks beginning so it is then necessary to move the peak's end by moving the cursor to its proper end and press F2.

**F4-Delete Peak**

This function will delete a numbered peak on the scan. After you press F4, the computer will ask which peak you want to delete. Just type in the peak number and [enter]. The computer will not allow you to delete more than 3 peaks. If you need to delete more than three peaks, you must delete all the peaks in the scan by holding the control button down while pressing F4. Then you will have to insert each peak youself using the F3 and F2 functions.

**F5-Zoom X**

This command will zoom in on the X coordinates to 2 times its original size around where the cursor is placed. This button can be pressed multiple times to zoom in 2x, 4x, 8x, and 16x, its original size.

**F6-Zoom Y**

This command will zoom in on the Y coordinates by amplifying everything in the y direction to 2 times its size. This button can be pressed multiple times to zoom in 2x, 4x, 8x, and 16x, its original size.

**F7-Unzoom**

This function allows you to undo the zoom functions to take the screen peaks back to their original size.

**F8-Recalc**

This function is very important because it will recalculate peak areas and percentages after any peak changes have been made. Be sure to hit F8 after changing any peaks and before you exit the peak edit section or your peak areas will not be modified.

**F9-Peak Data**

This function will display a peak data table reporting all the peaks, their location, the peak area and the area percent. Esc will take you back to the peak edit command screen.

**Esc-Quit**

This command will take you out of the peak edit and back to the main screen.

**F7-Computer Control <---> Scanner Control**

This function will toggle back and forth between computer control and scanner control each time you press the F7 key. To use the computer to control the scanner, the computer needs to be in scanner control. The F9 Download and F10 Auto scan functions, will turn from Red to Green when you switch from computer control to scanner control. You want it to be in scanner control

to set the scan parameters and run the auto scan.

**F8-Scan Parameters**

****Start X (mm)** 10.00
****Start Y (mm)** 15.00
****End Y (mm)** 93.00
****Number of Lanes** 10
****Distance between lanes (mm)** 10.00
**Delta Y (mm)** 0.10
**Swing Width (mm)** 5.00 **Zigzag mode only**
****Plate label** 1213a1
**Photo mode** reflectance
**Zero set** At start
**Scan mode** Linear
****Beam Size** 0.4 x 10.0
****Wavelength** 240
**Smoothing** 15 points
**PKF filter** 2
****Min. Width** 2
****Min. Area** 250

** I Needed to change the default values to those as listed here.
The yellow parameters mean you must enter the number and press [enter].
The red parameters mean you use the arrows to find the desired value or parameter and then press [enter].
Be sure to press [enter] after changing any parameters.

Only 10 lanes can be scanned at one time with this program.

**F9-Download**

This function will download the X and Y coordinates of where the light beam is on the scanner to the start X and start Y scan parameters under F8.

**F10-AutoScan**

This function will automatically scan the programmed number of lanes on your plate according to the programmed scan parameters in F8. When you press F10, the screen will show, "There is no going back form here. Start Scan?" Press **Y** if you are ready to start the autoscan. You cannot stop the autoscan unless you turn the computer or the scanner off. When the autoscan is finished, all the scanned lanes will appear at the top of the screen.

**Step by Step sample walk through to use the computer interface with the Shimadzu Scanner**

Turn the power on the Shimadzu with the blue button on the right side of the plate holder.

Turn the power on the monitor with the button at the lower right corner of the screen.

Move the TLC plate with the arrows on the left side of the TLC plate stage. For my work, I scanned the whole lane so the Y coordinate started at approximately 15 mm and scanned down to 93 mm. The X coordinate defines where the lane or spot is on the plate. My first lane started at approximately 10 mm and all other lanes were spaced evenly about 10 mm apart.

To use the computer interface make sure the RECORDER is OFF.

All settings on the Shimadzu should be correctly set before placing the Shimadzu in External Mode.

**Important Settings on the Shimadzu:**

Recorder (off)

Grid (off or on)

Ordi range (auto)

Lane Auto (light on)
single

Area (light on)
Conc

Operation (local or external mode) external mode is necessary for the computer interface. See instructions below.

Photo Mode (Reflectance)

Zero Set Mode (at start)

X position = <-------> side to side on the plate

Y position = up and down on the plate

Light Source = $D_2$ for wavelengths from 150-300 nm
for wavelengths from 300-600 nm

Move the plate with the arrows on the left of the stage (where the TLC plate is held).

-Record the desired X and Y coordinates as reported on the screen for the lane you want to scan

-Hit the [page] button to get back to the screen which will show you the X and Y coordinates

You also need the Shimadzu to be in external mode for the computer to control the scanner. To put the Shimadzu in EXTERNAL mode, press the operation button and then press the number 4 and finally [enter] to have it in external mode. There will not be a READY message at the lower right corner of the screen when it is in external mode.

**Computer**

To get into the scanner program,

At the c prompt type: **cd scanner**

c:\>**cd scanner** [enter]

Then type **scan**

c:\>scanner**scan** [enter]

You should come to a screen which lists all the functions at the bottom of the screen

| | | | |
|---|---|---|---|
| F1 - Load | F4 - View | F7 - Computer Control | F9 - Download |
| F2 - Save | F5- Print | F8 - Scan Parameters | F10-Auto Scan |
| F3 - Directory | F6 - Peak Edit | | |

**Press F7**. The "comp control" message should change to "scanner control" and the red F9-Download and F10-Autoscan should become green writing instead.

**Press F8** (-Scan Parameters). Enter all your desired parameters by typing in the numbers for the parameters written in yellow and then press [enter] and use the arrow keys for the parameters written in red and then press [enter] when your desired selection is shown.

**F10** (AutoScan) will show, "There is no going back from here. Start Scan?" Press Y and then [enter] if you are ready to start the scan.

The Scanner will then begin to scan. The scanner always prints out a "Command Format Error". Do not worry about this message.
When the scanner is finished scanning, all the scanned lanes will appear listed at the top of the screen.

**Esc** - Takes you back to the Main Menu

*****Warning*****
You can only scan 10 lanes at a time with this program.
There is also a memory limit to how long a lane can be. I hit this limit when I scanned more than approximately 80 mm. If the length of the lane is too long, the computer will start the autoscan

but will get caught and remain at “contemplating data-” indefinitely and will not continue scanning. If this happens, you will have to reboot the computer and turn off the Shimadzu scanner and start everything from the beginning. I also needed to reboot the computer after scanning 10 lanes or it would get caught and remain at “contemplating data-” indefinitely and will not continue scanning.

Press **F2 (save)** to save each scanned lane as its own file. If saving to the hard drive and the file name is correct, just hit [enter]. If you want to save the file to the a drive, backspace over the file name and type **a:**filename. Move the cursor down to the next lane and repeat the saving procedure.

To retrieve a file, from the c drive, press **F3 (Directory)** and type **c:scanner** [enter]. Then highlight the desired file and press [enter].
To retrieve a file from the a drive, press **F3 (Directory)** and type **a:** [enter]. Then highlight the desired file and press [enter].

**To edit the scanner file loaded**, press **F6 (peak edit)**. Then add or delete or change the start or finish of the various peaks with the various commands at the bottom of the screen.
You cannot delete more than 3 peaks. If you need to, hold the control button down while pressing the F4 key, which will delete ALL the peaks shown (which is what I had to do almost every time and then insert my own peaks).
Use the arrow keys to move the cursor. To move the cursor in larger jumps, hold the control button down while pressing the arrow key.
To insert a peak, move the cursor to the beginning of the peak and press **F3 (insert peak)**. Then you need to move the cursor to the end of the peak with the control button and arrow keys to the desired place and then press **F2 (move end)**. After editing all the peaks in that lane/file be sure to press **F8 (recalc)** before exiting the peak edit screen.

To exit the peak edit screen press the [esc] key.

**To save all the the changes you made to this file**, press **F2 (save)** and then hit [enter] if the file name is correct. If that file name already exists, the computer will write "File exists, Overwrite?" Type **[Y]** if you just want to update the file with the peak edits you performed.

After saving the updated file, you want to **print out you file Press F5 (Print)**. I generally pressed F1-"Start printing on an HP3", which will print the scanned and its peak data table. But you can choose whatever command suits your desire.

**To quit/exit the scan program**, press [esc] at the main menu and "Are you sure you want to quit?" will appear. Answer [Y] for yes and [N] for no.

**General Comments:**

You can only scan 10 lanes at a time with this program.

There is also a memory limit to how long a lane can be. I hit this limit when I scanned more that approximately 80 mm. If the length of the lane is too long, the computer will start the autoscan but it will get caught and remain at "contemplating data-" indefinitely and will not continue scanning. If this happens, you will have to reboot the computer and turn off the Shimadzu scanner and start everything from the beginning.

When the Scanner begins to scan, it always prints a "Command Format Error" when the Computer is controling the scanner. Do not worry about this message.

Make sure a Hewlett Packard Lasar Jet III printer is connected to the computer you are using to print your files.

You can only use the Old IBM to interface with the Shimadzu 9000 (i.e. to scan plates and save

the files.  I used the Ast 486 to edit my saved scan files and to print them out because this was faster but it is not necessary if the printer is connected to the old IBM.

To use the scan Program on the 486, type **cd scan** at the c:\>prompt.  Then type **scan** again once you are in the scan directory i.e..

c:\> **cd scan** [enter]

c:\> scan**scan**

When you are finished scanning,editing, and printing your data, turn off the computer and the Shimadzu.

## Vita

Tara Conti Gardner was born February 23, 1970 in West Chester, Pennsylvania. She is the third oldest of seven children. The author earned her Bachelor of Science in Chemical Engineering in 1992 from Lafayette College in Easton, Pennsylvania. She then began her graduate studies in Chemical Engineering at Virginia Polytechnic Institute and State University. Tara Gardner's studies and research at Virginia Polytechnic Institute and State University focused in the biochemical engineering area (Delipidation Treatments for Large Scale Protein Purification Processing) under the guidance and support of her advisor, Dr. William H. Velander.