A NEW PROCESS FOR THE SEPARATION AND PURIFICATION OF EGG YOLK ANTIBODIES

By

Edward A. Charter

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Department of Chemical Engineering
The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

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Abstract

A three stage process was developed for the separation of an antibody (IgY) from industrially separated chicken egg yolk. This included aqueous extraction of the water soluble fraction (WSF) from the yolk by dilution with distilled water and pH adjustment, separation of IgY from the WSF using a cation exchange column in an automated chromatography system, and finally purification of IgY using precipitation with sodium sulphate. The overall recovery of the process was approximately 50%, and the purity 95% or greater depending on the number of precipitation steps used. An automated liquid chromatography system was developed to allow efficient study of various chromatographic media for the separation of the antibody. Analysis of breakthrough curves indicates that superficial velocity is the governing parameter in the binding of IgY to the cation exchanger. Results of a pilot scale experiment involving the application of 46.5 litres of WSF containing approximately 0.8 mg/ml IgY to a 1500 ml column are presented.

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List of Abbreviations

AEC	Anion exchange chromatography
AIDS	Acquired immune deficiency syndrome
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CEC	Cation exchange chromatography
CV	Coefficient of variance
DEAE	Diethylaminoethyl-
ELISA	Enzyme linked immunosorbent assay
EYS	Egg yolk supernatant
HPLC	High performance liquid chromatograph
IEF	Isoelectric focussing
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
LDL	Low density lipoproteins
LPS	Lipopolysaccharide
LSF	Lipid soluble fraction
MCIC	Metal chelate interaction chromatography
NMWC	Nominal molecular weight cut-off
PB	Phosphate buffer (sodium form)
PBS	Phosphate buffered saline (sodium form)

PEG	Polyethylene glycol
pI	Isoelectric point
R^2	Coefficient of correlation
RID	Radial Immunodiffusion
S	Standard deviation
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TB	Tris buffer
TBS	Tris buffered saline
TMV	Tobacco mosaic virus
TP	Total protein
UF	Ultrafiltration
VDC	Volts direct current
$\overline{X}$	Mean (statistical)
WSF	Water soluble fraction

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Chapter 1

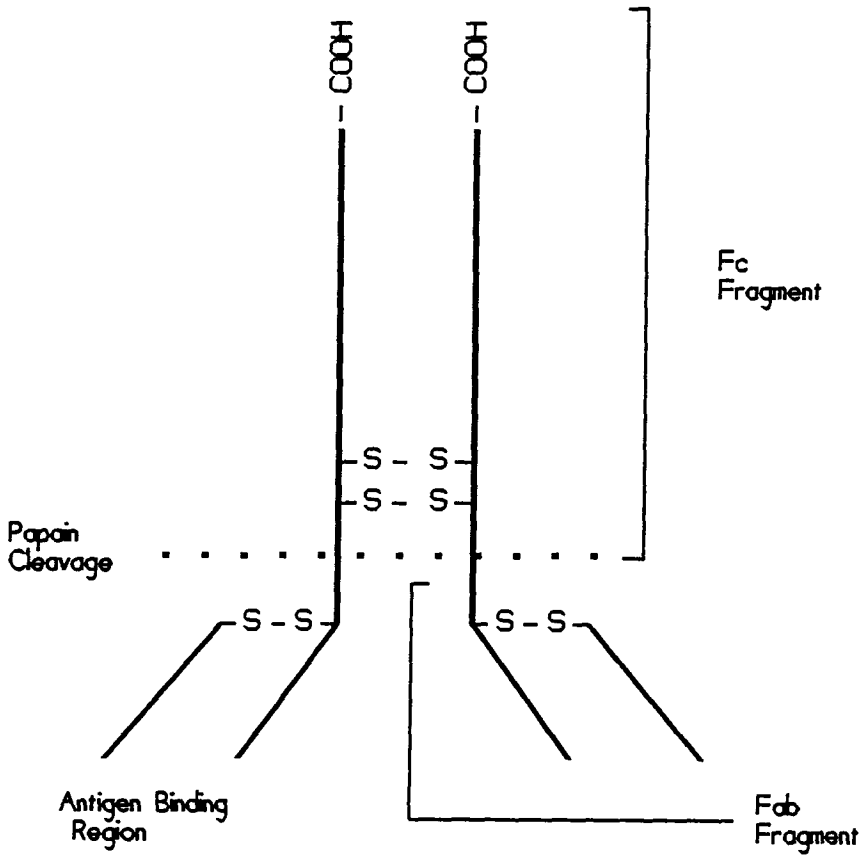
Introduction

This dissertation is principally concerned with the purification of an antibody found in chicken egg yolk. Antibodies, also termed immunoglobulins (abbreviated Ig's), are glycoproteins (Weir, 1988) which are produced in response to immunization with antigens, and which specifically react with the antigen (Sell, 1987). A schematic diagram of a typical immunoglobulin molecule is shown in Figure 1.1. Mammals, including humans, produce several classes of antibodies that reside in the blood and perform various functions in protecting the individual from pathogens.

Human beings have been plagued by diseases as far back as our recorded history can take us. In describing a plague in Athens nearly 2500 years ago, Thucidides, the famous Greek historian, noted that those who had recovered from the plague were able to tend the sick without fear of contracting the disease again - in other words, they were immune (Steward, 1984).

Today, so much is known about immunology and the function of the various immunological molecules, and so much is being published about this topic that it is virtually impossible to keep abreast of all of the new information. Fortunately, there are some similarities between the classes of antibody found in different animals and bird species, and many of the techniques for analyzing and utilizing these molecules are now well developed. Antibodies in the chicken have been studied for a century, but large scale use of chicken antibodies has been hindered by problems associated with acquisition and

Figure 1.1: Schematic Diagram of a Typical Immunoglobulin Molecule. S-S represents disulphide bonds. F_{ab} is the antigen binding portion of the molecule. F_c is the heavy chain portion of the molecule.



purification in large quantities. Similar problems exist in attempting to purify large quantities of mammalian antibodies. Antibodies are used in a host of scientific procedures, most involving some sort of assay for another protein, enzyme, virus or bacterium. Such procedures are termed immunoassays because they make use of the strong affinity of the immunoglobulin for a specific antigen. They can also be used as ligands for affinity chromatography columns, and interest in using purified antibodies in prophylaxis and therapy is also growing. Thus the demand for purified antibodies is also growing and present sources are not likely to be sufficient for future demand.

One of the most common mammalian antibody classes is known as γ-globulin or immunoglobulin-G (IgG). This molecule has a molecular weight of about 150 kDa, and in a purified state from various animal sources it is used extensively in the applications described above. A non-mammalian but roughly equivalent antibody with similar function is chicken γ-globulin, known as immunoglobulin-Y (IgY) to emphasize certain differences between it and its mammalian counterpart. IgY is a slightly larger molecule and has certain other charateristics that set it apart from IgG, but has been used successfully in many of the same applications. What is of especial interest about this molecule is that it is efficiently tranferred from chicken serum to egg yolk in order to provide passive immunity to the chick. Many researchers have considered chicken egg yolk as a potential source of antibodies. Much of the impetus for such research comes from the advantages of using egg yolk as a source of antibodies instead of serum from either birds or mammals.

This dissertation describes efforts to develop a practical purification process beginning with industrially separated egg yolk as raw material, and ending with a purified product that retains its biological activity. The emphasis has been on simplicity in order to develop a process that is not so complicated that it is difficult to scale up. Labour and capital intensive methods have been avoided where possible so as to develop a process

that is also economically viable.

The process consists of 3 basic stages. The first stage, extraction of IgY, involves the separation of the lipid soluble fraction (LSF) of egg yolk from the water soluble fraction (WSF), which contains IgY. The second stage, separation of IgY, involves the removal of the majority of the contaminants from the WSF. An automated chromatography system devised by the author is described. The third and final stage, purification of IgY, involves the isolation of the antibody to purities well in excess of 90%. An HPLC technique for molecular weight determination and quantification of IgY in aqueous solution is described and compared to radial immunodiffusion (RID). Following the process development, a study of factors affecting the scale-up of the chromatographic step is carried out, and the results of a pilot-scale experiment presented.

This work represents a pioneering effort in the use of cation exchange chromatography (CEC) to separate IgY from the WSF of ckicken egg yolk. As such, it is not meant to be the final word on the subject, but rather to demonstrate CEC as a potentially practical industrial method.

An attempt has been made to avoid the verbose style often used in large documents of this nature, and to this end gobbledygook has been avoided[1]. However, for sections where this was not achieved, the author sincerely apologizes.

[1]Gobbledygook has been defined as writing that suffers from a swelling of its parts, see Meyer, 1977, Reports full of gobbledygook, J. of Irreproducible Results, 22(4), 12.

Chapter 2

Literature Review

2.1 Introduction to IgY

IgY is a molecule which has received considerable attention in recent years, and yet still is not fully understood from an immunological perspective. In this first section of the review, a brief history of the study and use of the molecule is presented, along with some of its biochemical and physical characteristics, and a discussion of the other major constituents of egg yolk.

2.1.1 A History of the Study of IgY

According to Brambell (1970), the earliest published paper dealing with the transfer of passive immunity from mother to offspring in mammals was by Ehrlich (1892). A year later, Klemperer (1893) discussed the transfer of passive immunity in the fowl. Thus it has been only in the last 100 years that the modern scientific approach has been used to study the presence of immunological factors in the egg and young chick.

At the turn of the century, Osbourne and Campbell (1900) isolated what they termed "a large amount of protein" from egg yolk, and identified it as vitellin[1]. Plimmer (1908), upon repeating their work, was also able to isolate a water soluble protein in the yolk and named it "livetin", an anagram formed from the letters of vitellin (Burley and Vadehra, 1989). He also quoted Gross (1899) as having noted the presence of this water soluble

[1]The vitellin of egg yolk is today termed "lipovitellin" due to its lipoprotein structure.

protein.

Meanwhile, the ability of chicken serum to precipitate blood components was receiving some attention from medical researchers. Ewing (1903) first used fowl "precipitins" in blood identification, while Sutherland (1910) used this technique on a large scale. Hektoen (1918) reported on the immunization of chickens with human blood and serum, and determined that the maximum concentration of "anti-human precipitin" occurred 9 to 12 days after injection.

Kay and Marshall (1928) isolated the livetin fraction in a relatively pure form, and in 1932, Jukes and Kay determined that IgY and chicken IgG are either closely related or identical. The livetin fraction was separated into 3 proteins using electrophoresis by Shephard and Hottle (1949), and later by Martin et al. (1957), who named the three α-, β-, and γ-livetin.

Since the early 1960's, many papers have been published dealing with methods of isolation and purification of IgY, techniques for obtaining high antibody titers in serum and yolk, and new applications of the purified antibody. Perhaps the most notable purification procedure developed is that of Polson et al. (1980a), in which polyethyleneglycol (PEG) is used to precipitate IgY. Although this method, as well as two revised procedures (Polson et al., 1985; Polson, 1990) provide IgY of relatively high purity for laboratory scale use, they are not as appealing for large scale purification of IgY due to their labour-intensive steps, and the use of PEG, which may prevent the use of the product in food applications. For these reasons, numerous other procedures for IgY isolation and purification have been studied, and will be discussed in more detail later.

Table 2.1 presents some of the important milestones in IgY research over the last century.

Table 2.1: Milestones in the Study of IgY

Year	Author(s)	Event
1892	Ehrlich	studied transfer of passive immunity from mother to offspring
1893	Klemperer	discussed transfer of passive immunity in the fowl
1899	Gross	noted presence of a water soluble protein in yolk
1900	Osbourne & Campbell	isolated "a large amount of protein" from yolk and identified it as vitellin
1903	Ewing	first used fowl precipitins in blood identification
1908	Plimmer	isolated water soluble protein in yolk and named it livetin
1910	Sutherland	used fowl precipitins on a large scale in blood identification
1918	Hektoen	studied anti-human precipitins in chicken serum
1928	Kay & Marshall	isolated livetin fraction in a relatively pure form, but noted signs of heterogeneity
1932	Jukes & Kay	determined that IgY and chicken IgG are either closely related or identical
1949	Shepard & Hottle	isolated 3 proteins from livetin fraction using electrophoresis.
1950	Shmittle	detected antibodies to Newcastle disease in egg yolk
1957	Martin et al.	isolated the 3 livetins and named them α-, β- and γ-livetin.
1980	Polson et al.	developed the polyethylene glycol method for IgY purification

2.1.2 Characteristics of IgY

Identification as an Immunoglobulin

Although they were not aware of the divesity of proteins in the yolk livetin fraction, Jukes and Kay (1932) were convinced that livetin and serum globulin were one and the same. Nace (1953) noted that livetin absorbs antibodies from rabbit antisera to fowl serum albumin and fowl γ-globulin and therefore contains proteins that are similar to these.

Knight and Schechtman (1954) decided to study the ease with which proteins are transferred from the hen's serum to the yolk. They injected hens with rat serum, bovine albumin and γ-globulin, and lobster serum. They were able to detect all of these antigens in the yolk a few days after injection by precipitin reactions with rabbit antisera against the corresponding antigens. Bovine albumin and IgG appeared to be transferred in substantially unmodified form from the hen's blood into the ovarian egg.

Using immunoelectrophoresis and starch gel electrophoresis, Williams (1962) identified γ-livetin as γ-globulin. In 1969, Leslie and Clem suggested that fowl IgG be called IgY, because in their words "it does not align itself very well with any of the mammalian immunoglobulins". Leslie (1975) stated that IgY is without question the predominant immunoglobulin of yolk, and that most of it is transferred to embryonic circulation in the last 5 to 6 days of embryonation. Wang et al. (1986) found a mannose-binding subclass of chicken IgY that was identical in serum and yolk when compared by electrophoresis, gel filtration, amino acid composition and several other physical and chemical properties.

Based on sodium dodecylsulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focussing (IEF), Loeken and Roth (1983) determined that ovarian IgY receptors in the fowl selectively transport all subpopulations of maternal IgY, but not

Table 2.2: Some Early Papers Dealing with Antibody Activity in Egg Yolks

Source	Year	Author(s)	Antigen
chicken	1893	Klemperer	tetanus
"	1901	Dzierzgowski	diphtheria
"	1928a/b	Ramon	tetanus
"	1934	Jukes et al.	diphtheria
duck	1934	Fraser et al.	diphtheria
chicken	1946	Brandly et al.	Newcastle disease
"	1948	Briles	hemolytic activity
"	1952	Bornstein et al.	Newcastle disease
"	1951/52	Buxton	Salmonella

other immunoglobulins.

Thus the evidence is substantial to support the identification of yolk immunoglobulins as chicken IgY, originating from maternal serum.

Antibody Activity

Numerous authors have reported the presence of antibody activity in egg yolks appearing several days after immunization of hens with various antigens.

Schmittle (1950) tested 136 hens that had been exposed to *Tortor furens* (Newcastle disease virus) and found that the serums and egg yolk of 96.3% of the hens were positive (ie: precipitated the virus). He believed that contamination of the yolk with albumen caused some nonspecific hemagglutination inhibition. Table 2.2 lists a few more specific examples.

It seems that the antibody activity of the yolk is well maintained for at least 6 months in eggs stored at cooler temperatures (Brandly et al., 1946). However, the half-life of chicken IgG has been reported as about 35 h in an adult hen, and about 72 h in a newly

hatched chick (Patterson et al., 1962b).

Heller (1975) monitored the antibody production in hen serum and yolk after injection of the hens with a pathogenic E. coli strain. Yolk antibody titres were slightly lower than those found in the serum of the hens.

Otani et al. (1991) determined that IgY antibodies lost little of their antibody activity by incubation for 10 min at pH 7.2 below 60°C, or by incubation for 10 min at 40°C above pH 4.0. However, above 65°C or below pH 4.0 their antibody activity was greatly diminished.

Molecular Weight

Many authors have reported values for the molecular weight of the IgY monomer. Most have used methods such as sedimentation equilibrium or approach to equilibrium, although a few have reported using other methods such as gel filtration or SDS-PAGE. A summary of literature values is given in Table 2.3.

From the literature, it is apparent that the IgY monomer is larger than mammalian IgG, with a mass close to 170 kDa.

Two authors have reported estimations of the molecular weights of IgY aggregates. Hersh and Benedict (1966) reported a value of 550 kDa, and Van Orden and Treffers (1968) a value of 540 kDa. Thus, it is apparent that IgY has a tendency to form a trimer, since the expected molecular weight of the trimer, based on the size of the monomer, would be 510 kDa.

Concentration in Blood and Yolk

The amount of IgY in yolk relative to the other livetin proteins is reported to be quite variable, depending on the level of immunization, and the time since injection. Martin

Table 2.3: Survey of Molecular Weight Values Reported for IgY Monomer

Source	Reference	Method	MW kDa
yolk	Martin & Cook (1958)	Sedimentation Equilibrium	150
serum	Tenenhouse & Deutsch (1966)	Sedimentation Equilibrium	206
serum	Leslie & Clem (1969)	Sedimentation Equilibrium	170
yolk	Wang et al. (1986)	SDS-PAGE	169
yolk	Shirman et al. (1988)	Gel filtration	160
yolk	Hassl & Aspock (1988)	Gel filtration	175
yolk	Polson et al. (1980)	Various methods	165
yolk/serum	Loeken & Roth (1983)	SDS-PAGE	180
yolk/serum	Rose et al. (1974)	Sedimentation Equilibrium	174
serum	Van Orden & Treffers (1968)	Gel filtration	150
yolk	Akita & Nakai (1992)	SDS-PAGE	180
yolk	Fichtali et al. (1992)	SDS-PAGE	175
		$\overline{X} =$	171.2
		$S =$	15.0
		$CV =$	8.8%

and Cook (1958) were unable, at the time of publication of their work, to estimate the amount of IgY present in egg yolk, but suggested that it may constitute nearly half of the livetin based on the relative areas in electrophoretic and sedimentation diagrams. Polson et al. (1980a) determined that IgY comprised more than 70% of the total livetin proteins in yolk, seemingly in contradiction to the results of Shephard and Hottle (1949), who found the 3 livetin proteins to be present in relatively the same proportion. Polson et al. attributed this descrepancy to the fact that they used eggs of hyperimmunized hens, and suggested that the increase in the IgY component is due to an increased amount of antibody against the virus.

In general, the literature seems to indicate that the concentration of antibodies in egg yolk from immunized hens tends to be slightly lower than the corresponding concentration in hen's serum (Orlans, 1967), although the occasional paper will indicate a higher yolk than serum IgY concentration (Rose et al., 1974; Wallman et al., 1990). Since both serum and yolk levels of IgY first increase and later decrease after immunization of a hen, the concentration is usually in a state of flux. For example, Patterson et al. (1962a) compared serum and yolk antibody levels for hens immunized with a single injection of influenza virus. They found the peak serum level was nearly double that of the yolk, but yolk antibody levels remained near peak levels for a longer period of time (almost 6 days compared to 1 to 2 days for serum). Gardner and Kaye (1982) report that quantities of IgY corresponding to almost 500 ml of antiserum can be recovered from a hen's eggs in one month.

In terms of absolute concentration, Wang et al. (1986) reported yolk to have an IgY concentration of at least 9 mg/ml based on a semiquantitative double immunodiffusion method. Levels as high as 25 mg/ml (Rose et al., 1974) have been reported for the yolks of hyper-immunized chickens.

The amount of antibody produced is also a function of the type of antigen used to elicit the response. Polson et al. (1980b) immunized hens against several proteins and natural mixtures of proteins and found that hens produced significant quantities of antibodies to the high molecular weight antigens (>150,000 daltons), but did not react as strongly to the low molecular weight antigens (<30,000 daltons). "It is feasible that more avid antibodies to low molecular weight antigens could be elicited in hens by cross-linking them to bacteria or giant molecules such as haemocyanin." (Polson et al., 1980b).

Another factor that influences the amount of antibody produced is the physical state of the hen. Serum antibody levels have a direct influence on the resulting yolk levels, since it has been determined that IgY subpopulations are transported in proportion to their concentration in maternal serum (Loeken and Roth, 1983). It appears that the serum of a laying hen has a higher IgY concentration than the serum of a hen before its reproductive period, or of a rooster (Polson, 1980a).

Heterogeneity (subclasses)

A significant amount of evidence exists that supports the idea that IgY represents a heterogeneous population of molecules made up of several subclasses. Wilkinson and French (1968) used immunoelectrophoresis and radioimmunoelectrophoresis to identify 2 distinct heavy-chain subtypes. Using a radiochromatographic assay, Howell et al. (1973) also concluded that at least 2 major subpopulations exist[2]. Other authors have confirmed the existence of at least 2 major subclasses (Rose et al., 1974; Loeken and Roth, 1983), and others possibly even more[3].

[2]A specific IgG (anti-2,4- dinitrophenyl 7S antibody) contained 5.5% carbohydrate by weight compared to 6.0% for normal chicken 7S, indicating that at least 2 subclasses exist.

[3]Using IgY as an antigen to elicit antibodies in rabbit, and then carrying out 2 dimensional Laurell electrophoresis, Polson et al. (1980b) discovered that five antigenic components were recognized, supporting the idea that IgY has several subclasses.

The diffuse bands obtained in electrophoresis (McIndoe and Culbert, 1979), and isoelectric focussing of IgY (Awdeh et al., 1968; Rokhlin et al., 1975) are another indication of the heterogeneity of this molecule. Recently, Ternes (1989) carried out isoelectric focussing of the livetins and found 25 bands occurring in the pH range 4.3-7.6. He found the alpha and β-livetins concentrated in the ranges 4.3-4.6, 5.3 and 5.5, while the gamma livetin bands were centered around pH 4.5, 4.7 and 5.0. McCannel and Nakai (1990) presented evidence of subclasses based on variations in binding patterns on a copper loaded metal chelate interaction chromatography column.

Solubility and Aggregative Properties

Although soluble in aqueous solution, the solubility of IgY is highly dependent on ionic strength and changes in temperature.

While attempting to purify IgY, Martin et al. (1957) found it to be less stable than the other livetins. They found that once most of the other proteins had been removed, the solubility of IgY in water was reduced and an ionic strength of about 0.1 M was required to dissolve it. They noted that a marked loss in solubility occurred at each purification step, on freeze drying, and even on standing. In 1958, Martin and Cook reported that on purification, γ-livetin has the solubility behaviour of a euglobulin but loses its solubility irreversibly on prolonged dialysis against water or freeze-drying. These findings have been reiterated by numerous authors throughout the literature, who have noted the tendency of IgY to form aggregates and even precipitate under conditions of both very low and very high ionic strengths.

Most of the literature dealing with the aggregative properties of IgY has been focussed on antibody purified from chicken serum. As far back as 1918, Hektoen found that chicken antihuman serum had a tendency to precipitate if stored in 0.9% NaCl solution,

and especially on rapid transfer from lower to higher temperature. He found that this precipitation could be avoided if 1.9% NaCl solutions were used instead.

At still higher salt concentrations, IgY has a tendency to aggregate. The salt induced aggregation of IgY into trimers or tetramers has been studied by Hersh and Benedict (1966) and Van Orden and Treffers (1968a). In using gel diffusion techniques, Polson et al. (1980b) found that IgY formed dimers when the concentration of NaCl was 1.5 M, and remained as monomers in gels containing 0.15 M NaCl. They further state that "Although it is unknown which chains of the IgY are involved in the mutual linkage, at least two of the four antigen binding sites of the dimer remain reactive, and are thus able to bind antigens to form a lattice".

Cser et al. (1982) used a low IgY concentration of 0.62% w/v in X-ray small-angle scattering experiments to avoid aggregation. They still had to use Sephadex G-200 to remove IgY aggregates from solution[4] just prior to their experiments. Thus the strong tendency of IgY to form aggregates and come out of solution means care must be taken in its handling.

Miscellaneous Properties

IgY possesses considerable acid and temperature resistance (Lösch et al., 1986). The isoelectric point of IgY has been variously determined to be: 4.8-5.0 (Kay and Marshall, 1928, for livetins); 5.2 (Tenenhouse and Deutsch, 1966); 6.5-9.0 (Loeken and Roth, 1983); and 4.5-5.0 (Ternes, 1989).

The extinction coefficient has been determined to be 13.5 (Tenenhouse and Deutsch, 1966) and 12.74-14.42 (Leslie and Clem, 1969) depending on the buffer. Tenenhouse

[4]The solution used was PBS - pH 7.4, 3.186 g $Na_2HPO_4 \cdot H_2O$, 0.1533 g KH_2PO_4 and 8.765 g NaCl per 1000 ml of H_2O

and Deutsch (1966) also determined the partial specific volume to be 0.718 cm^3/g, the nitrogen content to be 14.8%, and the diffusion coefficient, $D_{20,w}$ ($x10^{-7}$ cm^2), to be 3.21-3.24 for the monomer. The diffusion coefficient has also been reported as 2.2-2.4 in 8% NaCl, where aggregates are certain to be present (Van Orden and Treffers, 1968a).

Cser et al. (1982) determined the radius of gyration to be 6.13 nm, somewhat lower than rabbit (6.68 nm), pig (6.78 nm) or human (7.4 nm).

Finally, Wang et al. (1986) have determined that the optimal pH for IgY binding is pH 5 to 9 (based on a mannose binding subclass of IgY).

2.1.3 Other Constituents of Egg Yolk

IgY is only one protein in a complex mixture of proteins, lipids and lipoproteins. A review by Powrie and Nakai (1985) provides a thorough discussion of the major components. Fresh yolk has a solids content of 52 to 53%, of which the principal constituents are proteins and lipids. The lipid fraction includes triacylglycerol (66%), phospholipid (28%), cholesterol (5%), as well as minor lipids. The proteins (and lipoproteins) can be divided between those found in the granules (phosvitin, lipovitellins, low density lipoproteins (LDL), and myelin figures), and those present in the plasma (ie: the WSF). The WSF proteins include α-, β-, and γ-livetin. The molecular weight of α-livetin is about 80,000 Da, and that of β-livetin approximately 45,000 Da. Two components of low-density lipoprotein are also present in the WSF, with molecular weights of several million Daltons.

There has not been as much interest in studying α- and β-livetin and not too much is known about their characteristics compared to IgY. However, Martin et al. (1957) determined the extinction coefficients of α- and β-livetin to be $E_{o,1\%}$ at λ=2800 $\mathring{A}$ (280 nm) = 7.38 and 9.44 respectively.

2.2 Potential Applications of IgY

In the last decade there has been a flurry of publications dealing with specific uses of IgY. Table 2.4 lists several papers published since 1980 dealing with some of these applications.

2.2.1 Immunoassays

By far the most prevalent use of the purified antibody is in immunoassays of various kinds including RID (radial immunodiffusion), various ELISA's (enzyme linked immunosorbent assays), radioimmunoassays, latex agglutination, hemagglutination inhibition tests, immunoblotting and various forms of immunoelectrophoresis, to name just a few. Enteroviruses such as poliomyelitis, Coxsackie, hepatitis A and rotaviruses have a significant role in the development of many human and animal diseases (Shirman et al., 1988). Traditional methods of detection based on tissue cultures are time consuming and not always effective for detecting viruses, such as hepatitis A and rotavirus, with weak cytopathic effect. ELISA's are becoming a common method of detection because of their simplicity, high sensitivity and specificity.

Immunoassays are also rapidly becoming important for the detection and quantification of various components in food (McCannel and Nakai, 1990). Not only are food immunoassays easy to perform, sensitive, specific and relatively inexpensive, but there are many potential applications in the determination of food additives, meat species, fungal and bacterial contamination, antinutritional factors, pesticide residues and hormones (Allen and Smith, 1987). Immunotechniques have also been used to trace proteins through the technological processes of brewing, and to detect contaminants, yeast proteins and additives in beer (Vaag and Munck, 1987).

The potential for the use of IgY in immunoassays is therefore quite significant, but

Table 2.4: Examples of Recent Papers on Applications of IgY

Reference	Application	Antigen
Bar-Joseph & Malikson (1980)	ELISA	citrus tristeza virus tobacco mosaic virus
Hau et al. (1980)	IE	pregnancy associated murine protein-2 and human pregnancy-specific β_1-glycoprotein
Fertel et al. (1981)	RIA	human parathyroid hormone
Devergne et al. (1981)	ELISA	Cucumoviruses
Gardner & Kaye (1982)	IA	various influenzae, adenovirus group antigen, rotavirus
Carroll & Stollar (1983)	ELISA	Calf Thymus RNA Polymerase II
Vieira et al. (1984)	RIA	human parathyroid hormone
Piela et al. (1984)	ELISA/HI	Newcastle disease, Infectious bronchitis, Mycoplasma gallisepticum
Al Moudallal et al. (1984)	ELISA	monoclonal antibodies
Altschuh et al. (1984)	RIE	serum IgG & IgM
Burger et al. (1985)	RID	Human plasma kallikrein
Song et al. (1985)	AR/DIP	α-subunit of insulin receptor
Bauwens et al. (1987)	RIA	1α,25-dihydroxyvitamin D
Ricke et al. (1988)	ELISA	Ruminal bacteria
Shirman et al. (1988)	EIA	Enteroviruses
Yolken et al. (1988)	PI	rotaviruses
Kuhlmann et al. (1988)	PI	procine enteropathogenic E. coli
Larsson & Sjönquist (1988)	LA/ELISA	Rheumatoid factor positive sera
Bauwens et al. (1988)	RIA	1α,25-dihydroxyvitamin D
Schmidt et al. (1988)	CIEIA	Organophosphorous compounds
Schmidt et al. (1989)	IHC	Canine distemper virus
Meisel (1990)	ELISA	Bovine lactoferrin
Kunz et al. (1991)	IHC	chlamydiae
Otake et al. (1991)	PI	dental caries
Wiedemann et al. (1991)	PI	porcine-enterotoxic E. coli
Kang & Ho (1991)	IB	Microcystis aeruginosa inorganic pyrophosphatase
Polson (1991)	ZE	human sperm
Yazawa et al. (1991)	HI	human type O red blood cells
Sturmer et al. (1992)	ELISA	native & recombinant α-amidating enzyme

Table 2.5: Summary of Abbreviations for Table 2.4

Abbreviation	Full Name
AR	Autoradiography
CIEIA	Competitive Inhibition Enzyme Immunoassay
DIP	Double Immunoprecipitation
ELISA	Enzyme Linked Immunosorbent Assay
HI	Hemagglutination Inhibition
IA	Immunoassay
IB	Immunoblotting
IE	Immunoelectrophoresis
IHC	Immunohistochemistry
LA	Latex Agglutination
PI	Passive Immunity
RIA	Radioimmunoassay
RID	Radial Immunodiffusion
RIE	Rocket Immunoelectrophoresis
ZE	Zone Electrophoresis

requires careful control of the purity and specificity of the antibody.

2.2.2 Therapeutic Applications

Therapeutic applications, such as passive immunization are also being explored and present a potentially large-scale application of IgY (Losch et al., 1986). Chicken antibodies are acid- and heat-resistant and might be used orally to prevent or cure infectious intestinal diseases in young animals or humans (Gassmann et al., 1990). For example, Wiedemann et al. (1991) concluded that IgY was a good alternative to antibiotic therapy in pigs for enterotoxic E. coli. Hatta et al. (1990) stated that the effects of oral administration of antigen-specific IgY have been reported as a promising method for prevention of gastro-intestinal or dental infections.

Kuhlmann et al. (1988) mentioned a variety of specific uses for IgY such as the treatment of infectious diarrhea in foals, puppies, lambs and zoo animals; protection against epidemics (typhoid, cholera) after natural catastrophes; protection of low birth weight or immunodeficient infants; and the treatment of diarrhea of AIDS patients or women during pregnancy.

2.2.3 Infant Formula

Another potential use of IgY is as a supplement for infant formula to prevent intestinal infections (Shimizu et al., 1988). It has been shown that breast fed infants have fewer occurrences of infection than those that are bottle fed (Ballabriga, 1982; Friend et al., 1983), and the effectiveness of oral administration of bovine immunoglobulins in reducing gastrointestinal infections in infants has also been demonstrated by Ballabriga (1982). The only serious concern is the possibility of allergenicity. Some cases of hypersensitivity to bird egg yolk livetins has been reported (Mandallaz et al., 1988; de Maat-Bleeker et al.,

1988). This concern would require further study before large-scale use of IgY in infant formula would be possible.

2.2.4 Immunoaffinity Chromatography

One potential application of interest to the biotechnology community is the use of IgY as a ligand for immunoaffinity chromatography. It is likely that IgY will be used in the future for the separation of bioactive compounds from fermentation culture media as well as natural resources due to the simplicity of such separation procedures (Nakai et al., 1992). Key concerns in developing such applications are the cost of both purified IgY and the supporting gel materials on which to immobilize the antibody.

2.3 Relative Merits of Using IgY in Lieu of other Antibodies

2.3.1 Advantages

The numerous advantages of using IgY in lieu of serum IgG's from various animals are what have spurred on research in this area. The major advantages include:

- Convenience: The ease with which eggs can be collected compared to bleeding of laboratory animals (Eichler and Rubach, 1986; Shirman et al., 1988; Polson et al., 1980a) means that IgY can be harvested and processed in large batches (Gardner and Kaye, 1982). The animals used most commonly for producing immune sera for diagnostic purposes are rabbits and guinea pigs. Hens require less attention than these animals (Polson et al., 1980a).

- The relatively high concentration of IgY present in the yolk and its low cost: Chickens are more efficient antibody producers, since via the yolk they produce approximately 20 times more antibody per kg of bodyweight than a cow does in colostrum

(Kuhlmann et al., 1988). Several authors have noted that a heightened level of IgY activity can be found in the yolk[5], and that this level remains high long after immunization has ceased (Polson et al., 1980a; Shirman et al., 1988).

- Compatibility with animal protection regulations: Several authors have noted that collecting eggs is obviously much less of a strain on the birds than bleeding (Jensenius et al., 1981; Polson et al., 1980; Rose et al., 1974).

- Health of the flock: Hens are less susceptible to diseases than laboratory animals (Polson et al., 1980a).

- IgY does not bind strongly to protein A or Fc receptors: This means that fewer non-specific effects should be encountered (Gardner and Kaye, 1982). Also, Shirman et al. (1988) noted that the use of IgY in the indirect sandwich variant used in enzyme immunoassays is especially convenient because, according to Al Moudallal et al. (1984), chicken antibodies do not give cross-serological reactions with mammalian immunoglobulins.

- Immunization is efficient: The quantity of antigen required for an immune response is only 20 to 30 μg (Gassmann et al., 1990).

2.3.2 Disadvantages

There are, of course, some disadvantages to using IgY. Chicken antibodies do not fix complement and therefore cannot be used in tests employing complement fixation (Gardner

[5]Shirman et al. (1988), using a variation of the method of Polson et al. (1980a), were able to obtain 50 to 100 mg of IgY from one egg of an immunized hen. Thus they claim that in one month they could obtain the same amount of immunoglobulins from one laying hen as could be obtained from 300 ml of serum.

and Kaye, 1982). As well, problems of isolation and purification have hindered wider useage of IgY reagents. Although many different isolation procedures have been described, none is as easy as the purification of most mammalian IgG antibodies using affinity chromatography with Protein A (Hassl and Aspock, 1988). Although several methods exist for the isolation and purification of IgY, most of these methods are either tedious and difficult to scale-up, or do not lend themselves to food applications (Fichtali et al., 1992).

Thus the development of a process to isolate and purify IgY from egg yolk that can be easily scaled-up to an industrial scale would be of great benefit.

2.4 Methods of Isolation and Purification

Several methods have been assessed for the isolation and purification of IgY from egg yolk. These have been divided into three basic parts for review. The first deals with the initial extraction of the water soluble protein fraction (WSF) of yolk, which is generally considered to be necessary step before various separation methods can be applied. The second concerns the separation of IgY from the WSF. Finally, methods for purification of IgY to relatively high levels of purity (ie: over 90%) are considered.

Very few authors report attempting direct separation of IgY from egg yolk without the preceding WSF extraction step. McBee and Cotterill (1979) used DEAE cellulose to fractionate egg yolk proteins into 18 bands, and stated that removal of lipids was not necessary prior to chromatography. Later Woodward and Cotterill (1983) used DEAE-Sephacel to obtain 18 peaks, but many showed multiple protein bands in polyacrylamide gel electrophoresis (PAGE) indicating incomplete resolution. IgY was apparently not well separated from the other proteins. As well, the difficulties involved in using raw yolk, such as its viscosity and tendency to rapidly solidify, have deterred most researchers from attempting to apply yolk directly to a chromatography column such as can be done more

readily with egg albumen in lysozyme purification.

2.4.1 Extraction of the Water Soluble Fraction from Yolk

The water soluble fraction can be isolated from the lipid soluble fraction (LSF) by either extracting the WSF in aqueous solution, or extracting the LSF using organic solvents.

Aqueous Extraction

Numerous authors have used dilution with aqueous solution to precipitate lipids and lipoproteins from yolk. The lipid granules have a strong tendency to aggregate and settle in aqueous solution, and this effect is promoted by large dilution factors. Various salts, natural gums, and other additives have been used to enhance the aggregation phenomenon. Some examples are given below.

Prior to gel filtration chromatography, Burley and Vadehra (1979) used dilution of yolk 1:1 with 0.16 M NaCl followed by centrifugation at 100,000 x g and 10°C for 30 minutes. They then resuspended the sedimented granules in twice their volume of salt solution and centrifuged again. The supernatants, containing the WSF, were then combined for further processing.

Jensenius et al. (1981) compared two methods of aqueous extraction involving dilution with Tris buffered saline (TBS), and 10 times dilution with water and pH adjustment to 7 with 0.1 N NaOH.

Hatta et al. (1988) used the simplex-centroid method (Nakai, 1988) to optimize the separation of egg yolk into the LSF and WSF by varying pH, concentration of sodium alginate and NaCl. The optimum conditions obtained were: sodium alginate - 0.1%; pH 5.8 - 6.4; no NaCl. Hatta et al. (1990) separated the WSF in yolk using dilution with water and precipitation of the lipoproteins using λ-carrageenan. Delipidation efficiency

is reported as 99.6% for this method.

Kwan et al. (1991) developed a method for fractionation of both water-soluble and water-insoluble components from egg yolk with maximum retention of biological and functional properties. The water soluble proteins were separated from the lipoproteins by simple dilution with distilled water, and were thus concentrated in the supernatant. The yolk pellet was found to be useable in food applications (for instance mayonnaise preparation) and was further fractionated to recover other valuable components. They noted that the efficiency of separation of lipids and phopholipids from the WSF using simple dilution of egg yolk with distilled water increased with the age of the egg.

Simple dilution with distilled water and pH adjustment has also been used by Akita and Nakai (1992) with optimal recovery of IgY in the WSF (and minimal residual lipids) reported to occur with 10x dilution and pH 5.2.

Extraction of Lipid Soluble Fraction (LSF) with Organic Solvents

A method developed by Martin et al. (1957) used ethyl ether and carbon tetrachloride to extract the LSF.

Polson et al. (1980a) used polyethylene glycol (PEG) to precipitate IgY from a diluted egg yolk mixture. Polson (1985) developed an improved PEG procedure in which contaminating PEG in the final product can be removed by diluting in 25% ethanol at -20°C. This yield was almost double the former technique. Traces of ethanol were removed by evaporation or by dialysis of IgY solutions against an appropriate buffer. Polson (1990) developed a new improved method of IgY isolation in which yolk homogenate was mixed with an equal volume of chloroform and then spun at 1000 x g for 30 minutes. The IgY concentrated in a watery phase on the top of the centrifuge tube, and was decanted prior to the use of 12% PEG Mr 6000 to precipitate IgY. He claimed that the yield of IgY

increased by a factor of 2.57 compared to the previous method.

Bade and Stegemann (1984) used 4 extractions with pre-cooled -20°C propane-2-ol, followed by 2 extractions with pre-cooled acetone to remove the LSF, resulting in a final precipitate containing IgY. This precipitate was then dissolved in 0.01 M PB (pH 7.5, 0.1 M NaCl, 0.01% NaN_3) and centrifuged at 25,000 x g, 10°C, for 15 minutes.

2.4.2 Separation of IgY from WSF

Salt Precipitation

Although a labour intensive, often repetitive process, salt precipitation has been used successfully to obtain IgY of high purity. The reported methods generally employed precipitation using ammonium sulphate (Martin and Cook, 1958; Wallmann et al., 1990), sodium sulphate (Kekwick, 1940; Hatta et al., 1988), or dextran sulphate (Jensenius et al., 1981). This technique is more practical, both from an economical and technical point of view, to use as a final polishing step in industrial applications since towards the end of a purification process the product volume is generally smaller and the concentration of the target protein much higher than in the initial stages.

Ultrafiltration

Akita and Nakai (1992) were able to improve IgY purity from 30% to above 93% using a hollow fibre ultrafiltration cartridge with 100 kDa nominal MWC. However it was necessary to first separate crude IgY by ammonium sulphate precipitation. Otherwise, clogging of the membrane with lipid-containing mucous materials would prevent efficient separation. Recently (Nakai et al., 1992) were successful in applying ultrafiltration directly to the WSF by adjusting the pH to 9.0. The resulting recovery and purity were 95% and 80% respectively.

Protein-A Columns

It is not possible to use protein-A columns to purify IgY, as is done for mammalian IgG. Ansari and Chang (1983), using a protein-A Sepharose column, found that IgY does not bind to protein-A molecules. They concluded that the reasons for the non-binding properties of chicken IgY may be:

- the presence of protein which binds to the protein-A molecule and inhibits the binding of protein-A to IgY
- the amino acid configuration of the F_c region of chicken IgY is different from mammalian IgG and has different binding properties

Precipitation with Antigen

Polson et al. (1980a) raised antibodies to tobacco mosaic virus (TMV) in hens and purified this specific IgY using absorption to the virus and removal of the nonspecific IgY by centrifugation and discarding of the supernatant. They determined that 15-18% of the total IgY was directed to TMV.

Schram et al. (1971) immunized hens against bovine albumin and recovered IgY from the other water soluble proteins by precipitation with BSA. The IgY was then purified using a Sephadex G-200 gel filtration procedure.

Chromatography

Due to recent innovations in the development of chromatographic media, industrial grade media are now available with greatly improved capacities and flow properties, and at reasonable prices. Several forms of chromatography have been used in IgY separation and purification.

Anion Exchange To date, anion exchange chromatography (AEC) has been the preferred chromatographic method for purification of IgY. In 1960, Mandeles used DEAE-cellulose with an acidic gradient elution profile to separate the WSF of the yolk. The resulting chromatogram consisted of 10 peaks, a larger number of components than usually obtained using electrophoresis. Although many researchers have made use of AEC, not all agree on its effectiveness. For instance, Higgins (1976) used DEAE Sephadex A-50 with a linear gradient to fractionate chicken serum. He concluded that ion-exchange chromatography has little application in the separation of fowl immunoglobulins since all three Ig classes (IgG, IgM and IgA) eluted throughout most of the linear salt gradient.

Using DEAE-Sephacel anion exchange media, McCannel and Nakai (1990) were able to obtain a purity of only about 36% for a recovery of 60%. The highest purity obtained was about 60%, but for a fraction representing only 15.8% of the IgY applied. However, the first eluted fraction, although containing only about 20% of the IgY applied, represented the majority of the specific IgY based on ELISA analysis. It follows that this method could be used to selectively purify IgY that is specific for the antigens used in the study, namely E. Coli LPS (lipopolysaccharide) and β-lactoglobulin.

McCannel and Nakai (1990) state that "Separation of immunoglobulins by ion- exchange chromatography may be due to amino-acid sequence differences occurring in the variable region of the heavy chain, which dictates the specificity of antibodies." Thus as a separation step, early in the process of IgY purification, AEC holds the potential for differentiating IgY subclasses.

Gel Filtration A table is given in the introduction of Chapter 7 which lists many of the published papers in which gel filtration of IgY has been reported. Gel filtration is generally used in the final stages of IgY purification. It has been used as a polishing

step, as well as a method for estimating the molecular weight and concentration of the molecule.

High Performance Liquid Chromatography One paper only (Burley and Back, 1987) has been found dealing with the use of an HPLC column to fractionate yolk proteins. However, this was done only on an analytical basis.

Hydrophobic Interaction Chromatography Hassl and Aspock (1988) developed a 2 step chromatographic procedure using hydrophobic interaction chromatography followed by gel filtration. The major advantage of the technique is that a relatively high purity is obtained with only one initial batch precipitation step, followed by 2 chromatographic steps. Therefore the process is rapid, there are potential time and labour savings since it could easily be automated, and antibody activity is preserved. Their procedure, however, has a relatively low yield, less than $\frac{1}{3}$ the yield for the method of Polson et al. (1985) with a purity of 85% or greater.

Immunoaffinity Chromatography Otani et al. (1991) used immunoaffinity chromatography to purify IgY produced in the yolk of hens hyper-immunized against α-s_1-casein. The casein was first bound to a column of Sepharose 4B, the anti-α-s_1-casein egg yolk IgY[6] applied to the column, and the IgY eluted with 0.5M glycine-HCl buffer, pH 2.3, and then 4M guanadine-HCl, pH 7.0, in a cold room.

Metal Chelate Interaction Chromatography Copper-loaded MCIC has been used for the separation of bovine IgG from blood and cheese whey (Lee et al., 1988). McCannel and Nakai (1989) used this technique to separate IgY from the other livetin proteins, and

[6]Previously purified according to the method of Polson et al. (1985), followed by DEAE Sephacel chromatography

found that the majority of IgY eluted in the unbound fraction. For moderate loading of the gel (60 mg of protein applied per ml of MCIC gel), it was possible to pool the unbound fraction and obtain a recovery of 74%, but with a purity of only about 50%. A purity of about 67% was possible, but with a much reduced recovery of only 36%. Considering the rather high cost of this gel in comparison with ion exchangers, it is unlikely that this process would prove as economical.

Cation Exchange Very few authors report the use of cation exchangers to separate IgY from other yolk proteins. Most of the published work involves whole egg or egg yolk.

Dreesman and Benedict (1965) used carboxymethyl-cellulose (CMC) to fractionate papain digested chicken antisera. "Immunoelectrophoresis of digested antibovine serum albumin IgG revealed 2 antigenically distinct fragments" that they termed electrophoretically-slow and fast. They concluded that the fragments separated poorly on CMC at pH 5.4, and a better separation could be achieved by chromatography on DEAE cellulose and by starch block electrophoresis.

Seideman and Cotterill (1965) fractionated yolk proteins on CMC and noted that the fractions obtained did not completely correspond with proteins prepared by other investigators. They suggested that these fractions may represent combinations or complexes of proteins or lipoproteins.

Parkinson (1967) applied whole egg, egg white and egg yolk to DEAE-cellulose (DE52) and whole egg to CMC (CM52) in order to fractionate the proteins. He concluded that DEAE-cellulose was more suitable than CMC, but that even using the anion exchanger, most of the fractions contained more than one protein, and there was considerable tailing in some of the fractions.

Seideman et al. (1969) investigated the use of ion exchange cellulose for the separation

of egg yolk proteins and lipoproteins. They determined that DEAE cellulose showed little promise for separating yolk proteins and lipoproteins, and likewise for CM cellulose using acetate buffers. However, they were able to obtain a separation using a citrate-phosphate buffer system. Using a column equilibrated to between pH 4.5 and 5.0, and then stored in a starting buffer of pH 5.4, application of native chicken egg yolk apparently resulted in the α-, β-, and γ-livetin passing directly through the column, along with lipid contamination of about 4.2% (a free lipid or lipoprotein). The other yolk proteins and lipoproteins were eluted by increasing the pH.

Parkinson (1972) used CMC to fractionate yolk proteins into a large number of peaks following elution with an NaCl gradient and other salt solutions.

Thus papers dealing with cation exchange chromatography of undiluted egg yolk and liquid whole egg are concerned more with identification of various fractions rather than practical purification of any of the proteins.

Chapter 3

Assembly of an Automated Chromatography System

3.1 Introduction

Since the goal of this work was to determine the potential for large scale purification of IgY using some form of chromatography, it was necessary to consider what type of equipment would be most beneficial in carrying out first small scale tests at the laboratory or bench scale, and later pilot scale experiments. Because it was considered likely that numerous experiments at the lab scale would be required in order to test different types and brands of chromatographic media, and to investigate the effect of changes in various process parameters such as flowrate, pH, ionic strength, etc., an automated system seemed essential. In order to simplify the work of scaling up the process to pilot scale, it was also considered advantageous if the same system could be adapted to run the larger experimental trials as well. Although many companies carry automated systems for laboratory scale chromatography, it is not as easy to find fully automated process scale equipment with a variety of interchangeable components (Johansson et al., 1988). Since different types of chromatographic media might require the monitoring of different parameters, the preparation and delivery of buffers of various elution profiles (linear vs step gradients in pH, ionic strength, etc.), or widely differing flowrates, it was necessary that types of various pumps, valves, and other equipment be easily interchangeable. Thus the design of a highly flexible chromatography system is an important step for purification process development.

3.2 Initial System Design

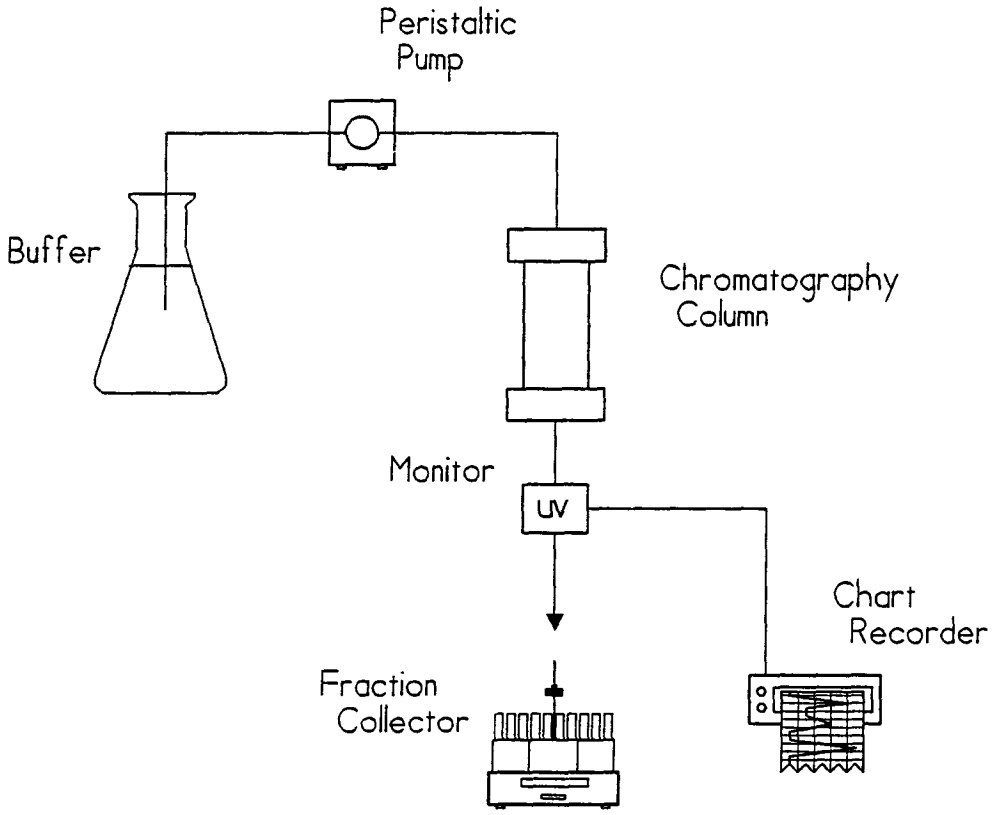
The author, having had no prior experience in operating chromatography equipment, sought the advice of those with practical experience in operating such equipment, as well as information available in scientific catalogues, manuals and textbooks on practical biochemistry. The idea was to determine a simple but proven starting point for the design of the system. Using primarily equipment already available in the laboratory, an automated system was pieced together as shown in Figure 3.2. As can be seen, a peristaltic pump is used to maintain a constant flow of sample or buffer through the chromatography column. Upon exiting from the column, the solution flows through an in-line UV monitor, and finally to a fraction collector. The chart recorder produces hardcopy of the chromatograms which must then be digitized for computer analysis, or integrated using other methods.

The system is crude and unsophisticated, but it gets the job done - as long as there are not too many buffers involved, no elution gradients (other than steps) are required, and the operator has the time to hang around and look after switching buffers and turning equipment on and off. In many laboratories, and for many small experiments, this type of system is quite adequate. However, since an anion exchanger requiring linear gradient elution was one of the first media tested for purifying IgY, a slightly more sophisticated system was quickly assembled.

3.3 Improved System with Process Controller

The next permutation included a second peristaltic pump and mixing chamber (formed by placing a flask on a stirring hot plate) to allow the formation of linear salt or pH gradients, several valves and buffer tanks, and a Pharmacia C3 dedicated controller.

Figure 3.2: Simple Laboratory Chromatography System



The control unit provided timed on/off control of the solenoid valves, and on/off power control for the pumps, mixing chamber and fraction collector. The chromatograms were still registered by the chart recorder. A schematic diagram of this system is shown in Figure 3.3.

This system was used for the first series of experiments in which an anion exchange column was used to separate IgY from the other water soluble proteins in diluted egg yolk supernatant (to be described in Chapter 4). Although the full potential of the C3 controller was not used[1], it was still limited in its ability to collect and analyze chromatograms. Therefore, a microcomputer based controller was sought which could be interfaced easily with the equipment already available and provide rapid data acquisition and analysis.

3.4 Final System Design

Figure 3.4 is a schematic diagram of the automated chromatography system which was first designed for bench-scale separation. The system includes a 2.5 cm inside diameter column, buffer and sample tanks, two remote control Ismatec peristaltic pumps (Cole-Parmer, Chicago, USA), an in-line Pharmacia UV detector and monitor (Pharmacia-LKB, Upsalla, Sweden), an ISCO Retriever II fraction collector (ISCO Inc., Lincoln, Nebraska), several solenoid valves (Burkert, Germany), an IBM compatible personal computer and monitor, as well as tubing and fittings.

An ISCO ChemResearch data management/system controller specifically designed for HPLC was adapted for use with the low pressure liquid chromatography system used in this work. The software is menu driven, and includes routines to allow calibration and

[1]The C3 is capable of controlling processes involving more complex elution gradients, but requires a gradient former. As well, the C3 can accept input from an in-line flow meter, and can trigger the collection of individual peaks based on sudden changes in the monitored baseline.

Figure 3.3: Improved Chromatography System

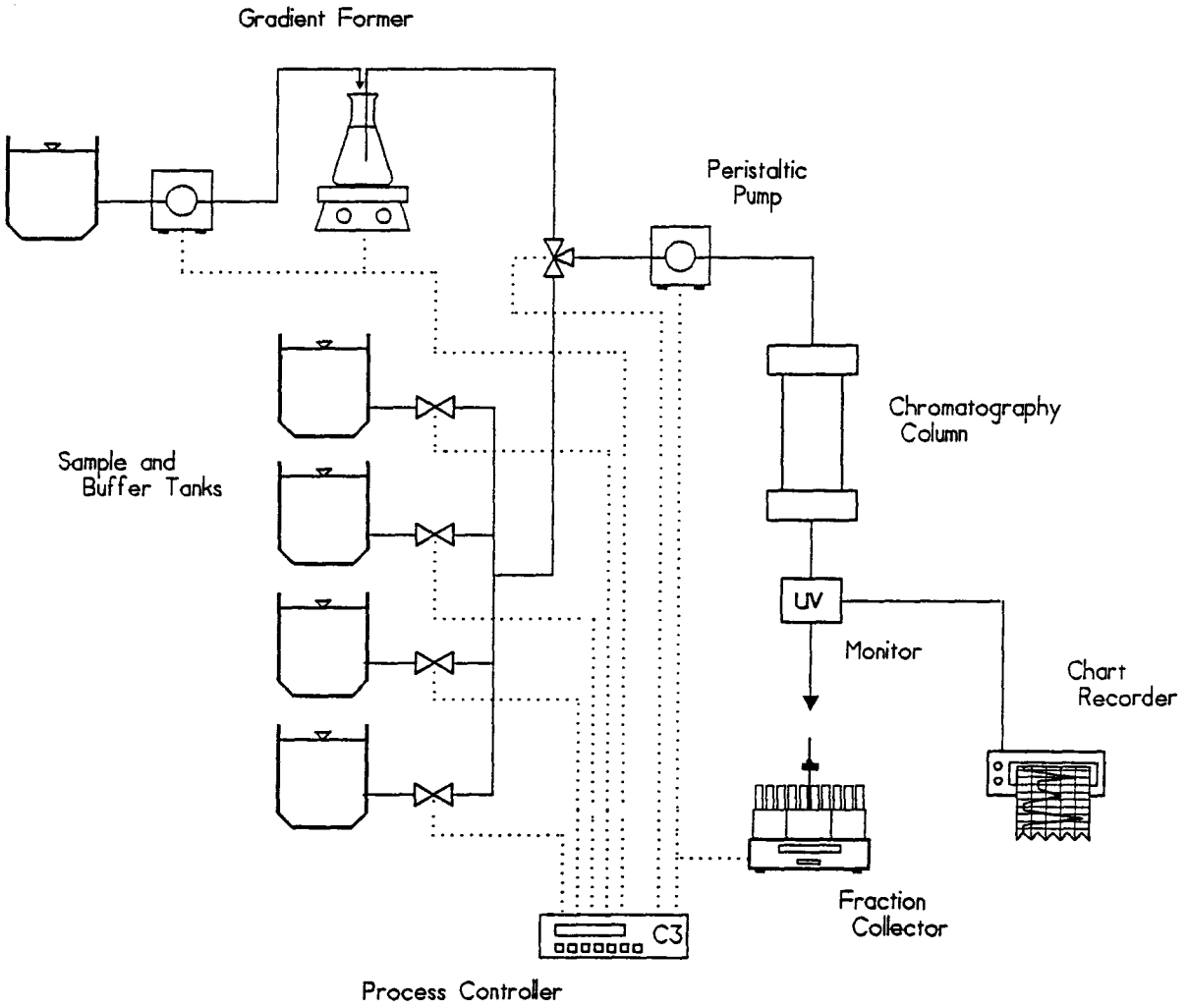
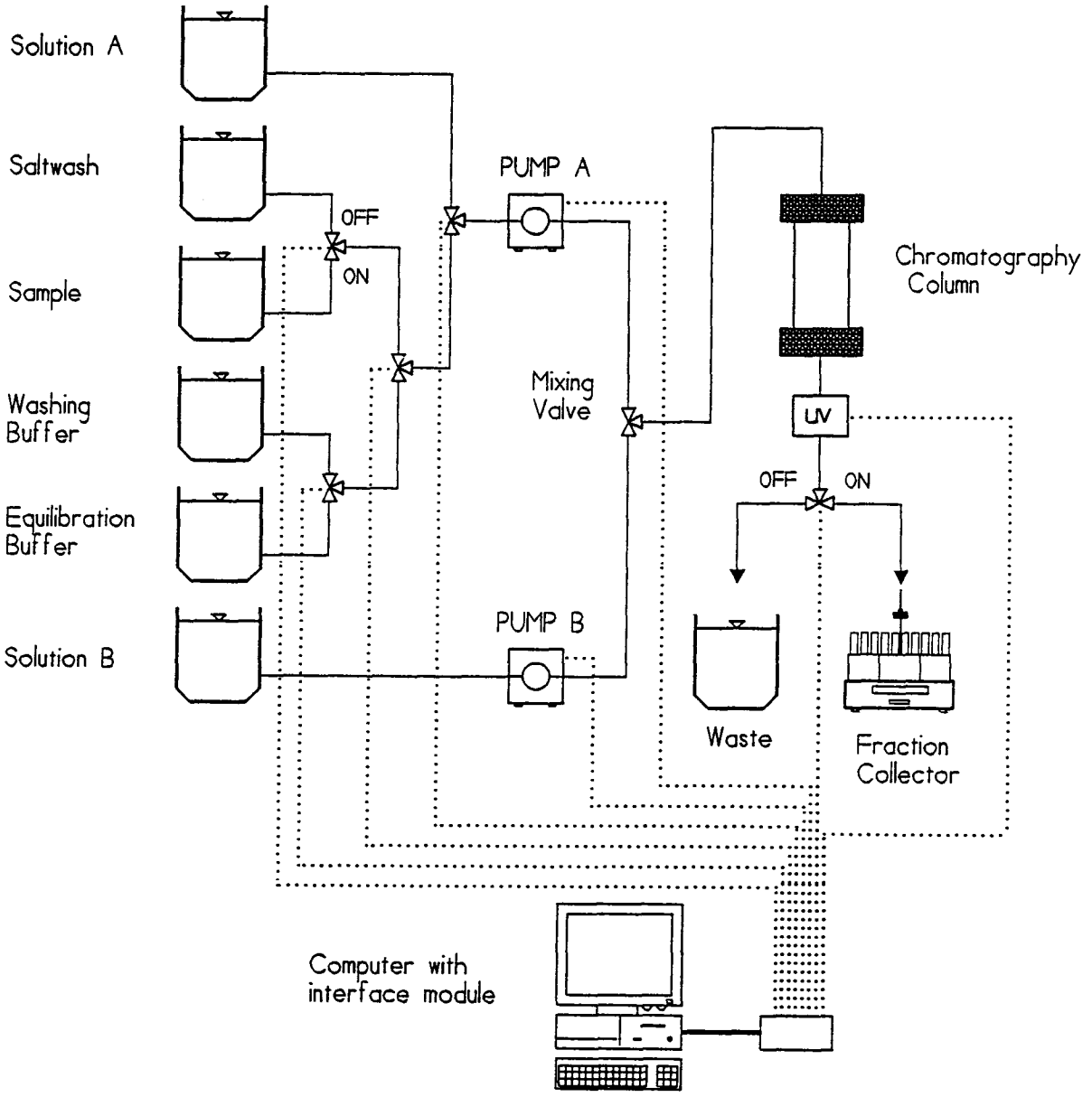


Figure 3.4: Computer Controlled Chromatography System



control of devices (i.e. pumps, valves), data acquisition, analysis and graphic display. An external distribution interface module into which externally controlled instruments and accessories are connected is included in the system. However, it was necessary to use a custom designed and built control interface unit to actuate the process scale solenoid valves. The unit amplifies the low/high (0/5VDC) signal from the ISCO interface module by using a Darlington transistor arrangement powered by a 24 VDC power supply[2]. Tygon food grade tubing (B-44-4X, 6.4 mm outside diameter, 3.2 mm inside diameter) was connected to the valves by nylon fittings.

3.5 Conclusions

The automated system developed offers several advantages for carrying out repetitive chromatography experiments. The data acquisition software allows for routine organization and saving of equipment settings and parameter values, and rapid analysis of chromatograms. It is relatively easy to transfer the data to a graphing program in order to produce publication quality graphs. Time savings are substantial since many operations are accurately controlled and carried out. It is possible to run experiments overnight, which in turn allows more efficient use of laboratory equipment. Finally, experiments can be accurately controlled and easily reproduced.

[2]See Appendix A for a schematic diagram of the interface.

Chapter 4

Extraction of the Water Soluble Proteins

4.1 Introduction

As mentioned in the literature review, there are two basic approaches used to separate the water and lipid soluble fractions: aqueous extraction of the WSF or the use of organic solvents to extract the LSF. Since other authors have reported good separation of the WSF from manually separated yolk using only dilution with H_2O and pH adjustment with diluted HCl or NaOH (Kwan et al., 1991), it was decided to study the aqueous extraction of industrially separated yolk using different dilution ratios at various values of pH.

Such a procedure meets the goal of developing a process yielding purified IgY of food quality. This would result in a fairly simple extraction that meets with the requirements of developing a simple process for future scale-up.

4.2 Materials and Methods

4.2.1 Raw Material

Industrially separated egg yolk obtained from a local egg breaking plant (Vanderpols Eggs Ltd., Abbotsford, B.C.) was stored at 4°C with 0.02% sodium azide until use. Eggs were also obtained from a local market and the yolk separated manually for comparison.

4.2.2 Separation of Lipoproteins

Simple water dilution, followed by sedimentation, was used for lipoprotein separation from egg yolk water soluble proteins. Industrially separated fresh egg yolk was diluted 10x with distilled water, the pH adjusted with 0.1N HCl/0.1N NaOH, and the solution further mixed with magnetic stirrer for about 10 min. The effect of pH on residual lipids in the supernatant and IgY recovery after 24 h sedimentation of lipoproteins was studied for pH ranging from 5.0 to 7.0. Three pH values (5.0, 5.25, and 5.5) were considered in studying the effect of pH and time on lipoprotein sedimentation and IgY recovery. The solutions prepared under the specified conditions were allowed to settle at 4°C in one litre Imhoff cylinders, and the precipitate volume and IgY concentration in the supernatant measured as a function of time up to 7 days. The effect of a second dilution/extraction step on the IgY recovery after 24 hours sedimentation, using 10x dilution and pH 5.5, was also investigated. All solutions were prepared in duplicate, and the average experimental results reported for each treatment.

4.2.3 Analytical Procedures

Non-denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Pharmacia Phast System using a 10-15% gradient PhastGel and Coomassie brilliant blue stain according to the manufacturer's recommendations (Pharmacia-LKB, Upsalla, Sweden). Molecular weight standards (BioRad, Redmond, CA) were used to estimate the molecular weights of proteins in the WSF.

Total protein was determined using the BCA method (Pierce, Rockford, Il.). In this procedure, 0.1 ml of unknown protein sample is mixed with 2.0 ml of working reagent containing bicinchoninic acid and Cu^{2+} ions. The protein present in the sample reduces

Cu^{2+} to Cu^{1+} (the biuret reaction) which then reacts with BCA to produce a purple reaction product. Spectrophotometric quantitation of the protein is possible by measuring the absorbance of the solution at 562 nm after incubating the sample for 30 minutes at 37°C.

The total lipids content of the supernatant samples was determined using solvent extraction (see Hatta et al., 1990).

Radial immunodiffusion (Kwan et al, 1991) using chicken serum IgG (ICN, Cleveland, OH) as a standard was used for quantitative analysis of IgY in aqueous solution. Rabbit anti-chicken antibody (0.15 ml) was added to 1.85 ml phosphate buffer (0.05 M, pH 7.5), and the test tube then warmed in a 56°C water bath. Agarose (0.07 g) (Sigma, St. Louis) was added to 4.6 ml PB and 0.4 ml 0.35% sodium azide. This mixture was heated in a boiling water bath until the agarose had dissolved, and was then placed in the 56°C bath. After about 10 minutes, the two solutions were mixed and poured into radial immunodiffusion plates. After approximately 15 minutes at room temperature, the plates were transferred to a sealed container to which moist paper towel had been added. The container was then stored at 4°C until use. Wells were cut in the gels prior to use, and 4 μl samples applied to these wells. After storage for 3 days in the moist chamber at 4°C, measurements of ring diameters were taken using a digital micrometer. IgY purity was determined by dividing IgY concentration determined using RID by the total protein of the sample, as determined by the BCA method.

4.3 Results and Discussion

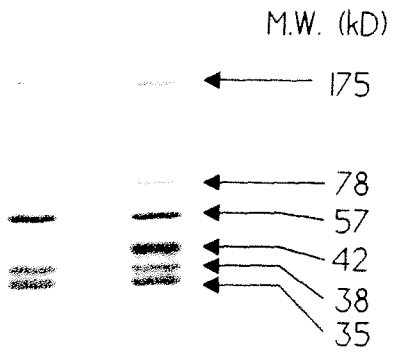
4.3.1 Comparison of Industrially and Manually Separated Egg Yolk

Figure 4.5 shows the SDS-PAGE of supernatants obtained from the industrially separated egg yolk used in our study (column A) and from manually separated egg yolk (column B). BioRad molecular weight standards were used to determine the molecular weight of the bands obtained in non- denaturing SDS-PAGE of the WSF from manually vs. industrially separated yolk. The slowest moving band was IgY with an estimated molecular weight of 175 kDa. Next came 2 bands at 78 and 57 kDa, which have been identified by other authors as α- and β-livetin, respectively. Both samples contained two smaller bands at 35 and 38 kDa which have not been identified. One major additional band appears in the supernatant obtained from the industrial egg yolk. Since the industrial separation of the yolk from the white is not complete (the separated yolk typically containing 22% egg white on wet basis or 5.5% on dry basis), and since the molecular weight of the additional band is in the vicinity of 42 kD, it would appear that this additional protein is ovalbumin. Electrophoresis of purified ovalbumin standard revealed a band in a similar position on the gel. Thus the purification of IgY from industrial egg yolk is somewhat more difficult than from manually separated yolk.

4.3.2 Separation of Lipoproteins from Egg Yolk Soluble Protein

The first step in the purification process is the separation of the granular lipoproteins from the soluble fraction consisting mainly of livetins (Hatta et al., 1990). The effect of pH and dilution on lipid removal and IgY recovery has already been studied using laboratory separated egg yolk as the raw material and using centrifugation for separation (Akita and Nakai, 1992). As the objective was to develop a process useful for large scale

Figure 4.5: SDS-PAGE of Manually versus Industrially Separated Egg Yolk



purification of IgY, industrial egg yolk was used instead, and sedimentation rather than centrifugation was considered in order to decrease the overall cost of the process.

Figure 4.6 shows the effect of pH and dilution on residual lipids (Figure 4.6a) and IgY recovery (Figure 4.6b). For the case of 5x dilution, the IgY recovery at pH 5 and 5.5 has not been reported as the separation was poor and often inconsistent, perhaps due to the low dilution ratio. At pH 5.0 the effect of dilution on residual lipids is negligible, but at higher pH the residual lipids drops significantly with increasing dilution. At pH 6.0, the maximum recovery of IgY occurs for all dilutions (around 80%), but this also corresponds to the highest lipid residual for the conditions studied. Only at pH 5.0 for all dilutions, and pH 5.5 for 10 times dilution, is the residual lipids below 10%.

Under these conditions, where the residual lipid content is less than 10%, the best recovery was obtained for 10x dilution at pH 5.5 (recovery of 53%). Having settled on a 10x dilution ratio, two additional pH's were tested (pH 5.25 and 5.75) and the residual lipids and IgY recovery after 24 h presented in Table 4.6. This table shows that residual lipids increases sharply when the pH is greater than 5.5. The experiment was carried out once, and the residual lipids and IgY concentration determined in duplicate.

Figure 4.7 shows a 1 litre Imhoff cylinder of 10x diluted yolk adjusted to pH 5.5 and allowed to sit at 4°C overnight. A relatively clear supernatant layer (50 - 60% of the total volume) forms above a fluffy yellow precipitate. A thin lipid layer also forms over the top of the aqueous supernatant.

In order to follow the sedimentation of the LSF over a period of time, 10x diluted yolk adjusted to pH 5.0, 5.25, and 5.5 was allowed to settle in 1 litre Imhoff cylinders for several days at 4°C. Figure 4.8 shows the sedimentation curves (Fig. 4.8a) and IgY recovery (Fig. 4.8b) as a function of time with the experimental sedimentation data fitted to the following model:

Figure 4.6: Effect of pH and Dilution on Residual Lipids (a) and IgY Recovery (b)

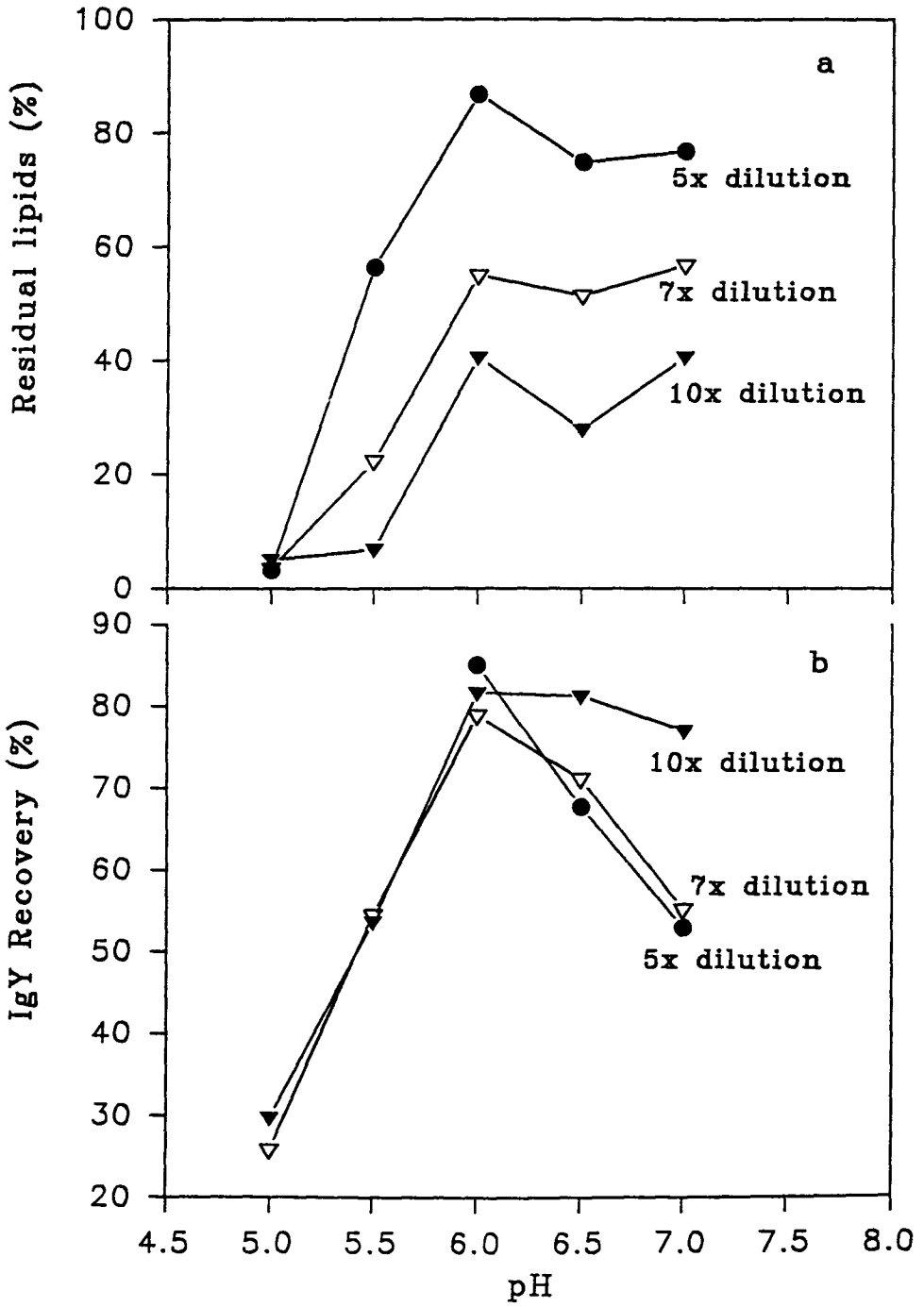


Figure 4.7: Diluted Yolk after 24 hours at 4°C

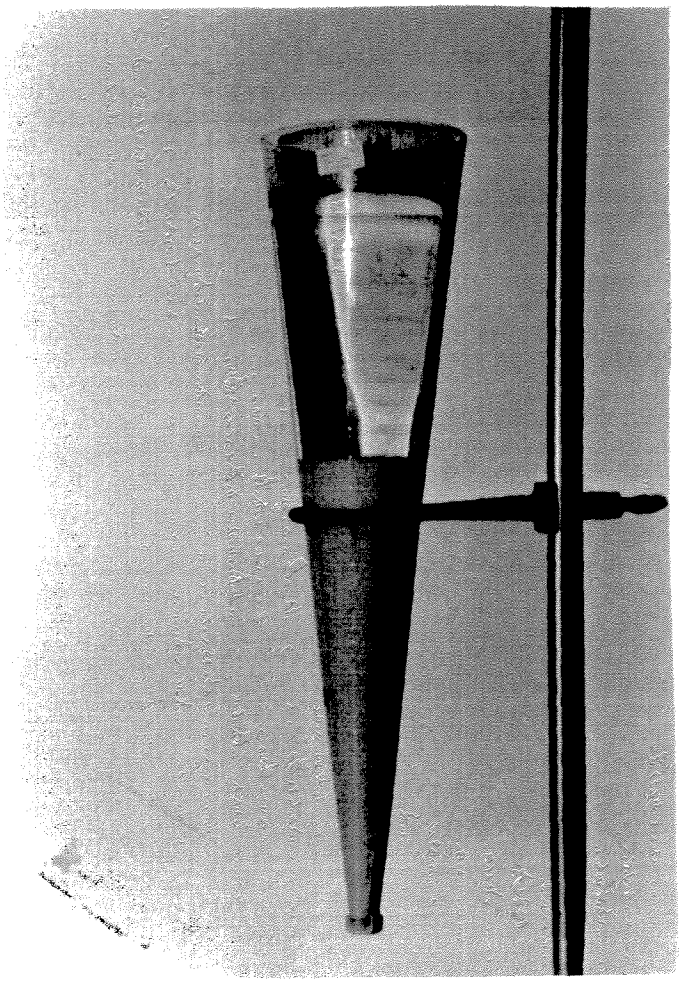


Figure 4.8: Effect of pH and Time on Volume of Sedimented Lipoprotein and IgY Recovery

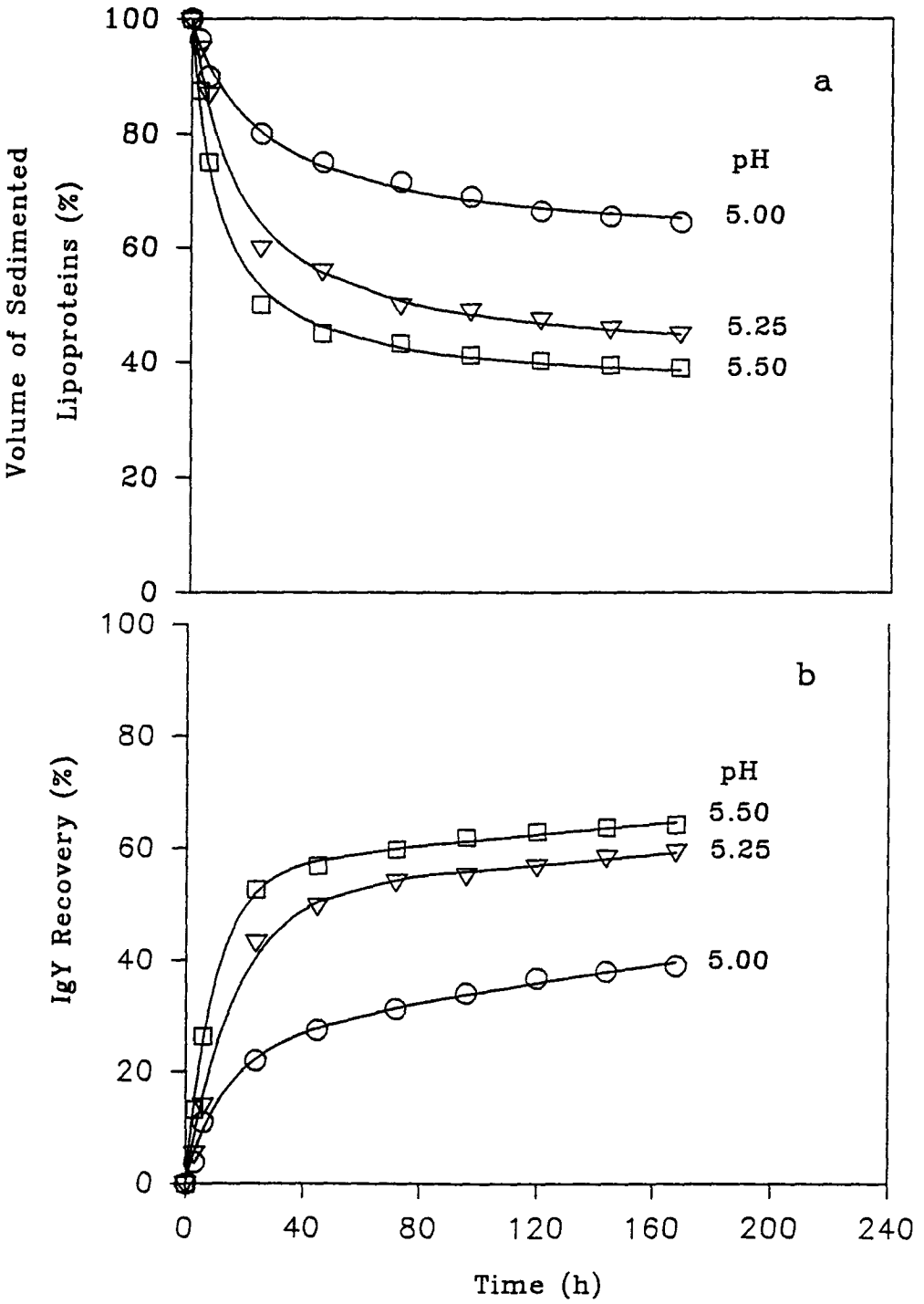


Table 4.6: Effect of pH on residual lipids and IgY recovery after 24 h sedimentation using 10x dilution

pH	Residual lipids (%)	IgY recovery (%)
5.0	5	22
5.25	5	43
5.5	6	53
5.75	16	61
6.0	41	82
6.5	28	81
7.0	41	77

$$s = a/(t + b) + c$$

and IgY recovery data fitted to the following model:

$$r = d(1 - e^{-ft}) + gt^{1/h}$$

where s is the percent volume of sedimented lipoproteins; t is the time; r is the percent IgY recovery; and a, b, c, d, f, g and h are empirical parameters determined to best fit each model. These models are strictly7 empirical best fits to the data determined by trial and error and a standard curve fitting routine.

The yield under the chosen conditions (10x dilution, pH 5.5) is 53% after 24 h sedimentation, and slightly higher (57%) after 48 h sedimentation (Figure 4.8b). An additional dilution/extraction step under the same conditions improved the recovery from 53% to 79% after 24 h sedimentation. However, this recovery is still lower than what could be obtained using centrifugation (>90% using 10x dilution and pH 5.2) according to Akita and Nakai (1992).

4.4 Conclusions

Although the use of additives such as sodium alginate (Hatta et al, 1988b) and λ-carageenan (Hatta et al., 1990), centrifugation of the settled yolk mixture (Ibid., Akita and Nakai, 1992), or filtration (Akita and Nakai, 1992) can result in significantly higher recoveries and/or better lipids removal in the first step, simple water dilution and pH adjustment is still a valuable low cost technique for recovery of significant quantities of the WSF. In addition, the remaining water-insoluble yolk fraction could still be used in food applications or for the separation of other biologically active components (Kwan et al., 1991). The specific economics of a particular large-scale operation would dictate if the added benefits are worth the additional cost of materials and equipment (such as the gums and a continuous centrifuge).

In the experiments described in the following chapters, unless otherwise stated, the supernatant containing the WSF was prepared by 10 times dilution with distilled water, pH adjustment to 5.5, and settling for 24 h at 4°C.

Chapter 5

Separation of IgY

5.1 General Introduction

Although many techniques have been developed to isolate and purify IgY from the WSF of egg yolk (see literature review in Chapter 2), the vast majority have only been attempted at the laboratory scale, and using yolk carefully separated by hand from the yolk sac so as to minimize albumen contamination. For large-scale isolation of IgY, a technique is required which would be practical to use with the large volume of dilute protein solution obtained after aqueous extraction.

Ultrafiltration is a method that permits both concentration and fractionation of proteins in aqueous solution[1]. It is also easily scaled-up, although the energy costs can be significant compared to other methods (Kroner et al., 1984).

Anion exchange chromatography has been used by many authors for IgY separation using laboratory exchangers such as DEAE-Sephacel (Pharmacia, Uppsala, Sweden), and since industrial grade exchangers are readily available it was considered a viable possibility.

Finally, although no previous publications mentioned the use of cation exchange chromatography (CEC) to separate IgY from the WSF, CEC has been used to fractionate liquid whole egg (Parkinson, 1967), yolk (Seideman and Cotterill, 1965; Parkinson, 1967; Seideman et al., 1969; Parkinson, 1972), and the papain digested fragments of the livetin

[1]Provided the difference in mass of the molecules to be separated is sufficiently large.

fraction (Dreesman and Benedict, 1965).

Ion exchange chromatography is used economically at the large scale for other chemical and food industry separations (Streat, 1988; Barker and Ganetsos, 1988) and so both anion and cation exchange chromatography were tested for applicability to separate IgY from industrial yolk.

5.2 Ultrafiltration

5.2.1 Introduction

The technology of cross-flow filtration dates from the early 1960's, when the porous membranes used were initially developed for water desalination. Cross-flow filtration can be used to fractionate liquid streams from three digit molecular weight to submicron levels (Davis, 1987). There are four basic classes of membrane separation used today: microfiltration, ultrafiltration, reverse osmosis and nanofiltration. Ultrafiltration can achieve simultaneous fractionation and concentration of solute in the range 0.002 to 0.2 microns, corresponding to a molecular weight range of 500 - 300,000 Daltons (Dziezak, 1990).

The use of ultrafiltration in the food and pharmaceutical industries to concentrate dilute protein solutions and to separate proteins from solutes or cells is now quite common. Its use for fractionating proteins according to size is not yet as common due to several problems (Ingham et al., 1980; Dziezak, 1990):

- membrane characteristics - non-uniform pore size and compaction problems
- solute/membrane interactions - concentration polarization and fouling
- protein-protein interactions - hetero- and self- association of proteins

Over the last decade, major improvements have been made by the manufacturers in membrane quality, uniformity of pore size and in the development of new membrane materials (Abelson, 1989; Kroner et al., 1984).

Concentration polarization refers to the increase in concentration of rejected species with decreasing distance from the membrane. At high enough concentrations, a gel layer forms which can lead to fouling, the build-up of proteins, fats and suspended solids that causes hydrodynamic resistance and interferes with the flux. The challenge for membrane designers is to develop a membrane that is hydrophilic, but has very little net charge. Concentration polarization, however, occurs regardless of whether or not there is any protein adsorption to the membrane (Ingham et al., 1980).

Protein-protein interactions such as hetero-interactions[2] can significantly reduce the efficiency of fractionation. Ingham et al. (1980) carried out ultrafiltration of a binary mixture of lysozyme (molecular weight approx. 14,000 Da) and albumin (molecular weight approx. 69,000 Da) using an Amicon PM-30 UF membrane in an Amicon TCF-10 spiral channel unit in diafiltration mode[3] In the absence of albumin and salt, lysozyme passed through the membrane and virtually all resided in the permeate after three volume changes. When excess albumin (50 mg/ml) was added to the initial solution, less than 10% of the lysozyme passed through the membrane due to hetero-association with albumin. The effect was strongly reversed by the presence of 0.25 M KCl, which partially inhibits complex formation.

Likewise, many proteins self-associate (Ingham et al., 1980) and may not pass through a filter even though the nominal molecular weight cut-off value of the filter is much higher than that of the protein in its monomeric form.

[2]Interactions between different molecular species.

[3]In diafiltration mode, the sample volume remains constant. Used in this way, the ultrafiltration apparatus is effectively replacing dialysis, with the added benefit of protein fractionation.

5.2.2 Materials and Methods

Experimental Methods

Initial experiments involving the concentration of diluted yolk supernatant were carried out using a Romicon PM-100 (nominal molecular weight cut-off (NMWC) 100,000) hollow fibre ultrafiltration cartridge (Romicon Inc., Woburn, Mass.), with tygon tubing and MASTERFLEX peristaltic pump (Cole Parmer, Chicago, IL). Yolk supernatant was prepared by diluting industrially separated yolk ten times with distilled water. The mixture (pH 6.2 to 6.4) was allowed to sit a minimum of 24 hours at 4°C before ultrafiltration.

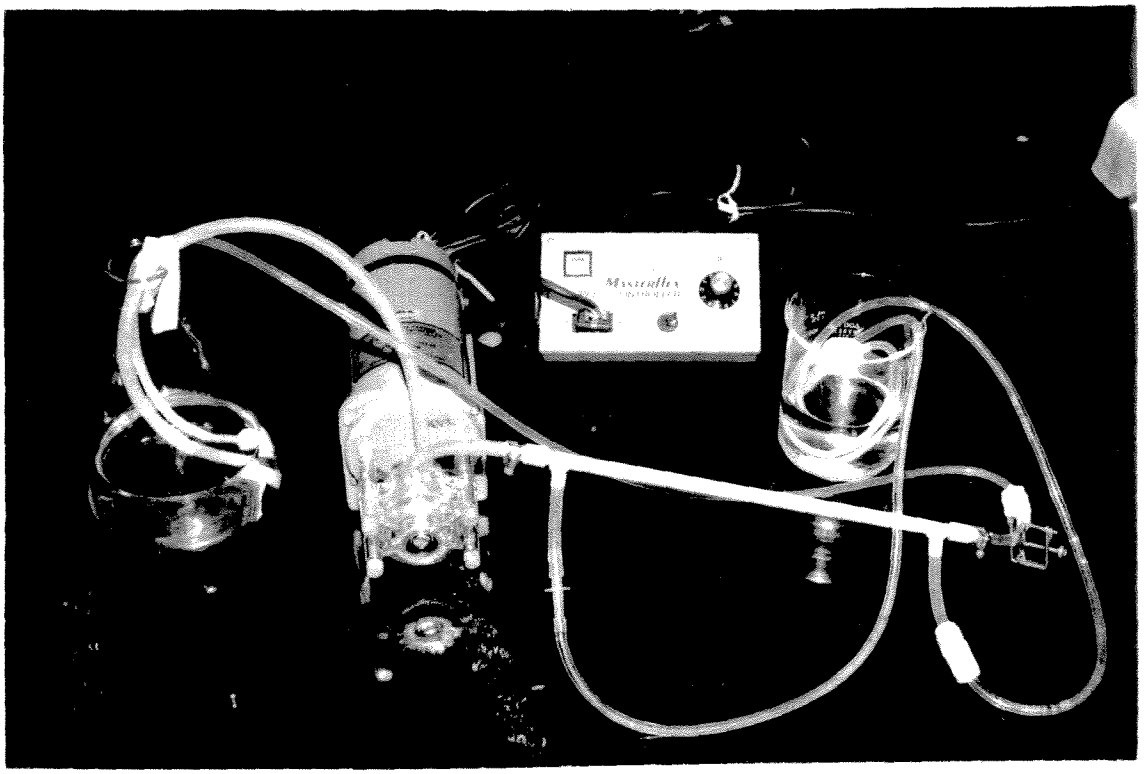
Later experiments involved the use of a lab-scale hollow fibre ultrafiltration cartridge (AG Technology Corporation, Needham, MA) of the same NMWC as above. Yolk was diluted ten times with distilled water and the pH adjusted to 5.0 with 0.1 N HCl. Settling was allowed to occur for one week at 4°C, and then the WSF filtered[4] and stored until use. One day prior to ultrafiltration, the pH was adjusted to 9.0 with 0.1 N NaOH, and the solution allowed to sit overnight at 4°C. After 0.45 μm filtration, the solution was diafiltered, the permeate volume being replaced occasionally with distilled water previously adjusted with 0.1 N NaOH to pH 9.0. The appartus used is shown in Figure 5.9.

Analytical Methods

Electrophoresis (SDS-PAGE) was carried out as described in Chapter 4, and HPLC analysis followed essentially the same procedure as that described in Chapter 7 except that the running buffer was PBS (0.01 M phosphate buffer pH 7.0 with 0.14 M NaCl and 0.02% NaN_3).

[4]See filtration of WSF in Chapter 4.

Figure 5.9: Ultrafiltration Apparatus



5.2.3 Results and Discussion

Initial Ultrafiltration Experiments

Even before the optimal conditions for the aqueous extraction step had been determined, ultrafiltration was considered as a possible separation step to follow. As mentioned previously, at that time the supernatant was being prepared at ten times dilution but without pH adjustment. The resulting pH was generally slightly acidic, pH 6.2 to 6.4, and as shown in Figure 4.6, the lipid residual was 30-40% under these conditions.

Since SDS-PAGE of the supernatant indicated the presence of 5 major contaminating proteins (of which alpha-livetin at a molecular weight of 80,000 is the largest), the idea was to retain only IgY and allow all 5 contaminants to pass through the filter by selecting an appropriate nominal molecular weight cut-off. Since a Romicon PM-100 (nominal MWC 100,000) was readily available[5], ultrafiltration was attempted with this cartridge.

SDS-PAGE results indicate that the apparatus functioned well in concentrating the supernatant, but no significant reduction in contaminating proteins occurred. This rather disconcerting result can be attributed to several possible causes:

- molecular weight cut-off too close to α-livetin
- fouling with lipids/lipoproteins
- protein-protein interactions

Since the molecular weight cut-off of the UF cartridge was close to the molecular weight of the largest contaminating protein, α-livetin, it is likely that a substantial amount of this protein would remain in the retentate. However, since all of the α-livetin appears to be retained, it is unlikely that this accounts solely for the lack of separation.

[5]Courtesy of Canadian Lysozyme Incorporated, Abbotsford, BC.

A significant amount of lipid is present in the supernatant, so fouling is definitely a possibility. Based on the data available in this study, it was impossible to determine whether or not protein-protein interactions were wholly or partially responsible for the retention of α-livetin. However, ovalbumin contamination of the industrially separated yolk is significant, and as noted previously, albumins (such as BSA) are known to form complexes with other proteins and hinder their passage across UF membranes. Thus this possibility also exists, and further studies are needed to elucidate the problem.

Later Ultrafiltration Experiments

Later in the work, another attempt was made to use ultrafiltration to separate IgY from other proteins in yolk supernatant. Mr. Emmanuel Akita of the Department of Food Science, UBC, reported obtaining excellent separation using a 100 kDa NMWC membrane (Akita, 1992, personal communication). Briefly, his starting material was manually separated yolk, and after 10 times dilution of the yolk and pH adjustment to pH 5.0-5.2, the mixture was allowed to sit for at least two hours. It was then either centrifuged at 10,000 x g for one hour at 4°C, or filtered through Whatman No.1 filter paper in the cold. After adjustment to pH 9.0, the supernatant was concentrated 5 times by ultrafiltration and then diafiltered using distilled water adjusted to pH 9.0.

The results obtained following Akita's method were only marginally better than those obtained without pH adjustment of the supernatant. An HPLC system was used to study the permeate and retentate. Using a gel filtration column under the conditions described in Chapter 7, it was determined that the two smalles contaminating peaks were almost completely removed after three volume changes.

Once again, α-livetin remained in the retentate. The two major differences between this experiment and those of Akita are that he used manually separated yolk free from

albumen contamination (compared to the albumen contaminated industrially separated yolk used here), and centrifuged the yolk mixture instead of using sedimentation at 4°C. The difference in results obtained might again be explained by lipid fouling of the membrane or complex formation caused by the presence of ovalbumin or other egg white proteins.

5.2.4 Conclusions

Ultrafiltration of the supernatant prepared by diluting egg yolk and allowing the mixture to settle overnight at 4°C failed to achieve significant separation of IgY from other contaminating proteins. Further work is necessary to investigate the possibility of using this technique for separation of IgY from industrially separated yolk.

5.3 Anion Exchange Chromatography

5.3.1 Introduction

Anion exchange chromatography is the chromatographic technique used most commonly for IgY separation and purification. Pioneering work was carried out in the 1960's and 70's by several authors (Mandeles, 1960; Benedict, 1967; Parkinson, 1967 and 1972; Burley and Vadehra, 1979) involving laboratory scale apparatus and most emphasizing the use of a linear salt gradient to elute a rather large number of peaks. Papers have continued to appear describing several variations of these protocols (Higgins, 1976; Gassmann et al., 1990; McCannel and Nakai, 1990; Otani et al., 1991).

In order to test the basic anion exchange protocol for its applicability to the separation of IgY from the WSF of industrially separated yolk, a number of small scale experiments were carried out.

5.3.2 Materials and Methods

A 50 ml column (i.d. 2.5 cm) was packed with DEAE-Sephacel (Pharmacia, Uppsala, Sweden) and used with the second generation semi-automated chromatography system described in Chapter 3 (shown in Figure 3.3). Two sets of buffer systems were utilized. The first set of experiments involved the use of Tris-buffer (TB). The column was equilibrated with at least 6 bed volumes[6] of 0.5 M TB (pH 7.5), and then washed with 4 bed volumes of 0.03 M TB, pH 7.5 (washing buffer). Sample was buffered to pH 7.5 with 0.03 M TB before application to the column. The column was then washed with washing buffer, and a multiple step or linear salt gradient formed using either a valving system or mixing chamber and 2 peristaltic pumps. A conductivity meter (Cole Parmer, Chicago, Illinois) was used to verify the ionic strength of the mixing chamber before and after each run, from which the NaCl molarity could be estimated for comparison with predicted values. A salt wash of 3 bed volumes of Tris buffered saline (TBS) pH 7.5, 0.5 M NaCl, was used to elute any unbound proteins and was followed by 1 to 2 bed volumes of washing buffer.

In later experiments, a phosphate buffer (PB) system was used, since this buffer is acceptable for food applications. The same procedure as described above was followed. Equilibration buffer was 0.2 M PB pH 8.0, washing buffer 0.02 M PB, pH 8.0, and saltwash 0.02 M PBS pH 8.0, 0.5 M NaCl.

[6]One bed volume is generally defined as the volume occupied by the exchanger in the column, including all pore spaces, ie: the total volume of the exchanger.

5.3.3 Results and Discussion

Initial experiments were carried out to study the extent to which IgY would bind to the DEAE-Sephacel column. A linear salt gradient was used for elution because one-step elution resulted in virtually all of the bound protein eluting from the column in a single peak. Using a linear gradient from 0 to 0.25 M NaCl, the majority of IgY (according to SDS-PAGE) was present in several peaks eluted in the range 0.1 to 0.15 M NaCl. However, several contaminating proteins were also present with IgY in these peaks. Significant "tailing" of peaks occurred in most but not all cases, indicating that a large number of proteins and possibly subgroups, with different isoelectric points are present.

The buffer system was then changed to phosphate buffer in order to ensure compatibility with food safety regulations. For most experiments, a linear elution profile from 0.02 to 0.18 M NaCl was used. The largest peak eluted when the salt concentration of the mobile phase was between 0.12 and 0.15 M, but again, IgY was spread across several peaks. The majority of adsorbed protein eluted near the beginning of the gradient as the ionic strength approached 0.10 M.

5.3.4 Conclusions

Although anion exchange chromatography has been used extensively by numerous researchers for laboratory scale purification of IgY, it appears to hold little hope for use on a large scale using industrially separated egg yolk as a starting material. This is not due to a lack of good industrial grade exchangers, but rather a lack of differentiation between IgY and other livetin proteins under the basic conditions of anion exchange.

5.4 Cation Exchange Chromatography

5.4.1 Introduction

Despite the extensive use of anion exchange chromatography to separate IgY, no attempt to use a weak cation exchanger to separate IgY from other yolk water soluble proteins at a preparative scale has yet been reported.

5.4.2 Materials and Methods

Chromatography Experiments

The automated system shown in Figure 3.4 was used with a column (2 cm inside diameter) packed with about 35 ml of CM-92 (Whatman Biosystems Inc., USA) cation exchange media. The column was equilibrated with 0.2 M Phosphate Buffer (PB) at pH 5.0 and then washed with 0.01 M PB at pH 5.5. Approximately 15 ml of filtered egg yolk supernatant (EYS) was applied to the column, the column washed again with 45 ml of 0.01 M PB and the bound proteins eluted using a linear gradient for 60 min with increasing phosphate buffer molarity from 0.01 to 0.2 M, followed by continued elution for 60 min with 0.2 M PB. After elution, the column was washed with 30 ml of 0.01 M PB/0.5M NaCl solution followed by 60 ml of 0.01 M PB. The flow rate was maintained at approximately 1.0 ml/min.

In later experiments, 50 ml of CM-92 was packed into a 2.5 cm i.d. column and the flowrate for washing, elution and saltwash was increased to approximately 2 ml/min.

Methods of Analysis

SDS-PAGE using a Phast System (Parmacia, Upsalla, Sweden) was used to obtain a qualitative determination of the IgY content of each peak.

5.4.3 Results and Discussion

The first attempt to bind IgY to a cation exchanger was carried out at pH 5.5 and with a washing buffer of 0.02 M PB pH 5.5. Unfortunately, even this low ionic strength buffer proved to be too strong to allow binding to occur. The washing buffer was then reduced in ionic strength to just 0.01 M PB, and an experiment carried out as described above using a 35 ml column of CM-92. Figure 5.10 shows the chromatogram obtained from the first successful experiment involving the use of CM-92.

As with the anion exchange experiments, a significant amount of protein is not bound and elutes with the mobile phase. The protein that is bound is held much more weakly than in the case of anion exchange since the majority of the bound proteins eluted early in the 0.01 to 0.2 M PB elution gradient. SDS-PAGE of the eluted peak showed that it was largely IgY, a result not entirely expected since the isoelectric points of the other livetin proteins are known to be in the same range as IgY. Thus this first experiment in the use of a cation exchanger to separate IgY from other water soluble proteins in egg yolk supernatant appeared to be very encouraging.

Figure 5.11 shows another cation exchange experiment carried out using Whatman CM-92 exchanger. In this case a 50 ml column was used and only 30 ml of EYS applied. This chromatogram includes the saltwash peak which indicates that under the conditions used, certain proteins are bound quite strongly to the column and must be eluted with relatively high ionic strength buffers (in this case 0.01 M PB/0.5 M NaCl).

5.4.4 Conclusions

The data obtained in this study indicate that cation exchange chromatography can potentially be used for the separation of egg yolk immunoglobulins. Under the conditions utilized, the majority of IgY is initially bound to the column, but can easily be eluted

Figure 5.10: Chromatogram from the first CM-92 Experiment

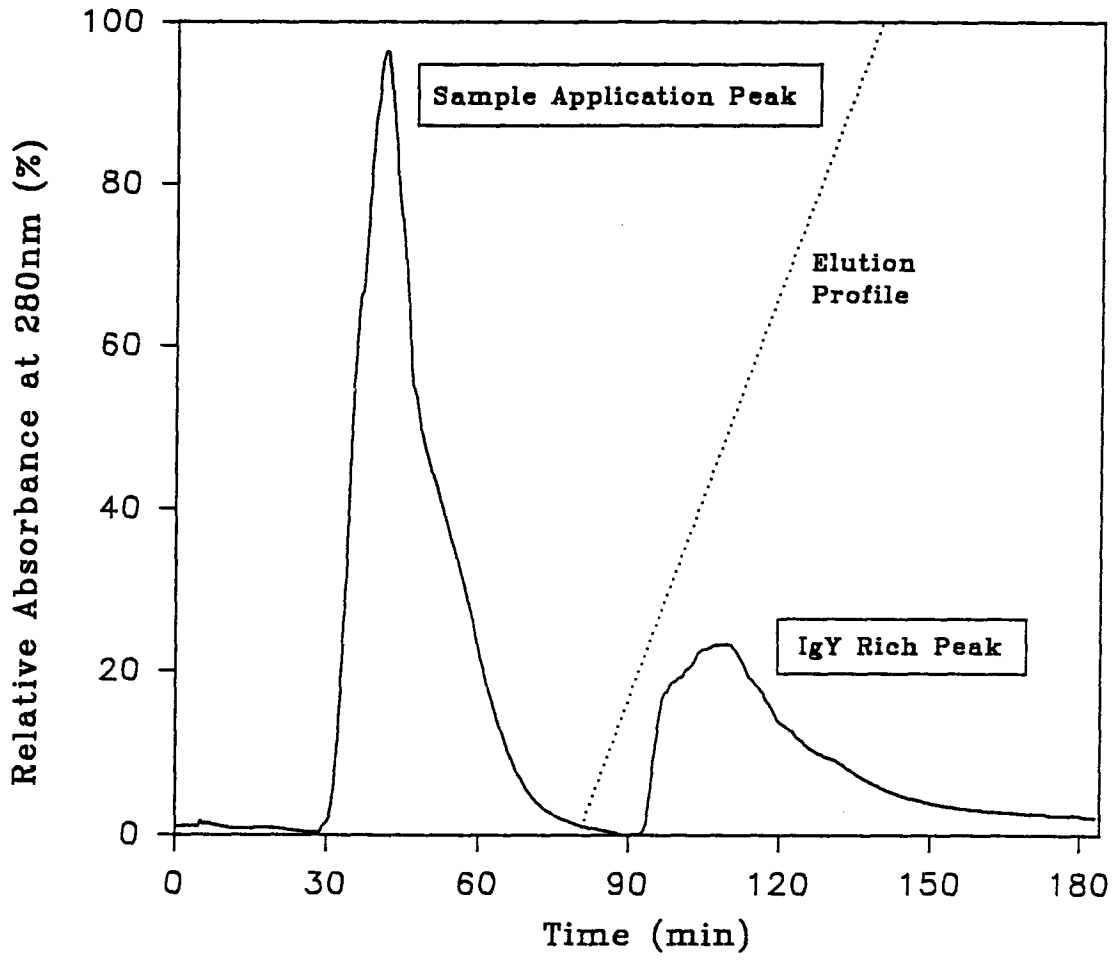
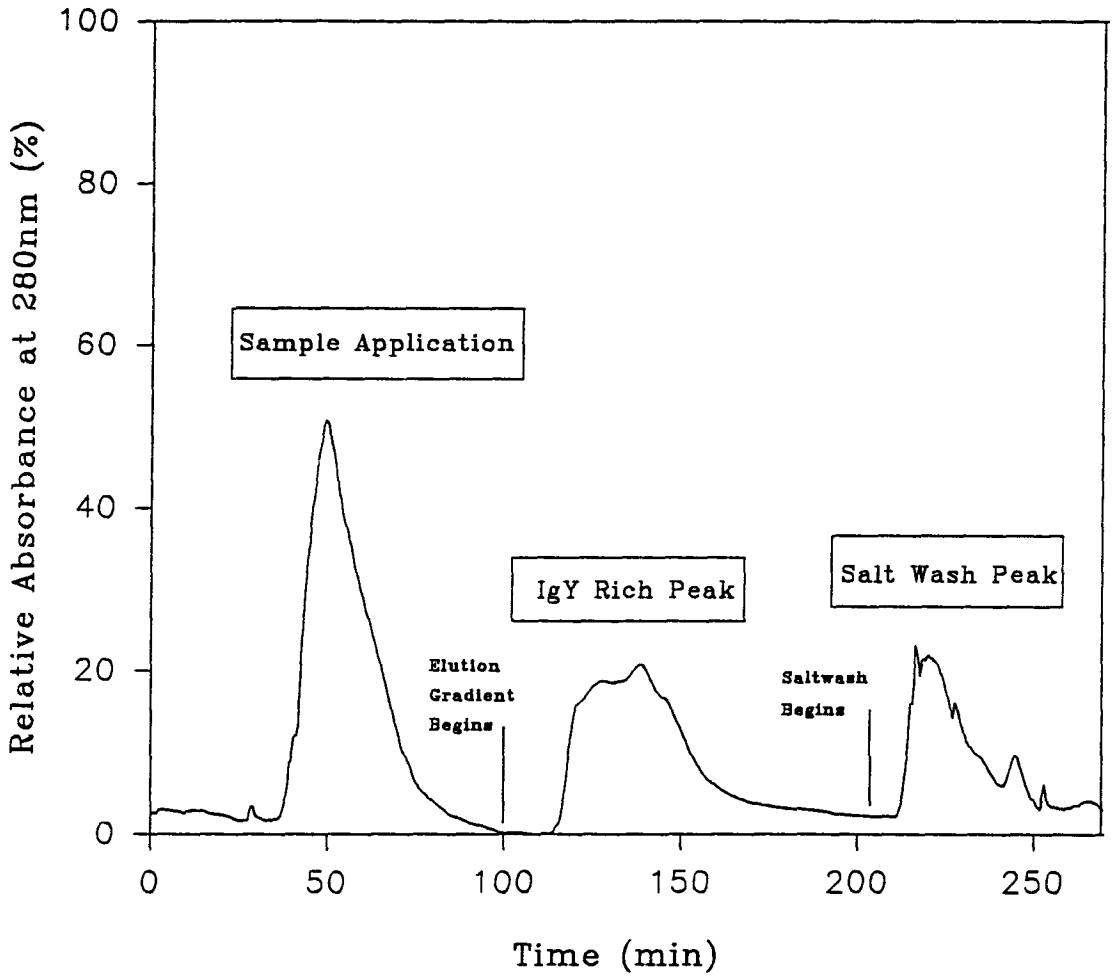


Figure 5.11: Chromatogram from a later CM-92 Experiment



with a relatively low ionic strength buffer.

5.5 General Conclusions

Both ultrafiltration and anion exchange chromatography failed to significantly improve the IgY purity in the WSF. Of the 3 techniques tested, cation exchange chromatography appears to hold the greatest promise for separating IgY from the WSF of supernatant prepared from industrially separated egg yolk. Under the conditions utilized in this study, it would appear that all of the ovalbumin (the principal egg white contaminant of industrial yolk) and the majority of the other contaminating proteins have been removed from the purified fraction, with the exception of a portion of the α-livetin. Further study of this technique with a view to incorporating it into a pilot-scale purification process is warranted.

Chapter 6

Comparison of Two Cation Exchangers

6.1 Introduction

Based on the conclusions of the work described in Chapter 5, the use of cation exchange chromatography for IgY separation was further investigated. Besides carrying out a more in depth study of the use of the CM-92 exchanger, an industrial grade exchanger (HC-2) was also tested and compared. It was decided that with a heavier sample loading[1] the effect of linear gradient could be compared to that of a single step elution profile for the elution of IgY from the column. This would indicate whether or not IgY elutes as a single peak, and if so, if it would be possible to separate it more efficiently from α-livetin by controlling the shape of the elution profile.

6.2 Materials and Methods

6.2.1 Cation Exchange Chromatography

The experimental equipment and buffers were as described in Section 5.4. The cation exchange media used were the weakly acidic carboxymethyl (CM) cellulose cation exchangers CM-92 (Whatman BIosystems Inc., USA) and HC-2 (Gibco CEL, New Zealand). CM-92 is a fibrous exchanger, which is supposed to allow faster flow rates. HC-2 is an

[1]The loading used in the preliminary CM-92 experiments was on the order of about 1 mg of IgY per ml of exchanger, of which about $\frac{2}{3}$ bound. Considering that today's ion- exchangers are generally capable of binding 50 to 100 mg of protein per ml, this is an fairly light loading, but sufficient for testing the exchanger.

industrial grade exchanger and contains particles with a range of diameters from 150 to 250 μm. It is based on a cellulose matrix.

Each exchanger was packed into a 2.5 cm i.d. column to a total bed volume of 50 ml. After equilibration with 3 to 4 bed volumes of equilibration buffer, approximately 3 bed volumes (140 ml) of yolk supernatant prepared from industrially separated yolk according to the protocol recommended in Chapter 4 was applied to the column. After a wash of 1 to 2 bed volumes, the bound proteins were eluted using either a linear gradient of PB with increasing molarity from 0.01 to 0.2 M, or a step change from 0.01 to 0.2 M PB. A saltwash followed elution. The flow rate was maintained at 1.95 ml/min with the exception of sample application where the flow rate was reduced to 0.93 ml/min. Fractions were collected for analysis every 7.5 min. during elution and salt washing, while the total volume was collected and measured after both sample application and washing, and representative samples kept for analysis. The collected samples were analyzed for total protein and IgY concentration, and the purity and recovery of each fraction were estimated.

6.2.2 Analytical Procedures

Total protein determination was carried out using the BCA method, non-denaturing electrophoresis (SDS-PAGE) with a Pharamacia Phast System using 10-15% gradient PhastGels and IgY concentration determination was by radial immunodiffusion. All of these techniques were applied as described previously.

6.3 Results and Discussion

Figures 6.12a and 6.12b are typical chromatograms obtained with the small column packed with CM-92 cation exchanger, using linear and step gradient elution, respectively. These experiments were repeated once for a total of 2 replicates. The resulting chromatograms were very repeatable. The first peak in each chromatogram occurs during sample application and washing, which represents the majority of impurities and about 30% of the IgY not initially bound to the column. The majority of the remaining IgY is split into two peaks using a linear gradient, while most is eluted in a single peak using step-wise elution.

The separation of the IgY rich peak in Figure 6.12a did not improve significantly either recovery or purity as the IgY concentration was high in both portions of the peak. Therefore, similar recoveries (about 60%) and purities (67-69%) were obtained for the total IgY peak using either linear or step-wise gradient.

Figure 6.13 shows the resulting elution chromatogram when a double step elution profile (steps of 0.1 M PB and 0.2 M PB) is used. In this case, despite the fact that two well separated peaks result, the IgY purity of each one is very similar. This also demonstrates the existence of different IgY subclasses, as previously noted. This technique might be useful if the separation of IgY into its subclasses is required. However, from the point of view of purifying the whole IgY population , there does not appear to be any great advantage in using linear or multi-step gradients compared to a single step elution profile. In all three cases, the saltwash peak that occurs at the end of the run contained very little IgY.

Figures 6.14 and 6.15 are typical chromatograms obtained using a column packed with HC-2 and using linear and step-wise elution profiles, respectively. Each experiment

Figure 6.12: Separation of IgY on a CM-92 Column using Linear (a) and Step (b) Elution Profiles

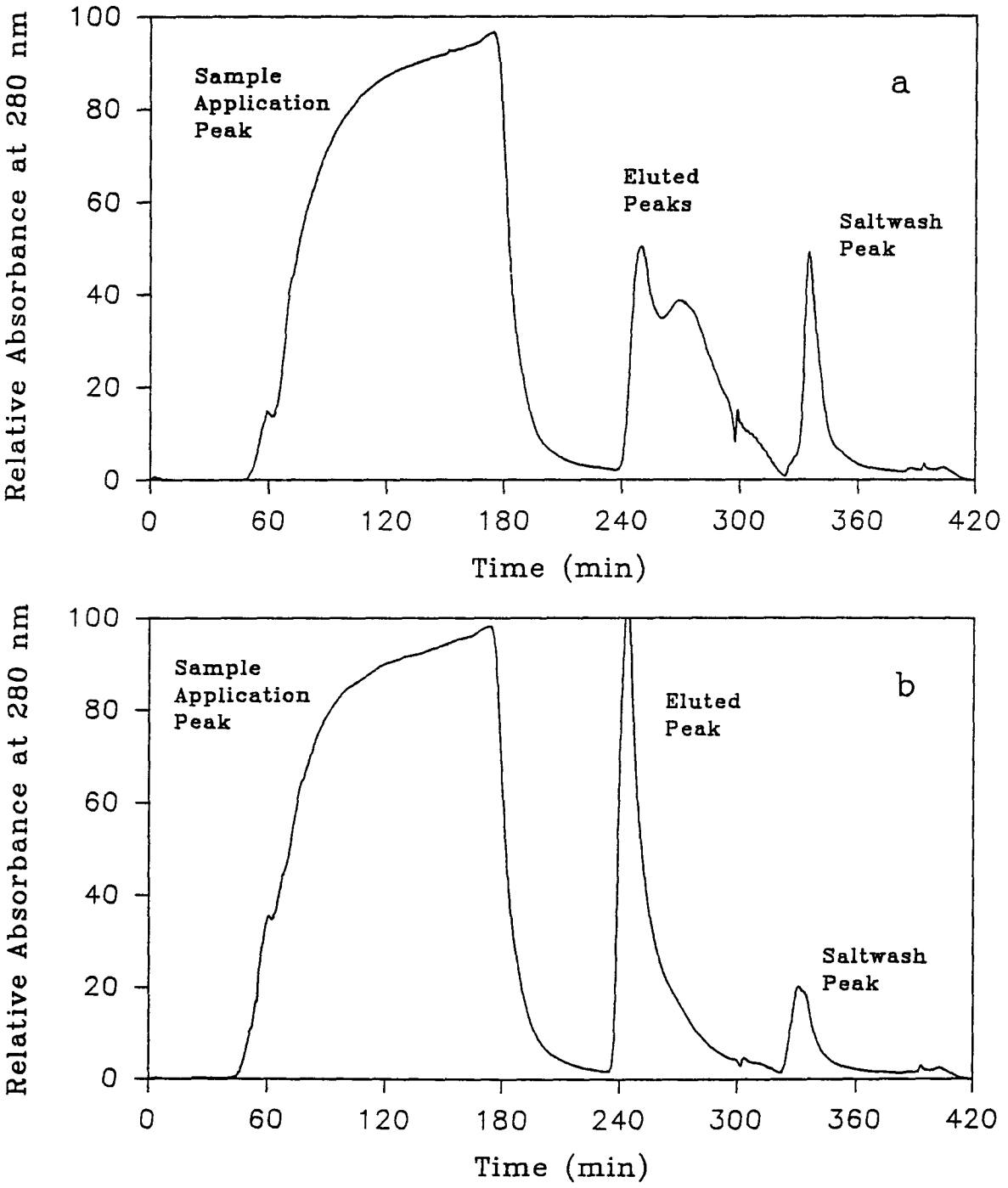


Figure 6.13: Separation of IgY on a CM-92 Column using Double Step Elution Profile. The sample application peak has been omitted for clarity.

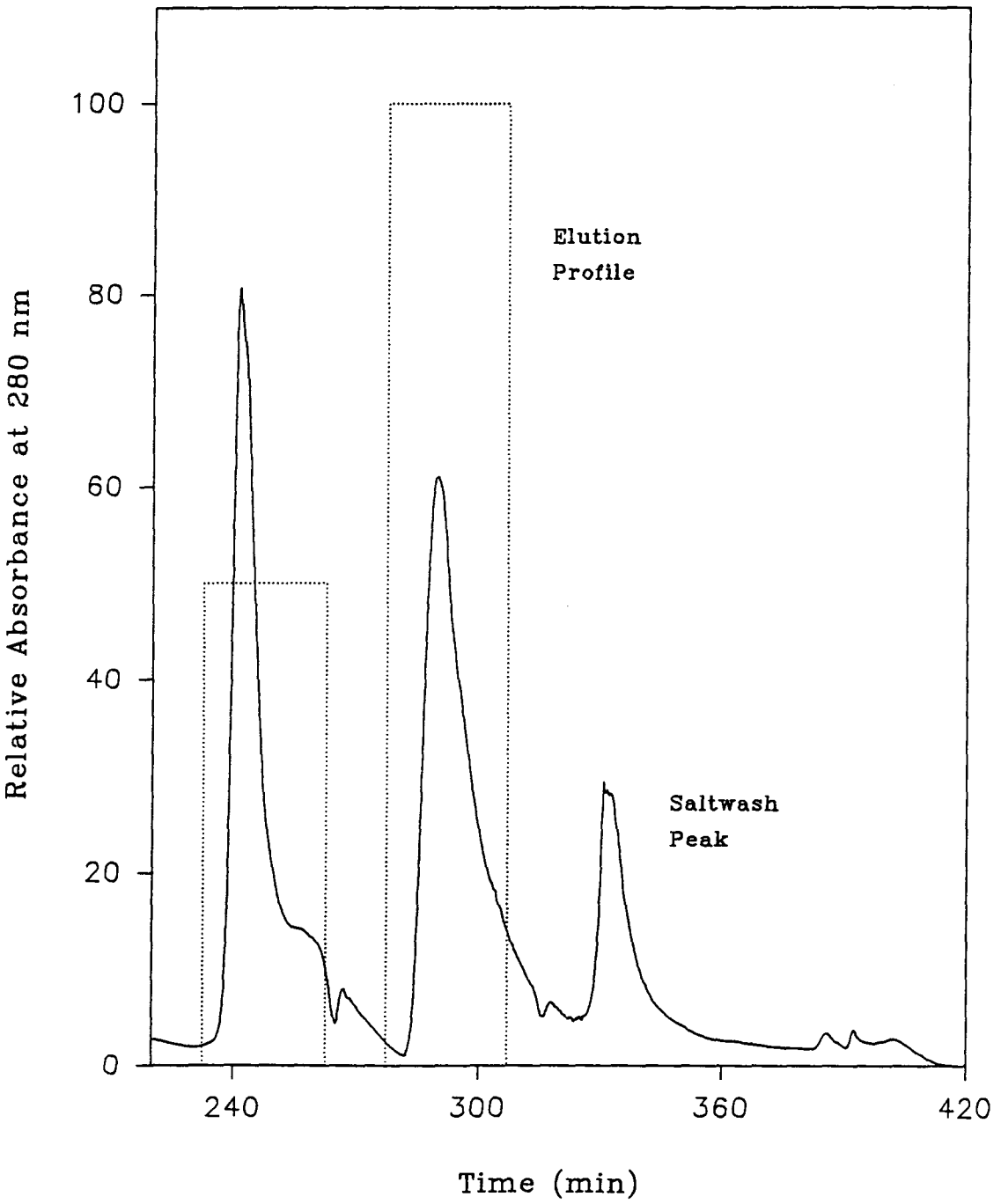


Table 6.7: IgY Recovery and Mass Balance using Linear Gradient and Step-wise Elution on a Column of HC-2 Cation Exchanger for the Representative Experiments shown in Figures 6.14 and 6.15.

	Linear gradient		Step-wise gradient	
Step	IgY (mg)	IgY (%)	IgY (mg)	IgY (%)
Sample application	22.5	22.2	20.1	20.3
Washing	8.8	8.7	9.8	9.9
Elution	64.8	63.9	67.4	68.0
Salt washing	6.7	6.6	3.6	3.6
Total	102.8	101.4	100.9	101.7
Total applied	101.4	100.0	99.1	100.0

was repeated twice for a total of 3 replicates In addition to showing the absorbance at 280 nm as a solid line, and the elution profile as a dashed line, these graphs also include histograms indicating the IgY concentration of individual fractions collected during the course of the experiments. The fractions with the highest IgY concentration occur in the IgY rich peak in both cases. Similar IgY concentration profiles were obtained for fractions from the two CM-92 experiments of Figures 6.12a and 6.12b. In the case of step elution, the HC-2 and CM-92 chromatograms are almost identical. In linear gradient elution, the HC-2 chromatogram contains only one major eluted peak instead of the two occurring with CM-92. The recoveries and mass balance calculations for HC-2 experiments are shown in Table 6.7. The close agreement in the mass balances obtained by comparing the sum of individual fractions to the total applied shows that all of the IgY is being accounted for and is not denatured throughout the course of the separation.

SDS-PAGE of a representative IgY rich peak is shown in Figure 6.16. Column A is the WSF from industrially separated yolk. Column B, the sample application and

Figure 6.14: Separation of IgY on an HC-2 Column using a Linear Elution Profile

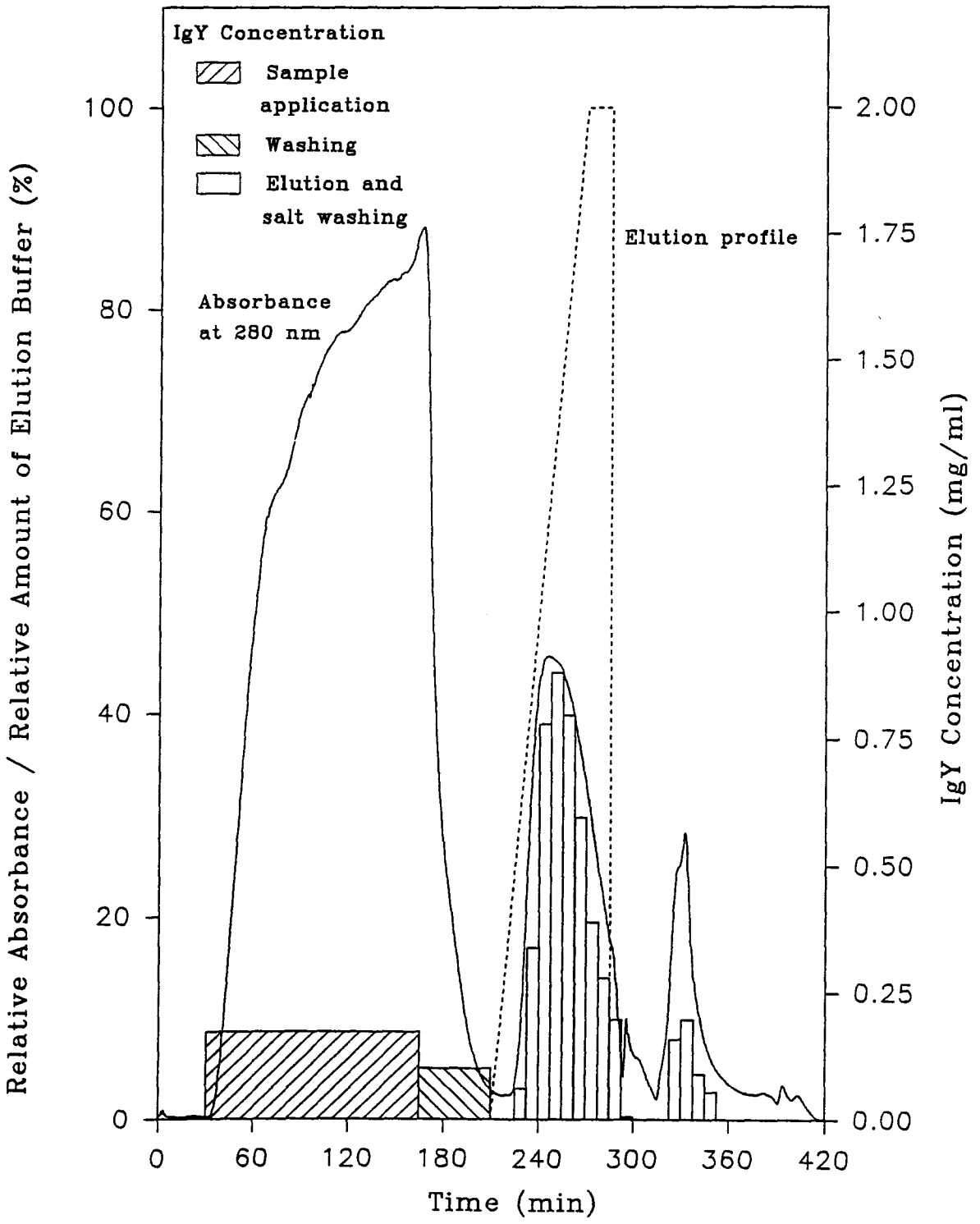
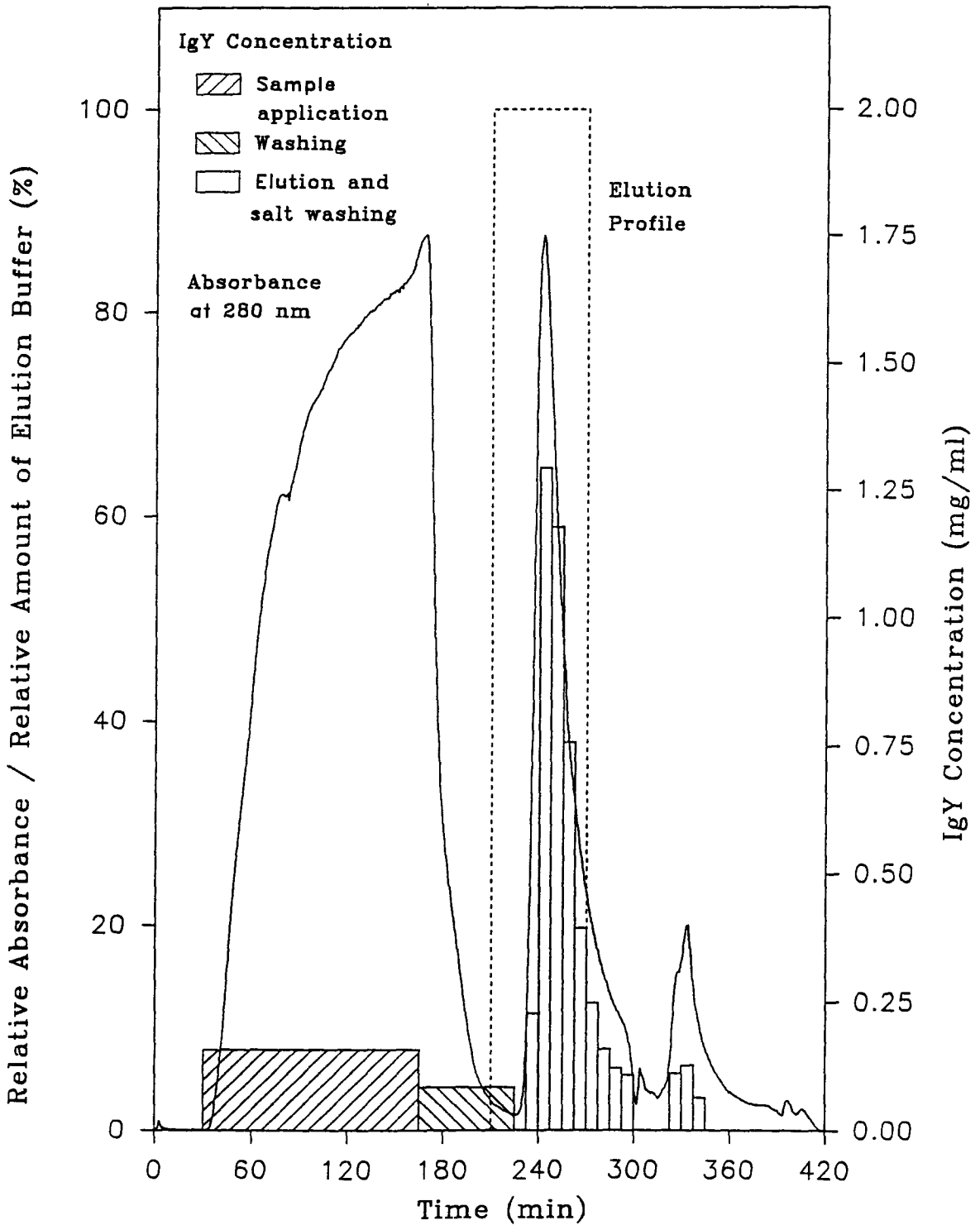


Figure 6.15: Separation of IgY on an HC-2 Column using a Step Elution Profile



washing peak, contains most of the β-livetin and smaller proteins. Column C is the IgY rich peak showing the strong IgY band which makes up almost $\frac{2}{3}$ of the fraction. The major contaminant, α-livetin, is also quite evident along with some lower molecular weight proteins. Column D, the saltwash peak, contains a small amount of IgY and the rest of the α-livetin and other proteins.

The IgY purity was also determined by dividing the IgY concentration by the total protein for each fraction. Figure 6.17 shows the resulting histograms for fractions eluted from the HC-2 column using linear gradient (Figure 6.17a) and step-wise (Figure 6.17b) elution profiles. Slightly higher purities were obtained for the corresponding CM-92 experiments. What is particularly interesting in these graphs is the fact that the fractions of highest purity are also the fractions of greatest total protein concentration. This is very helpful when sacrificing recovery in order to obtain higher purity.

A graph comparing the maximum purity obtainable as a function of recovery is shown in Figure 6.18. The data are based on the fractions collected from the CM-92 and HC-2 columns operating under identical conditions and using linear elution gradients. The curves are obtained by starting in each case with the fraction of greatest purity, and adding fractions of successively lower purity. Overall, the use of the CM-92 exchanger results in a somewhat higher purity for the same recovery. However, the price of HC-2 is considerably lower than that of CM-92 ($60 vs. $250 US/kg), and a large amount (10 kg) of HC-2 exchanger was already available. As well, the pressure drop across the HC-2 column was found to be quite low, even at relatively high flow rates. It was decided, therefore, to carry out further work with HC-2.

Figure 6.19 shows a similar plot of purity as a function of recovery for the linear gradient and step elution profile experiments with the HC-2 column. There appears to be little or no difference between results obtained with the different elution profiles. It

Figure 6.16: Non-Denaturing SDS-PAGE of Fractions from HC-2 Experiments. The lanes represent A - WSF prepared from industrially separated yolk; B - Unbound fraction; C - Eluted protein - IgY rich fraction; D - Saltwash peak.

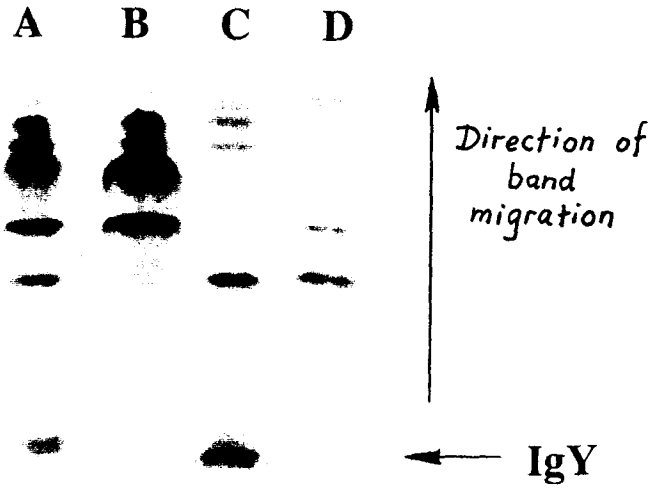


Figure 6.17: Purity of Individual Fractions Eluted from an HC-2 Column

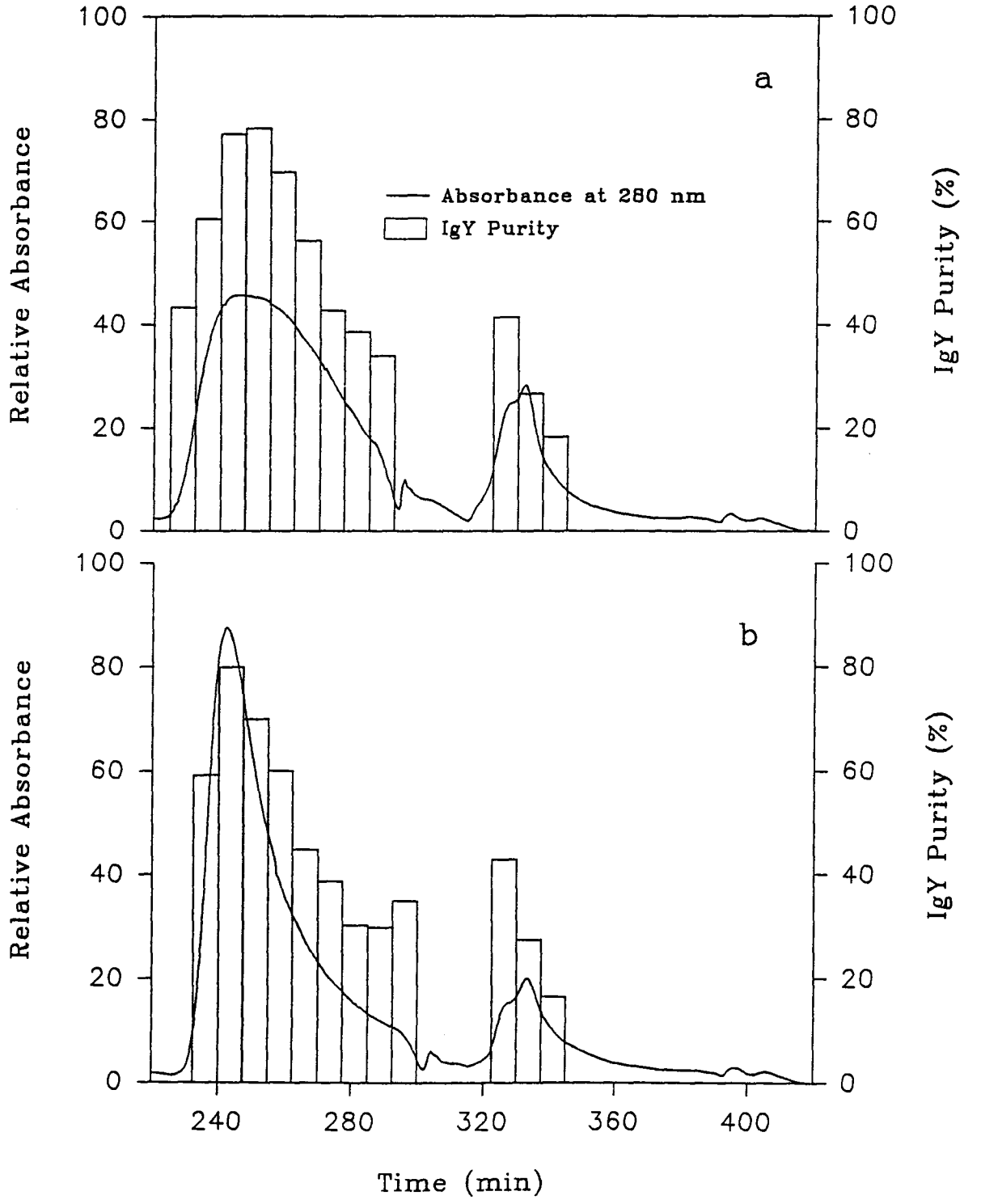
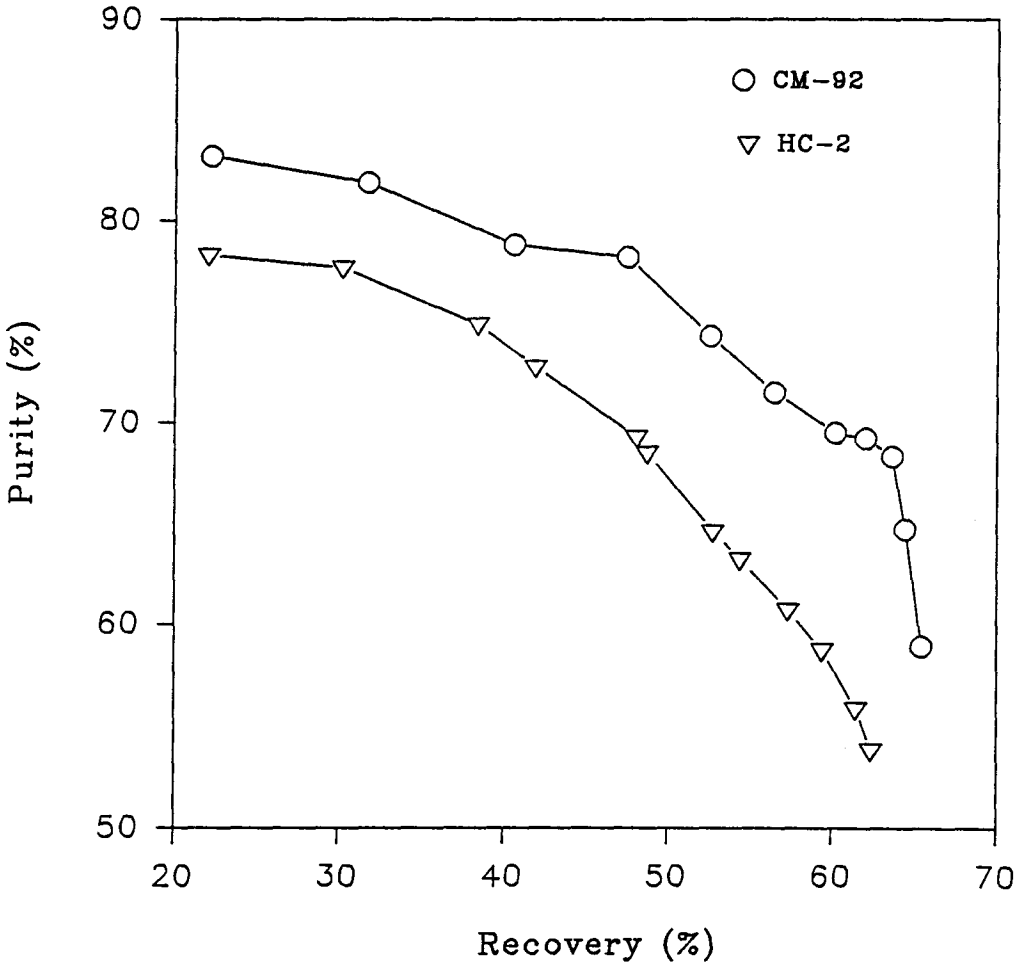


Figure 6.18: Purity as a Function of Recovery using Linear Gradient Elution



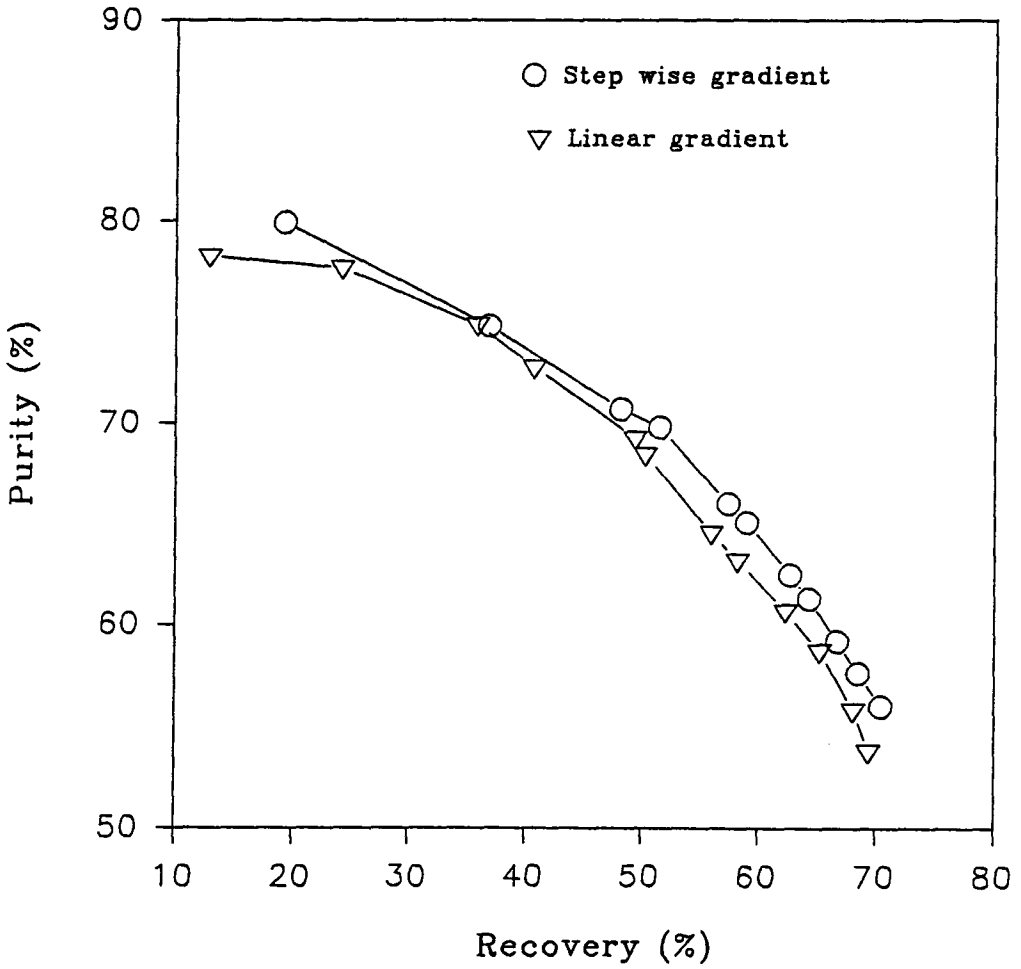
is much simpler to use a step-wise elution profile in practice.

6.4 Conclusions

It does not appear that the shape of the elution profile has any significant effect on the overall IgY recovery and purity that can be achieved using cation exchange chromatography. From analysis of individual fractions in the IgY rich peak, it was determined that IgY is not separated from α-livetin, but appears to elute over the entire peak.

Of the two exchangers tested, Whatman CM-92 consistently bound a larger amount of IgY per ml of exchanger. However, considering the exchanger cost, the difference in binding ability does not appear to be significant enough to warrant its use on a large-scale in place of the industrial cation exchanger HC-2.

Figure 6.19: Purity as a Function of Recovery for HC-2 Experiments



Chapter 7

HPLC Analysis of IgY

7.1 Introduction

Numerous authors have made use of gel filtration media either as a means of identifying or purifying chicken antibodies. Gel filtration has been used to purify chicken serum IgY, and similar methods have been used to fractionate chicken yolk IgY. A summary of literature references pertaining to the use of gel filtration in the study of chicken serum or egg yolk IgY is given in Table 7.8. This list is by no means comprehensive, but is a good selection of many papers that mention gel filtration. In almost all of these papers, gel filtration was used either to fractionate IgY from other water soluble proteins, or as final polishing step in a purification scheme. In three of the more recent papers (Yamamoto et al., 1975; Hassl et al., 1987; and Shirman et al., 1988) it was used to quantify the IgY present in solution. Van Orden and Treffers (1968) used gel filtration on Biogel P-200 to determine the molecular weight of serum IgY. Cser et al. (1982) used gel filtration on Sephadex G-200 as a final polishing step to remove IgY aggregates from solution prior to X-ray analysis of the monomer. The reported methods, however, have all made use of low pressure gel filtration columns packed with media such as Sephadex G-100 or G-200, Sephacryl S-200 or S-300 Superfine, or Bio-Gel P-200. No-one, as yet, has reported the use of an HPLC gel filtration column to either purify or quantify IgY. One paper only has been encountered, that of Burley and Back (1987), which refers to the use of an HPLC column in the study of the livetin fraction of egg yolk. The column used was an Ultropac

Table 7.8: References in the literature to gel filtration of chicken antibodies

Reference	Year	Media	Source
Flodin and Killander	1962	Sephadex G-200	serum
Dreesman and Benedict	1965	Sephadex G-200, G-50	serum
Hersh and Benedict	1966	Sephadex G-200	serum
Tenenhouse and Deutsch	1966	Sephadex G-200, G-100	serum
Van Orden and Treffers	1968	Biogel P-200	serum
Leslie and Clem	1969	Sephadex G-200	serum
Wilkinson and French	1969	Sephadex G-200, G-75	yolk
Leslie and Clem	1970	Sephadex G-200	serum
Orlans and Rose	1972	Sephadex G-200	yolk
Rose et al.	1974	Sephadex G-200	yolk
Yamamoto et al.	1975	Sephadex G-200	yolk
Higgins	1976	Sephadex G-200	yolk
Burley and Vadehra	1979	Ultragel AcA 34	yolk
Cser et al.	1982	Sephadex G-200	yolk
Wang et al.	1986	Fractogel TSK HW-55 (F)	yolk
"	"	Bio-Gel A-1.5m	"
Hassl et al.	1987	Sephacryl S-300	yolk
Hassl and Aspöck	1988	Sephacryl S-300 Superfine	yolk
Shirman et al.	1988	Sephacryl S-300	yolk
Akita and Nakai	1992	Sephacryl S-200 Superfine	yolk

DEAE-5PW, 10 μm column (7.5 mm x 7.5 cm).

Since the goal of this work was to study IgY purification with a view to developing a process viable for industrial scale production of the antibody, HPLC gel filtration was not considered as part of the separation process. Even today's so-called large-scale HPLC is still far from being large-scale when it comes to producing significant quantities of product for food use. However, HPLC appeared to be an interesting method to consider for analyzing and quantifying purified samples of IgY, and potentially for on-line monitoring of a separation process.

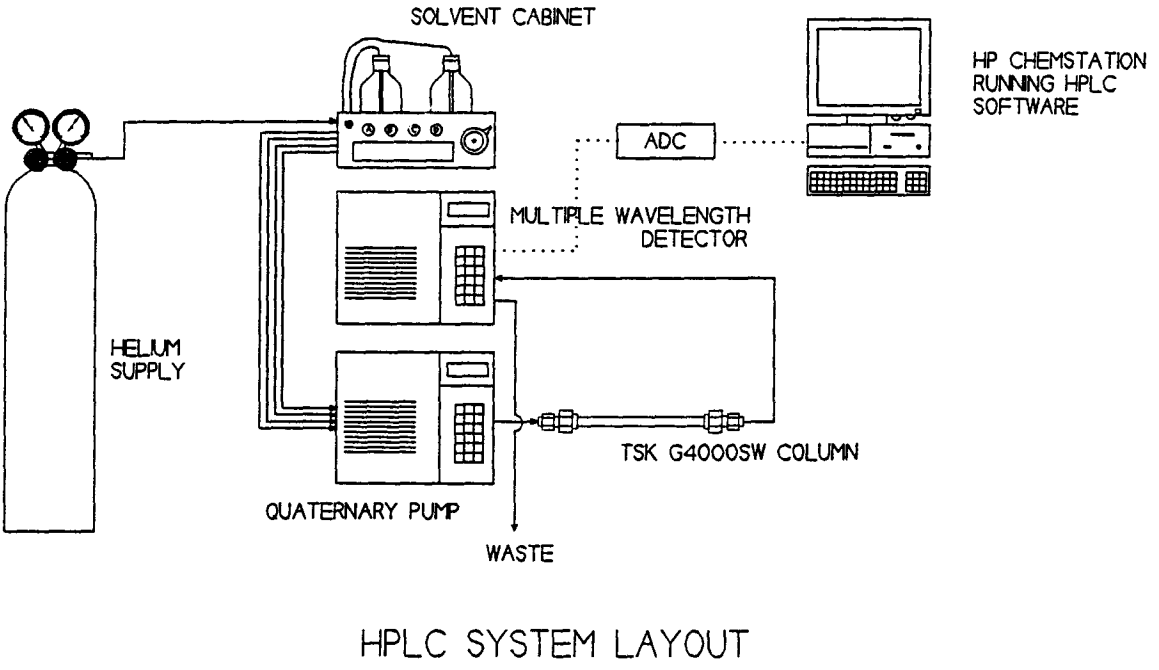
One of the drawbacks of the RID method used for IgY concentration determination is the delay of 3 days before obtaining the results of the analysis. As well, RID is subject to significant errors due to errors in pipetting, distortion of precipitin rings due to edge effects and uneven gel thickness, and errors in reading the precipitin ring diameters. HPLC analysis, on the other hand, allows for a much more rapid analysis time using a larger sample volume (20 μl applied by HPLC syringe compared to 4 μl for RID), decreasing the influence of pipetting errors. In this work, it was carried out using an almost fully automated system with computerized data acquisition, and once equlibrated with running buffer, the operator need only inject the sample and turn a valve in order to start the analysis. In other words, the equipment is easy to operate, ideal for quality control situations where technicians with minimal training on HPLC equipment could use the technique with confidence.

7.2 Materials and Methods

A 30 cm long TSK-G4000SW gel filtration HPLC column (Tosoh Corporation, Japan) was used with 0.1 M PB (pH 5.4 with 0.05% sodium azide as preservative) as running buffer. The column was connected to a Hewlett Packard HPLC system including a Series 1050 solvent cabinet with injection valve, quaternary pump and multiple wavelength detector. A Hewlett Packard ChemStation running HPLC software was used to acquire and analyze the chromatograms. The running buffer was degassed with helium prior to and during all runs, and samples were filtered prior to injection with a 13 mm diameter 0.45 μm (cellulose acetate membrane) disposable sterile syringe filter (Corning, NY). A schematic diagram of the HPLC system is presented in Figure 7.20.

The multiple wavelength detector has a diode array type light source that allows the user to program the wavelength and bandwidth of the source, as well as a reference

Figure 7.20: HPLC System Layout



wavelength and bandwidth. The wavelength used for all HPLC analysis was 280 nm and a narrow bandwidth of 4 nm, with a reference wavelength of 450 nm and 80 nm bandwidth.

In the case of purity determination, a flowrate of 0.25 ml/min was used resulting in 60 minute runs. For molecular weight and concentration determination, a flowrate of 0.5 ml/min was used resulting in 30 minute runs.

7.3 Results and Discussion

7.3.1 Molecular Weight Determination

Gel filtration has been used to determine the molecular weight of IgY. Using Sephacryl S-300 Superfine, Hassl and Aspock (1987) obtained a value of 175 kDa, and Shirman et al. (1988) a value of 160 kDa.

In order to obtain an estimate of the molecular weight of the IgY being purified in the separation process, proteins of various molecular weights were applied to the TSK-G4000SW column under the same conditions to be used for studying the purified samples. A total of 8 different protein standards were used, plus the dimers of two of the proteins, in order to obtain a curve relating the molecular weight of each protein to its retention time on the column. A summary of the molecular weights and corresponding retention times of the protein standards is given in Table 7.9. Sample chromatograms of some of the standards can be found in Appendix B. Figure 7.21a illustrates that as expected, an exponential relationship exists between the molecular weight of a protein and its retention time. Each point in the figure represents a single determination of the retention time for a purified sample. However, some of the samples were retested and the retention time was always within 0.5% of the previously determined value. Figure 7.21b is a plot of

Table 7.9: Summary of Molecular Weight Information

Data Point	Protein	Retention (min)	Molecular Weight (kDa)	Reference
1	Phosphorylase-α	17.37	370,000	a
2	Bovine IgG dimer	17.45	300,000	b
3	Bovine IgG monomer	19.32	150,000	b
4	BSA dimer	18.95	132,534	b
5	Conalbumin	20.56	76,000	c
6	BSA monomer	20.37	66,267	b
7	Ovalbumin	21.14	44,500	c
8	β-lactoglobulin	21.37	35,000	a
9	Carbonic anhydrase	22.19	30,000	a
10	Lysozyme	23.77	14,300	c

Reference	Source
a	Fasman, G.D., ed. 1989. CRC Practical Handbook of Biochemistry and Molecular Biology. CRC Press Inc., Boca Raton, Florida.
b	Fox, P.F., ed. 1989. Developments in Dairy Chemistry 4. Elsevier Science Publishers Ltd, London, UK.
c	Powrie, W. and S. Nakai. 1985. In: Food Chemistry, 2nd ed., Fennema, O.R., ed. Marcel Dekker, Inc., New York, NY, pp 829-855.

the natural logarithm of the molecular weight vs. retention time, and demonstrates that a strong relationship ($R^2 = 0.991$) results when a linear regression is performed on the transformed data.

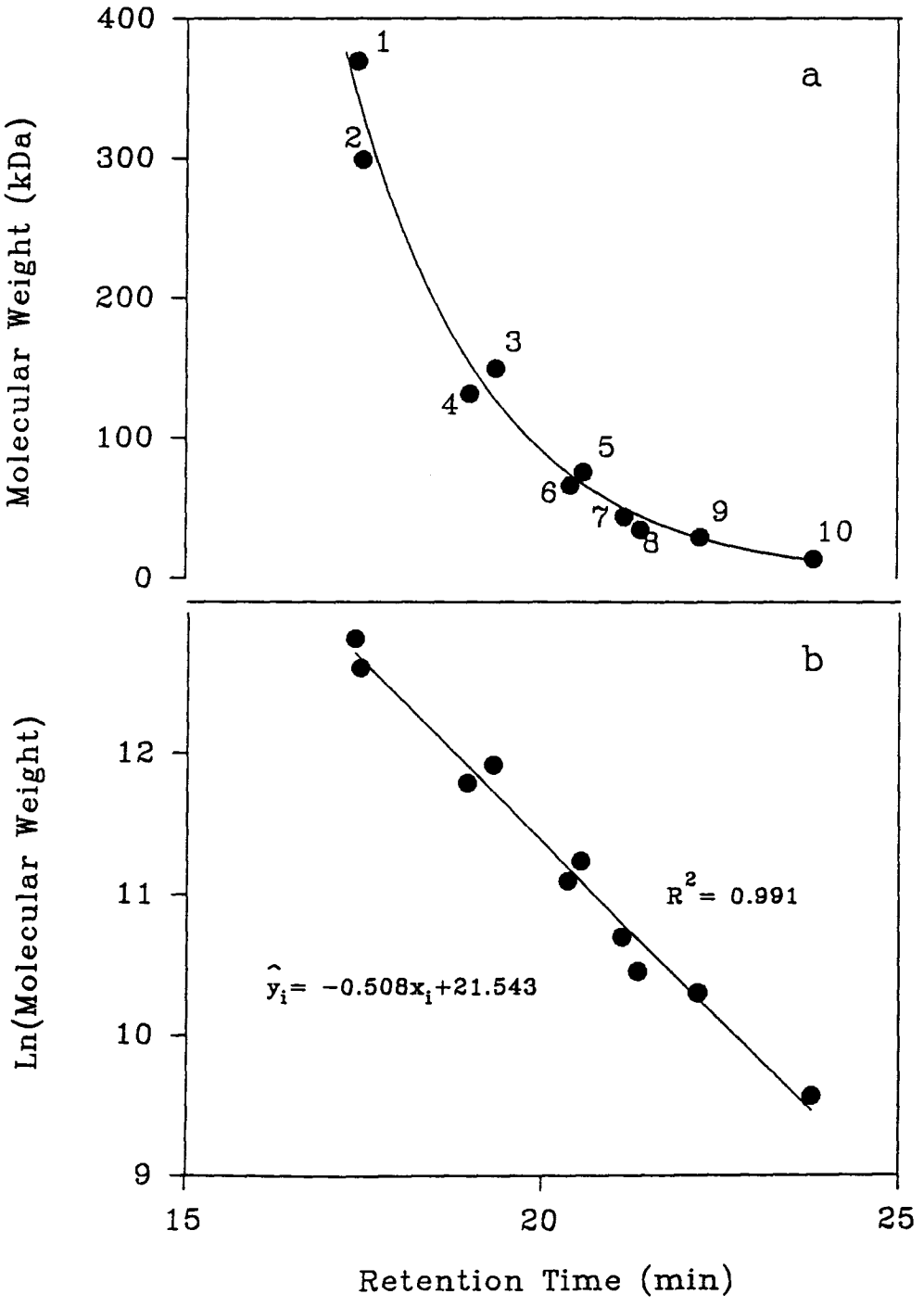
Using the resulting regression equation and the retention time for IgY monomer obtained from numerous experiments (approximately 18.87 minutes on average), the molecular weight of the purified IgY is determined to be in the vicinity of 156 kDa. This compares with the value of approximately 175 kDa obtained previously using electrophoresis. Bovine IgY, known to have a molecular weight of 150 kDa, is estimated by this procedure to be only 124 kDa. Thus it is clear that the error involved in this estimation is significant, and this is further supported by the range of values obtained for the molecular weight of IgY by other researchers[1] The estimation obtained nonetheless provides one means of verification when identifying the IgY peak in purified samples. The equation obtained also allows for the estimation of the molecular weights of impurities and antibody aggregates in these samples.

7.3.2 IgY Concentration Determination

The second use to which the gel column was put involved the determination of the IgY concentration of partially and highly purified samples. Unfortunately, proteins in the molecular weight range 30,000 to about 100,000 Daltons did not separate well from one another under various conditions of pH and PB concentration (pH 5 to 7, 0.05 to 0.2 M PB, 0 to 140 mM NaCl). Since there are at least three major impurities present in the

[1]One explanation for the large difference in estimated values is that gel filtreation separates on the basis of molecular size rather than molecular weight (hence the more correct, but not yet universally used term "size exclusion chromatography"). Although there is a direct relationship between these two characteristics of a molecule, the correlation is not perfect since some molecules have a more tightly packed structure than others, and this can also be affected by the molecule's micro-environment. Since it is known that the radius of gyration of IgY is somewhat smaller than expected based on it's molecular weight (Cser et al., 1982), it is not surprising the HPLC estimate is lower than expected.

Figure 7.21: Relationship Between Molecular Weight and Retention TIme



supernatant obtained from industrially separated yolk (α- and β-livetins, and ovalbumin) that fall within this range (80,000, 48,000 and 44,500 respectively), a very large peak with a retention time of around 21.0 minutes was obtained from supernatant samples applied to the gel filtration column. Since the retention time of ovalbumin was determined above to be 21.14 minutes, and since electrophoresis indicates the presence in yolk supernatant of a large proportion of ovalbumin, the large peak is apparently predominantly due to this protein. In samples of supernatant, this large impurity peak often distorts the IgY monomer peak (causing an increase in its calculated retention time) and seems to cause a reduction in the expected area of the IgY peak, perhaps due to matrix effects. Consequently, any attempt to use the TSK-G4000SW gel filtration to develop an IgY standard curve for concentration determination in impure samples appeared futile.

However, in relatively pure samples (IgY purities around 50% or higher), the effects discussed above do not appear to be significant. The only anomaly noted is the presence of what was at first assumed to be a dimer peak, present in both pure IgY standard (minimum 98% IgY according to Sigma) and the purified IgY. It is a well recognized fact that IgY has a tendency to form aggregates of 2, 3 or more monomer units[2] If the standard obtained from Sigma is as pure as the company claims, then this higher molecular weight peak must be primarily IgY.

Figure 7.22 shows the chromatograms obtained when samples of the standard (7.22a)

[2]Hassl et al. (1987) used gel filtration analysis to estimate the IgY concentration partially purified samples. Using a 60 x 1.6 cm column operating at room temperature and PBS as the running buffer, k, the extinction coefficient, was measured at 280 nm and the IgY content of samples determined by integration of peak areas. They compared IgY purification using 3 methods: precipitation with propanol as described by Bade and Stegemann (1984); precipitation with polyethylene glycol (PEG 6000) following the method of Polson et al. (1980); and an improved PEG method developed by Polson et al. (1985). In their gel filtration analysis of the IgY content of the purified fractions, significant amounts of lower molecular weight impurities (in the weight range of α-liveltin) were present in purified fractions for the first two methods, while a small amount of higher molecular weight impurity (a shoulder peak) was present in purified fractions using the third method. They suggested that this "shoulder" peak may represent IgY aggregates.

and purified IgY (7.22b) are analyzed. Using the regression equation obtained above to determine the molecular weight of the smaller "shoulder" ahead of the monomer peak, the molecular weight appears to be about 440 kDa for the IgY standard "shoulder" and 500 kDa for the IgY sample "shoulder". Thus it is apparent that the peak formerly thought to be a dimer is actually more likely a trimer of IgY. In order to integrate the area for concentration determination, it was decided to integrate both peaks in all analyses.

A stock solution of chicken IgY standard (Sigma Chemical Co., St. Louis) at concentration of 2.0 mg/ml was prepared in 0.1 M PB, pH 5.4, 0.05% NaN_3 and then passed through a 0.45 μm syringe filter. A series of standards were prepared and applied to the HPLC column at a flowrate of 0.5 ml/min. The known concentration was plotted against the area obtained on the chromatogram for each standard in Figure 7.23a, and the resulting regression equation found to be:

$$C = 0.00083(Area) - 0.03574$$

where C is the IgY concentration in mg/ml and $Area$ is the area of the IgY peak on the chromatogram. A very high regression coefficient ($R^2 = 0.9993$) was obtained. This equation was then used to determine IgY concentration in partially purified samples.

Since the standard method that had been used thus far in the work for IgY concentration determination was RID, a comparison was made of the two methods. A sample of purified IgY with a concentration in the vicinity of 1.2 mg/ml was diluted to obtain 5 "unknowns", which were then applied to both HPLC and RID using the chicken IgY standards prepared earlier. As well, a concentrated sample of partially purified IgY, eluted after the loading of a 15 cm HC-2 column with about 40 bed volumes of supernatant, was diluted 10x and both methods applied. Figure 7.23b demonstrates the

Figure 7.22: HPLC Analysis of Pure IgY Samples. The graphs show the chromatogram obtained for a sample of IgY standard (a) and a sample prepared by purification with Na_2SO_4 precipitation after extraction as described in Chapter 4, and separation as described in Chapter 6 (b).

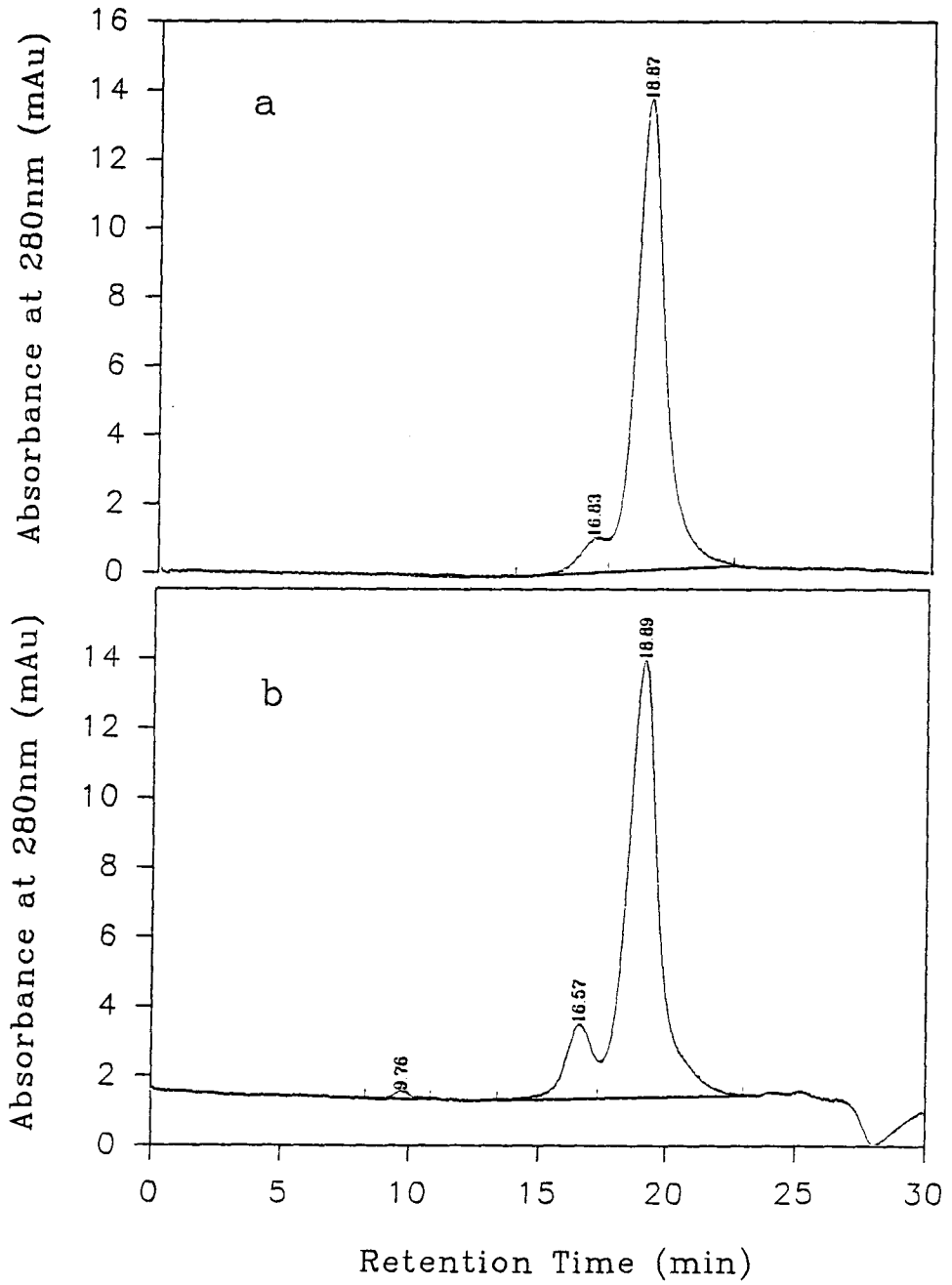
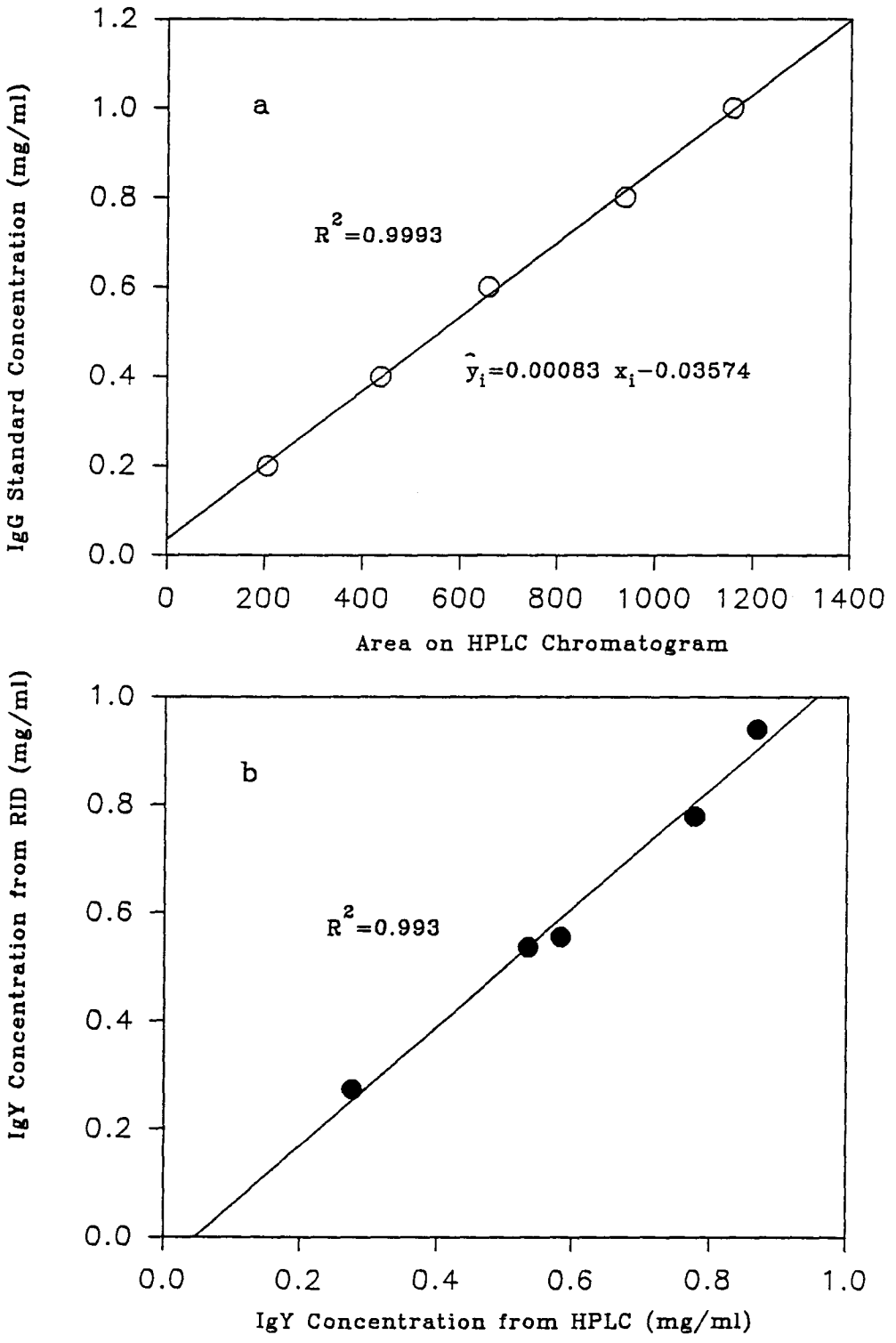


Figure 7.23: HPLC Standard Curve (a) and Comparison to RID (b)



resulting correlation between RID and HPLC results. Note that one of the unknowns was omitted in determining the regression due to the erroneously low RID result, leaving the four unknowns and the 10x diluted, partially purified IgY sample.

It is not clear from Figure 7.23b, but the variance in the results obtained by RID analysis is much greater than that for the HPLC results. Table 7.10 presents the concentrations determined by each method for each of the five "unknowns" tested (excluding the partially purified sample), as well as their dilution factors, calculated concentration of the original solution, and statistical parameters. From this data one can see that the coefficient of variance for RID was 4 times that of the HPLC technique. If one rejects completely the 3 times diluted sample in the RID portion, then the average estimate for the concentration of the original solution becomes identical to that estimated by HPLC, but with a coefficient of variance still more than twice that of the HPLC determined value (*CV* of 6.1% vs 2.7%). Numerous other analyses of IgY samples carried out with HPLC have shown a similar superior reproducibility in the results. Clearly then, for purified samples of IgY the HPLC method developed here is more rapid and reliable than RID.

7.3.3 IgY Purity Determination

A third potential use for HPLC gel filtration is in the determination of IgY purity. The primary method used previously involved RID. For solutions with relatively low antibody concentration, the purity is calculated by first determining the IgY concentration by RID, and then dividing this value by the total protein (TP) concentration of the sample, as determined by the BCA method. Using HPLC, the purity can be estimated by integrating the area of the IgY monomer and trimer peaks and comparing to the integrated area for the entire chromatogram. Although an error in the estimation will occur due to the inherent difference in extinction coefficient at 280 nm from protein to protein, this error

Table 7.10: Comparison of HPLC and RID Results

HPLC Results

HPLC (mg/ml)	Dilution Factor	Concentration (mg/ml)
0.867	1.33	1.15
0.777	1.5	1.17
0.583	2	1.17
0.398	3	1.19
0.277	4	1.11
	$\overline{X}$ =	1.157
	S =	0.031
	$CV(\%)$ =	2.71

RID Results including erroneous 3x dilution value

RID (mg/ml)	Dilution Factor	Concentration (mg/ml)
0.941	1.33	1.25
0.779	1.5	1.17
0.556	2	1.11
0.300	3	0.90
0.274	4	1.10
	$\overline{X}$ =	1.106
	S =	0.130
	$CV(\%)$ =	11.76

RID Results excluding erroneous 3x dilution value

RID (mg/ml)	Dilution Factor	Concentration (mg/ml)
0.941	1.33	1.25
0.779	1.5	1.17
0.556	2	1.11
0.274	4	1.10
	$\overline{X}$ =	1.157
	S =	0.070
	$CV(\%)$ =	6.07

will be greatly reduced as the sample is purified to a greater degree. Even for samples with relatively low IgY purity the method can provide a reliable estimate for purposes of comparison.

A flowrate of 0.25 ml/min was found to provide the best resolution of the peaks. Figure 7.24 shows the resulting chromatograms and Figure 7.25 the electrophoretogram obtained when samples containing IgY of 3 very different purities were applied to the HPLC column. Figure 7.24a is the chromatogram for a sample of egg yolk supernatant with IgY monomer eluting at 37.94 min. The integrated areas of the individual peaks are shown in Table 7.11. Based on the integrated areas, this sample is approximately 19% IgY (combining monomer and trimer peaks). This actually agrees well with the purity of IgY determined by the RID/TP technique, generally 20-22% for yolk supernatant.

A significant error may occur in the integration of the peaks. In the case of samples in the range of purity of 50 - 90 %, the size of peaks relative to baseline shift is generally large enough to keep this error fairly small. However, for very pure samples (especially >95%), it was necessary to set fixed limits for integration.

Figure 7.24b represents a sample of the IgY rich fraction eluted from an HC-2 cation exchange column. The purity as determined by HPLC is roughly 63%, which again compares favourably to the RID/TP estimate of 62%.

Finally, Figure 7.24c shows the chromatogram of a highly purified sample of IgY, following salt precipitation. It is in this case that the HPLC technique is of especial value, since the purity cannot be accurately estimated using RID/TP (even a 2% error in either IgY concentration or total protein can result in meaningless values for purity). The purity of this sample as estimated by HPLC is greater than 98%. Thus, the method developed provides an accurate means of determining IgY purity, especially for very pure samples.

Figure 7.24: Analysis Carried out at a Flowrate of 0.25 ml/min of Samples with Three Different IgY Purities: (a) - filtered yolk supernatant (WSF); (b) - the IgY rich fraction from cation exchange chromatography; (c) - IgY purified by precipitation with sodium sulphate precipitation.

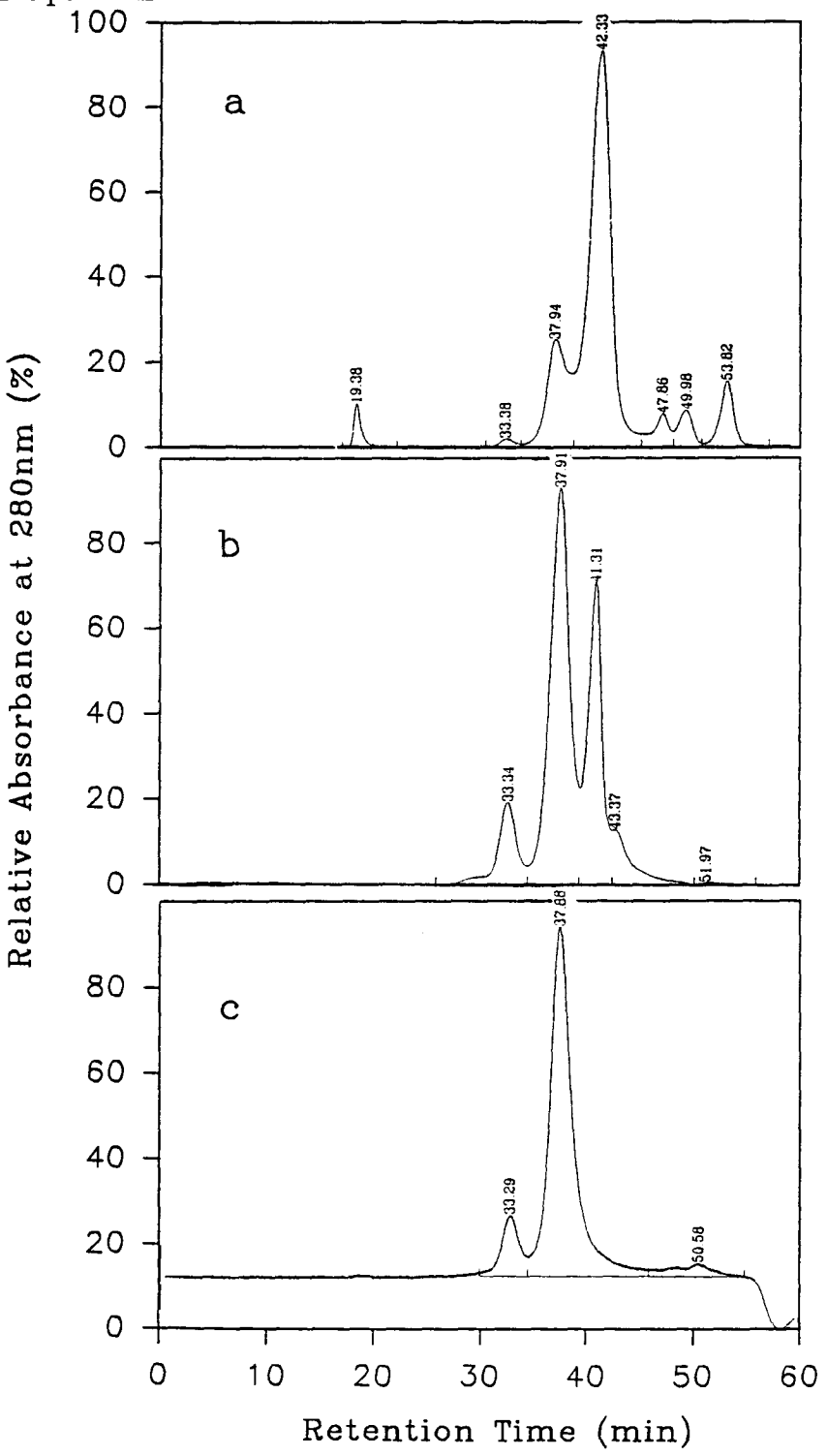


Figure 7.25: Electrophoresis of Samples with Three Different IgY Purities: A - the WSF; B - the IgY rich fraction from CEC; C - IgY purified by precipitation with sodium sulphate.

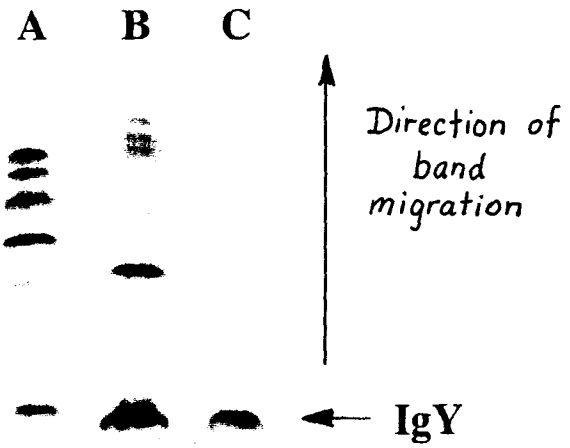


Table 7.11: Summary of HPLC Results for Figure 7.24

Sample: Egg Yolk Supernatant

Peak Number	Retention Time	Area %
1	19.38	2.94
2	33.38	1.12
3	37.94	17.34
4	42.33	63.90
5	47.86	4.03
6	49.98	3.91
7	53.82	6.76

Sample: Eluate from Cation Exchange Step

Peak Number	Retention Time	Area %
1	33.34	10.64
2	37.91	52.17
3	41.31	29.91
4	43.37	6.86
5	51.97	0.43

Sample: Purified IgY after salt precipitation

Peak Number	Retention Time	Area %
1	33.29	11.25
2	37.88	83.45
3	50.58	5.29

7.4 Conclusions

HPLC gel filtration is a convenient method for rapidly determining IgY concentration or purity in partially or very pure samples. Although analysis time for concentration and purity determination was 30 and 60 minutes respectively, this time could be cut in half for pure samples without significant loss of accuracy. This technique could be especially useful where rapid analysis of a small number of samples is required, such as in monitoring a purification process.

Chapter 8

Purification of IgY

8.1 Introduction

Of the techniques used to obtain very pure preparations of IgY, the most successful would appear to be gel filtration, salt precipitation and anion exchange chromatography. Gel filtration, although very effective in producing small quantities of very pure IgY, is at present generally considered impractical for large-scale purification of protein. Since the purification of IgY, which is the third step in the process being presented here, involves a smaller volume of solution of 60-70% purity, the use of a labour intensive batch technique such as salt precipitation was considered an economically feasible possibility for obtaining IgY of high purity for applications that would demand it. Likewise, anion exchange chromatography, having been used in previous publications as a polishing step, was considered a potentially feasible final purification step.

In addition to these two methods, and despite the failure to obtain good separation of IgY at the second stage of the process, ultrafiltration was considered as a potential method for purifying the IgY rich eluate from the cation exchange step. Thus, each of these methods was tested to further purify the eluate.

8.2 Materials and Methods

8.2.1 Anion Exchange Chromatography

A 50 ml column (2.5 cm diameter) was packed with HA-2 (Gibco CEL, New Zealand) anion exchanger. Like HC-2, HA-2 possesses excellent flow properties and is based on the same cellulose matrix. The column was calibrated with 0.2 M PB at pH 8.0 according to Hatta et al., (1990) and washed with 0.01 M PB at the same pH. A sample of 85 ml of the IgY rich peak collected in the first chromatographic step was adjusted to pH 8.0 using NaOH and applied to the column. After washing extensively with the 0.01 M PB (7 bed volumes), the IgY adsorbed was eluted with 0.2 M PB/pH 8.0 using step gradient. After elution, the column was washed with 0.01M PB/0.5 M NaCl solution followed by 0.01 M PB. The superficial velocity was maintained at 36 cm/h (flowrate of 3 ml/min) with the exception of sample application where the superficial velocity was reduced to half.

8.2.2 Salt Precipitation

The IgY rich peak fraction obtained from the cation exchange chromatography run was used for the salting out experiment according to the method described by Hatta et al., (1990). Aliquots were mixed with sodium sulfate at a concentration of 15% (w/v) and 20°C for 30 min. After 15 min centrifugation at 10,000 x g, superanatants were discarded and precipitates dissolved in PB (0.1 M, pH 8.0). This salting-out procedure was repeated twice. Two different starting pH's were investigated: pH 5.0 (no adjustment) and pH 8.0 (adjustment with NaOH).

8.2.3 Ultrafiltration

The same basic procedure as described in Chapter 5 was used, except that the pH of the sample eluate was not adjusted to 9.0.

8.2.4 Methods of Analysis

RID and SDS-PAGE were as described in Chapter 4. HPLC analysis was carried out according to the method developed in Chapter 7.

8.3 Results and Discussion

8.3.1 Anion Exchange Chromatography

The 60% pure IgY solution from the first chromatographic step was applied to a 50 ml column packed with HA-2 anion exchanger. Only a slight improvement in purity from 60% to 66% was achieved and SDS-PAGE revealed no major difference between the sample obtained after HC-2 chromatography and the one obtained after the additional step of HA-2 chromatography. Hatta et al., (1990) used DEAE-Sephacel chromatography after lipoprotein separation resulting in an increase in purity from 19% to 46%, while the overall recovery dropped from 86% to 76%. It seems that anion exchange chromatography would be more useful in the initial stages of purification. However, DEAE-Sephacel was also investigated as a final purification step by Akita and Nakai (1992) where the purity was improved from 93% to 99%.

HA-2 anion exchanger is much less expensive than DEAE-Sephacel and has superior flow properties. It is therefore generally more practical for large-scale purification processes. With the minor improvement in purity obtained here, however, it does not appear to be worth considering as part of the process being developed.

Table 8.12: Purities obtained in Salt Precipitation and Determined by HPLC Analysis

Salt precipitation	Purity†	Purity‡
step 1	94%	96%
step 2	98%	98%
step 3	99%	99%

† starting pH = 5.0
‡ starting pH = 8.0

8.3.2 Salt Precipitation

Salt precipitation was considered in an attempt to produce high purity IgY which might be needed for specific uses. Samples purified after one, two, and three steps of salting out were analyzed using RID, HPLC and electrophoresis. All the methods agreed on the high purity of the samples as shown in Table 8.12. Results obtained by HPLC were more consistent but slightly lower than RID data. Salt precipitation improved the IgY purity significantly from 60% to 94% and 96% at a starting pH of 5 and 8, respectively. A recovery of 99% is possible if care is taken in decanting the supernatant. Additional salt precipitation steps increased the purity to approximately 99%. A sample obtained from a batch separation (to be discussed in Chapter 10) was also used for the salting out experiment, and the purity after one step was 95%.

With heavier loading of the cation exchange column in the second stage of the process, IgY can be concentrated in the eluate. This in turn results in lower volumes to be processed by salt precipitation, which makes this technique appear quite feasible.

8.3.3 Ultrafiltration

HPLC analysis of ultrafiltered eluate showed no significant improvement in purity was obtained under the conditions used. A slight reduction in intensity of the two small peaks (see Figure 7.24) representing smaller molecular weight proteins occurred, but the ratio of IgY to α-livetin remained essentially unchanged. Thus under the conditions tested, α-livetin displays no tendency to pass through the membrane of the filter.

Further work would be necessary to determine whether or not ultrafiltration of the elutate from cation exchange chromatography could result in increased IgY purity. It may be that the lipid concentration in the eluate is still sufficiently high enough to cause fouling of the membrane. Also, some type of complex formation between α-livetin and some other protein, or perhaps even aggregation of α-livetin itself, may be preventing it from passing through the membrane.

8.4 Conclusions

Ultrafiltration and anion exchange chromatography can be used to improve the purity of the eluate obtained after the second step of the process only marginally. Salt precipitation using sodium sulphate, however, can improve IgY purity to around 95% after only one step, and is therefore a more appropriate technique to use as the third step in the purification protocol.

Chapter 9

Breakthrough Curve Analysis

9.1 Introduction

Much information useful for predicting large scale operation of a chromatographic column can be obtained by running small scale experiments (Gosling, 1987). Various mathematical models have been developed to take advantage of small-scale experimental data for predicting large-scale operation, but these models are often verified with ideal protein or sugar solutions involving molecular species with well defined iso-electric points and molecular weights. A somewhat less elegant, but nevertheless practical approach is to carry out small-scale experiments under conditions similar to those expected for the large-scale operation, and to vary key parameters in order to study their influence on the separation. In chromatographic processes in general, a doubling of column length results in a doubling of the pressure drop across the column but an improvement in the resolution between peaks by a factor of only $\sqrt{2}$. Increasing column diameter, on the other hand, generally results in a broadening of the peaks[1]. Thus the scale up of even a simple binary separation on a chromatography column can be quite complicated to predict, and as a result, in practical applications it is often easier to maintain a set bed height, increase column diameter to allow for increased capacity, and then connect several other "scaled up" columns in series in order to counteract peak broadening effects.

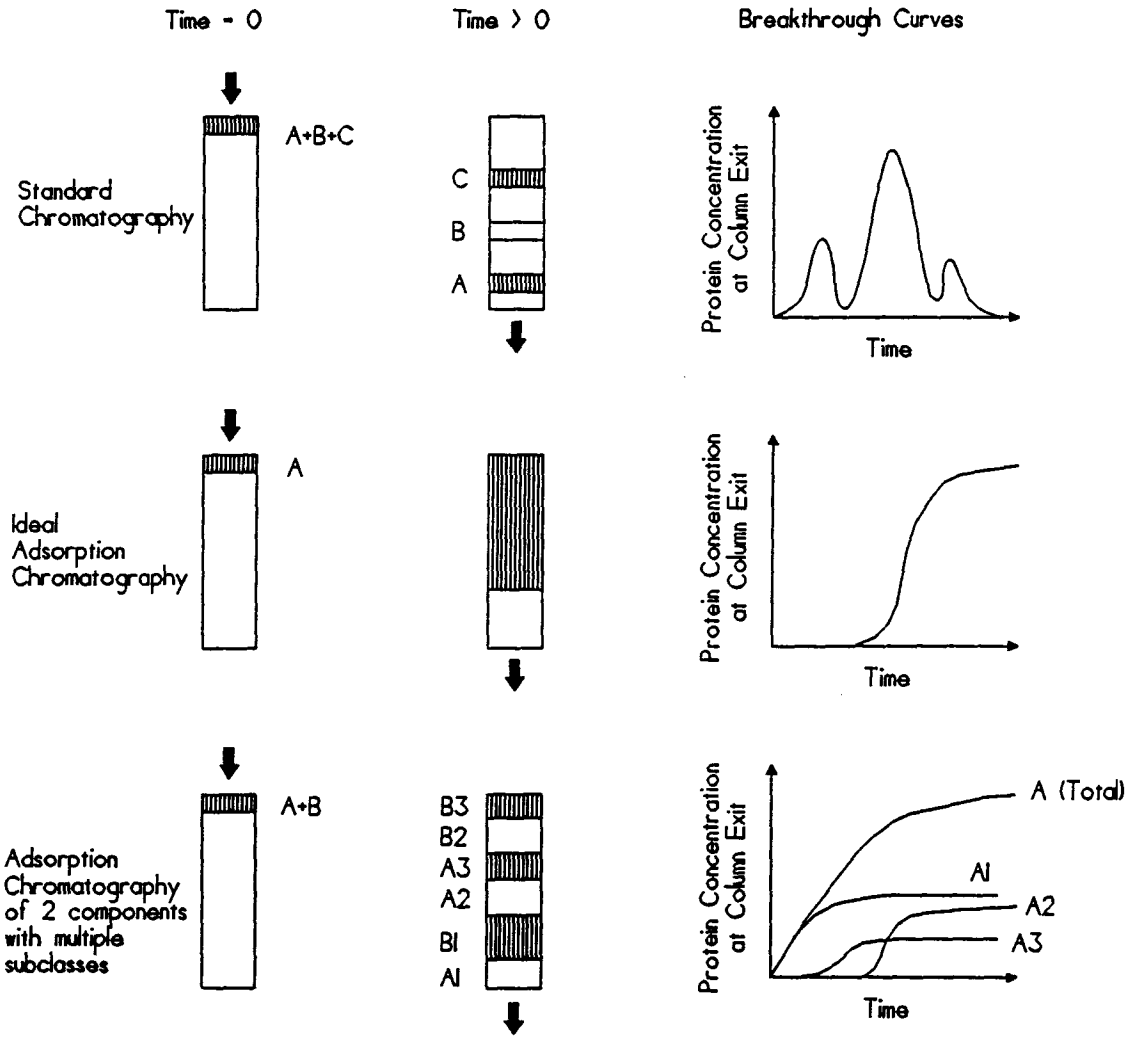
[1] This can be a complex function of a number of parameters involving flowrate, particle geometry, the presence of "short circuiting" channels in the column and other factors.

Adsorption chromatography involves a very different type of separation process. In adsorption chromatography, the desired molecule is usually preferentially bound to the column, while undesireable contaminants have little or no affinity for the column and pass through with the mobile phase. Whereas in standard chromatography only a small section of the upper portion of the column is used to bind the sample, in adsorption chromatography the majority of the column capacity can be used. Once the column is saturated, a step change in ionic strength or pH is used to elute the adsorbed species.

Although there are many published reports involving mathematical modelling of the adsorption of one species of protein molecule to different adsorbents, few have reported the more realistic situation of multicomponent adsorption involving more than one protein (Skidmore and Chase, 1990). In the case of the binding of IgY from the WSF to a cation exchanger (HC-2), the heterogeneity in the pI of the antibody, as well as the overlapping pI range of α-livetin results in a much more complex binding pattern than would be predicted by even the more complicated models involving adsorption from binary protein mixtures. Figure 9.26 illustrates graphically the expected binding patterns for IgY and α-livetin compared to standard chromatography with homogeneous molecular species. It is proposed that the strongly binding subclasses of each protein bind near the top of the column and gradually displace the weakly binding subclasses towards the bottom. Since the pI's of the two proteins overlap, it is likely that their subclasses are intermingled on the column during adsorption.

In adsorption chromatography, the simplest approach to modelling for purposes of scale-up is the analysis of breakthrough curves for the species to be purified. The shape of the breakthrough curve (Vermeulen and Hiester, 1959) provides information about the strength with which a protein binds to the exchanger (ie.: equilibrium and rate of binding), as well as the capacity the exchanger has to bind the protein of interest (ie.:

Figure 9.26: Schematic Representation of Protein Binding to Adsorption Column



the stoichiometry). The break through curve for a pilot of large-scale column is needed to determine the point at which it is no longer economical to continue the binding.

9.2 Materials and Methods

9.2.1 Cation Exchange Chromatography

In order to obtain breakthrough data for binding at different flowrates, 5 cc syringes (1.2 cm i.d.) were used as miniature chromatography columns. Figure 9.27 shows a photograph of one of these small columns packed with 3 ml of HC-2 exchanger. Glass wool was packed into the bottom of the syringe, followed by the equilibrated exchanger and excess equilibration buffer. The plunger with its rubber seal was used as the top cap for the column and a syringe needle was forced through the rubber seal to allow entry of buffer into the top of the column. Silicone tubing was used to carry buffer to the column, and was inserted into the top of the plastic funnel portion of the syringe needle. Solution exited the column under gravity and was collected by a fraction collector (not shown). Sample was applied at a controlled flowrate by an Ismatec peristaltic pump.

The same buffers as described previously were used for equilibration, washing, elution and saltwash. Sample was yolk supernatant at pH 5.5 prepared according to the protocol developed in Chapter 4.

Experiments were also carried out using a 1 cm i.d. column packed to a height of 15 cm (about 12 ml bed volume of exchanger). Figure 9.28 shows the experimental apparatus including the chromatography column, fraction collector, peristaltic pump and sample flask. Again, buffers were as described for the 3 ml column experiments.

All experiments were carried out at room temperature using WSF prepared according to the procedure described at the end of Chapter 4.

Figure 9.27: A Small Column Made from a Disposable 5 cc Syringe

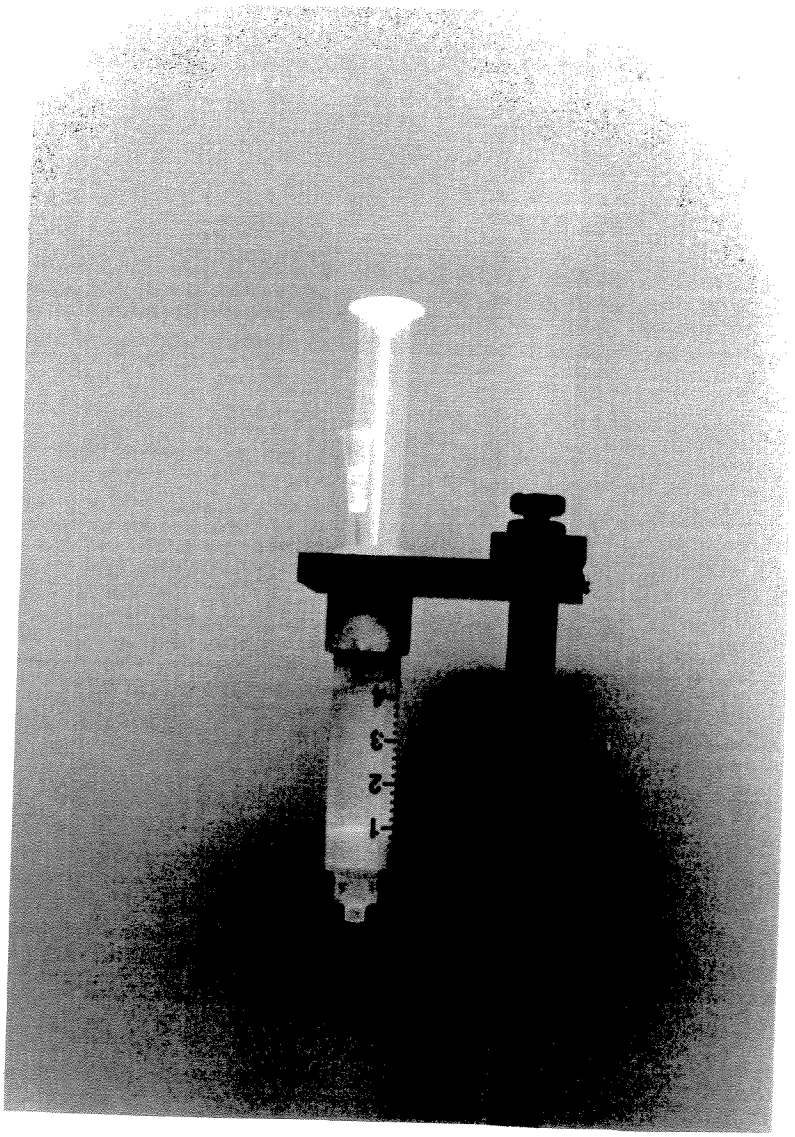
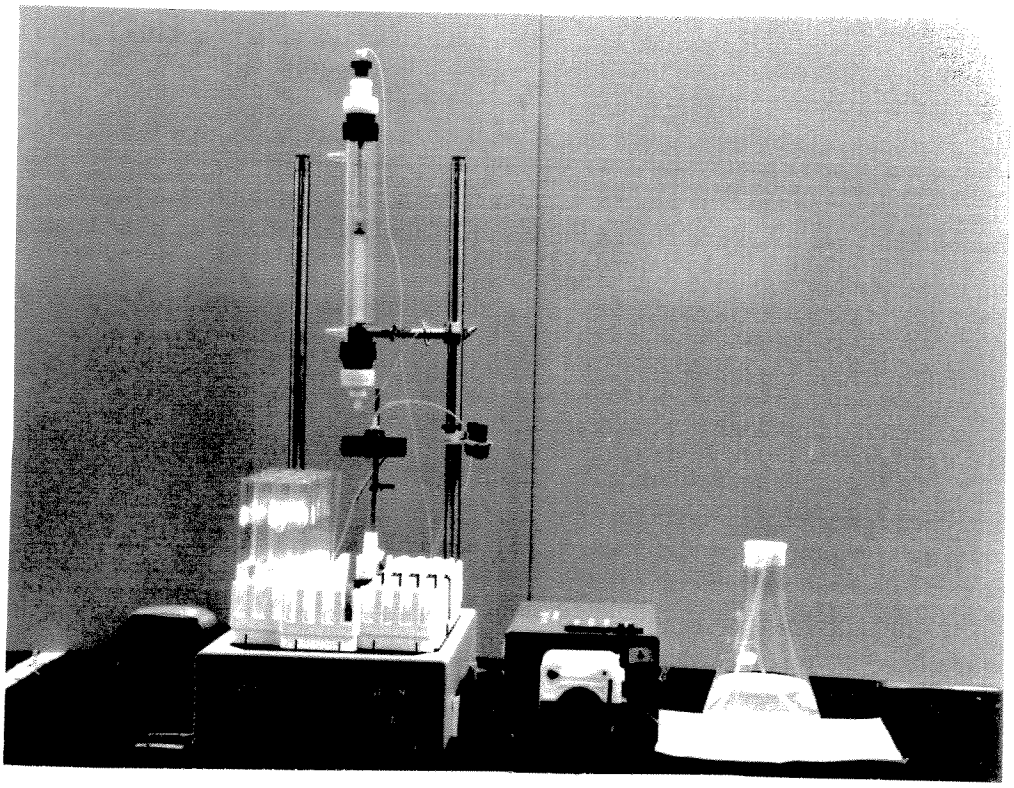


Figure 9.28: Apparatus used for Breakthrough Experiments



9.2.2 Methods of Analysis

RID as described in Chapter 4 was used to determine the IgY concentration of selected fractions.

9.3 Results and Discussion

At first the absorbance at 280 nm was monitored at the column exit in order to determine the breakthrough of IgY. However, this proved impractical because IgY is only one of many proteins in the applied sample, and no distinct increase in absorbance occurred. It was determined that only an assay specific for IgY, such as RID, would allow the monitoring of IgY breakthrough.

Figure 9.29 shows a typical breakthrough curve for IgY using one of the 3 ml columns of HC-2. The data points shown represent individual fractions collected from 2 identical experiments in which the flowrate was 1.0 ml/min, and the corresponding superficial velocity 53.1 cm/h.

Unlike the sigmoidal breakthrough curve that would be expected for an adsorbing species with a distinct isoelectric point, the curves obtained here show an early breakthrough of IgY. An attempt was made to fit the model of Arnold et al. (1985), which is a simple but practical model developed for affinity chromatography based on principles of specific adsorption, but this was quite unsuccessful. Another simple yet very practical model for adsorption chromatography developed by Skidmore and Chase (1988) was also considered, but rejected, since it also assumes that the desired species has a well-defined isoelectric point. An attempt to develop a more complex mathematical model capable of dealing with potential interactions between IgY and other proteins present, and taking into account the heterogeneity in the isoelectric point (pI) of the molecule would have

been beyond the resources of the author. Therefore, an empirical model that would closely approximate the breakthrough curve, and allow comparison of results from various experiments seemed preferable. Since the general shape of the breakthrough curve appeared similar to that of the Langmuir adsorption isotherm (Langmuir, 1918), this model was tested and found to provide an adequate fit. Thus the fitted curve is of the form:

$$Y = \frac{aX}{b+X}$$

where Y is the dimensionless IgY concentration, X is the number of equivalent bed volumes of WSF applied to the column, and a and b are empirical parameters chosen to best fit the curve to the data. Since the shape of the breakthrough curve is also a function of the flowrate, the parameters cannot be equated directly to those of an adsorption isotherm, which represents equilibrium binding conditions. However, the fitted curve is useful in comparing the results from experiments involving different flowrates (and therefore different superficial velocities).

Figure 9.30 shows the fitted curves for experiments carried out at 2.0 ml/min (Curve A) and 0.175 ml/min (Curve B) using the 3 ml column. It is clear that at the lower flowrate, more of the IgY is binding[2].

Experiments at 5 different flowrates (0.175, 0.225, 0.345, 1.0 and 2.0 ml/min) were carried out using the 3 ml HC-2 columns, and the resulting data points are shown in Figure 9.31, essentially bounded by the two curves of Figure 9.30 which represent the two extremes in flowrate used.

Since the binding of IgY is greatly influenced by both the amount of sample applied and the flowrate of sample application, a plot of the amount of the protein bound as

[2]The area above the curves is directly proportional to the quantity of IgY bound.

Figure 9.29: Break Through Analysis using 3 ml Column at 1.0 ml/min

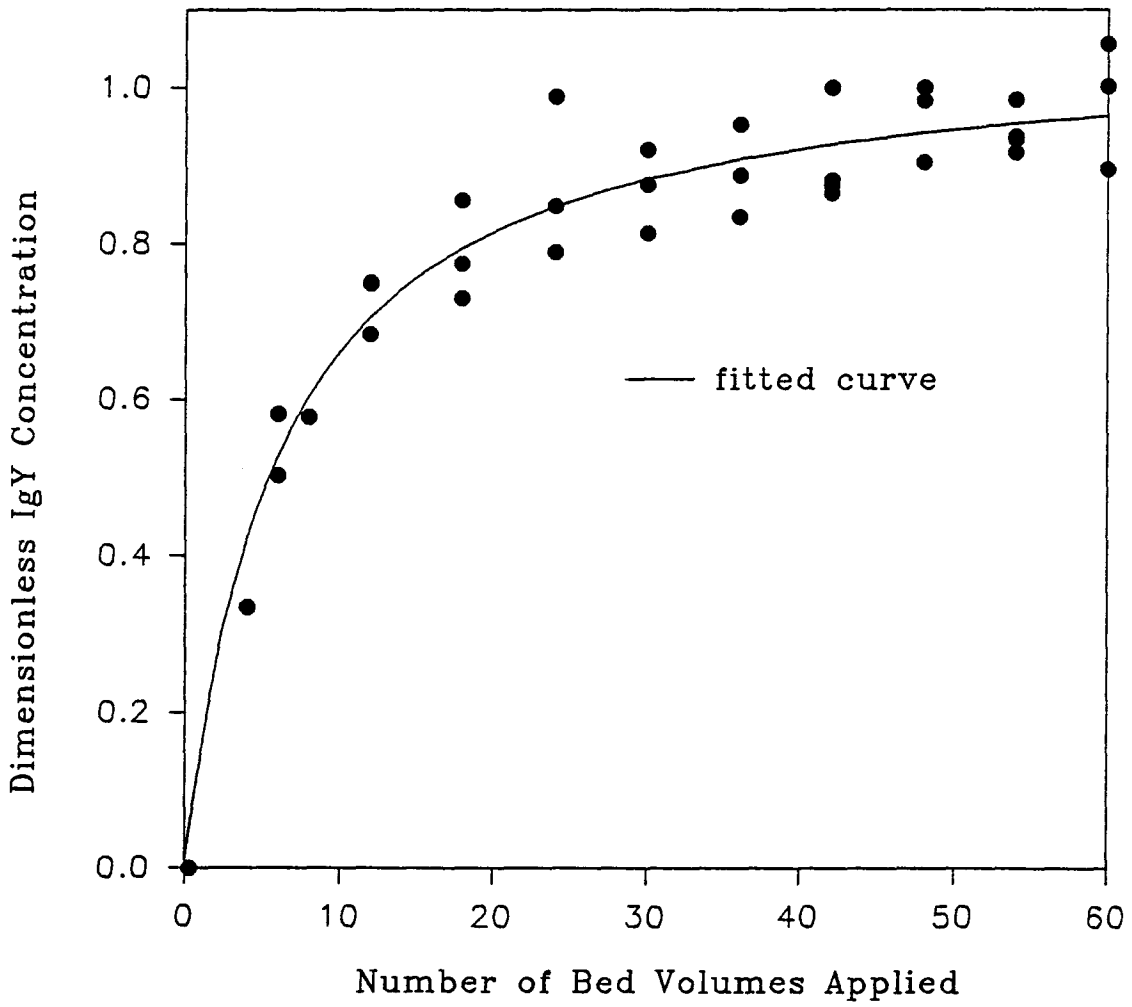


Figure 9.30: Comparison of Breakthrough Curves at Two Different Flowrates

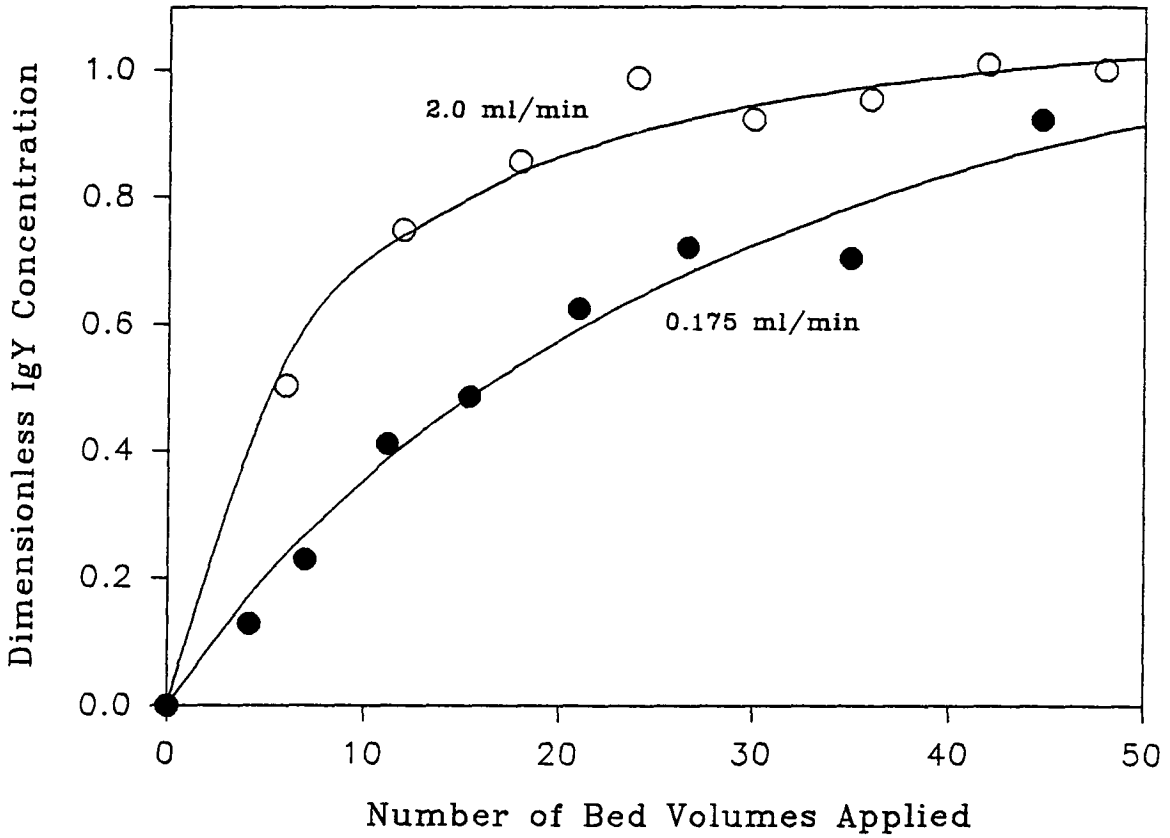
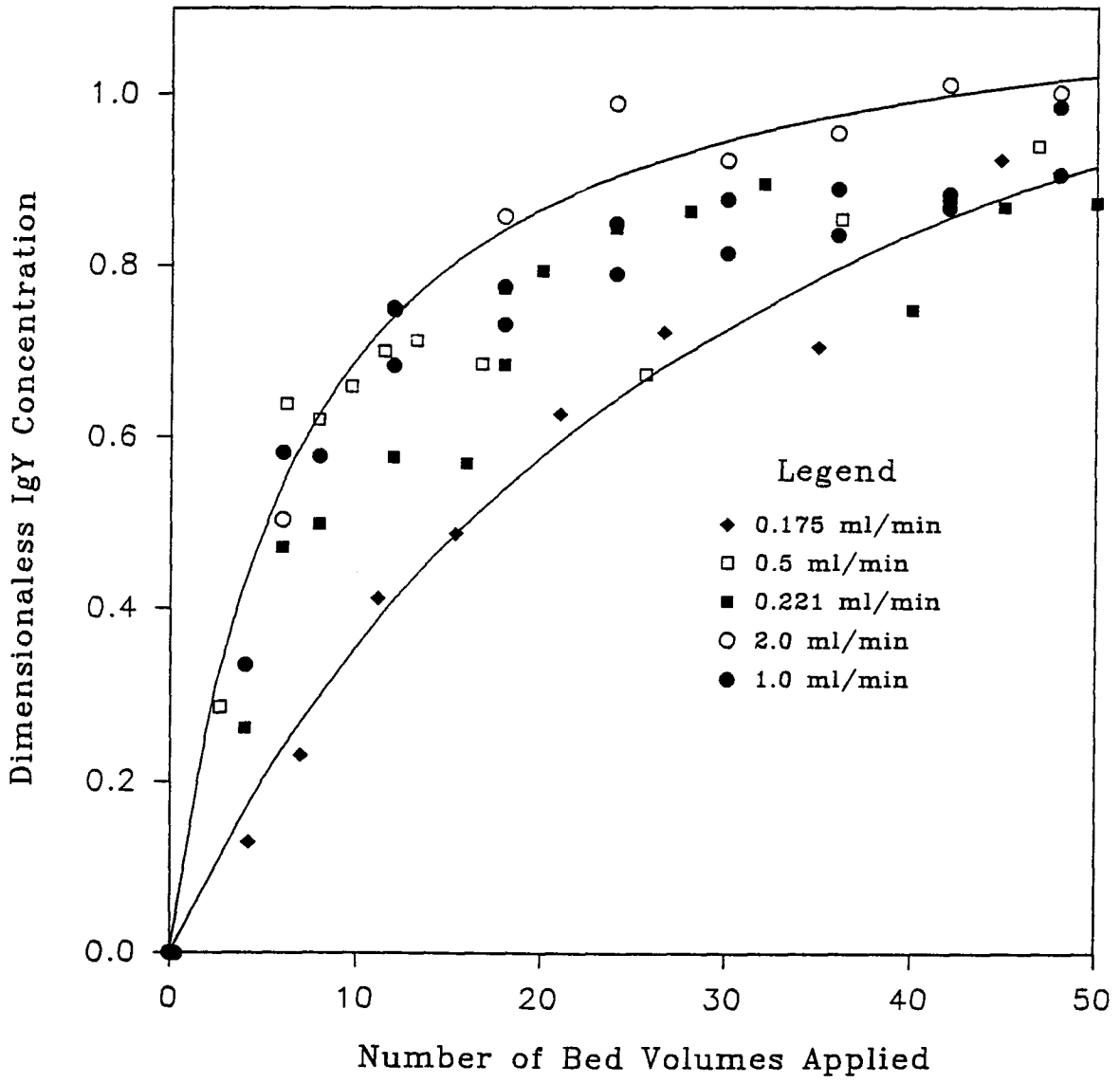


Figure 9.31: Plotted Data from Breakthrough Experiments at Five Different Flowrates (the solid lines represent curves fitted to data for the highest and lowest flowrates).

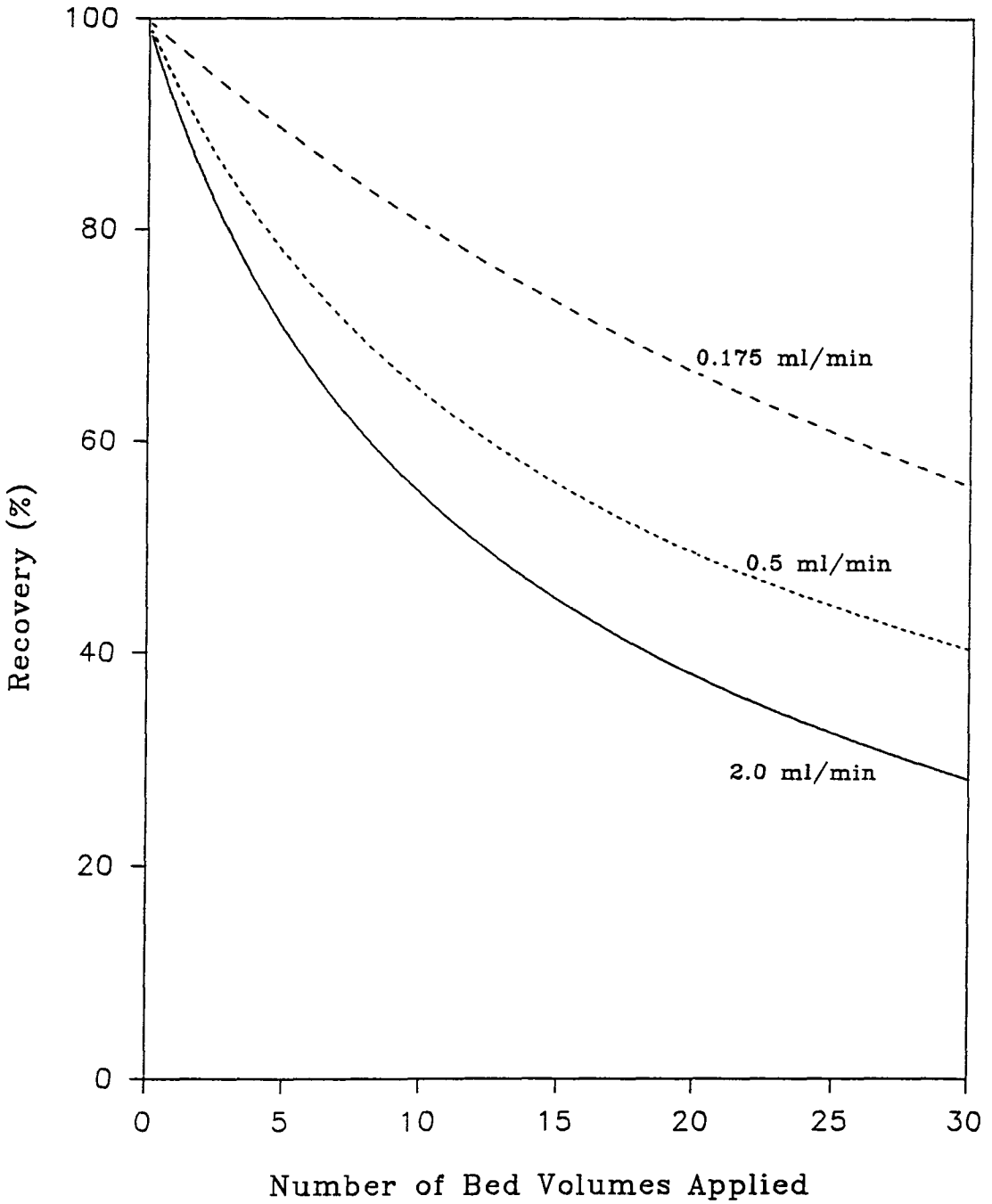


a function of bed volumes applied at various flowrates would provide a practical tool for estimating recovery. If curves are fitted at all flowrates, and the areas above each one integrated in order to estimate the amount of IgY bound, then the recovery as a function of the number of bed volumes applied can be estimated by dividing the total amount bound by the total amount applied. The resulting curves (fitted using the model described above) are shown for 3 flowrates in Figure 9.32. In all three cases, the recovery drops off rapidly with increased loading. However, it is clear that as the loading rate in decreased, a significant improvement in recovery occurs. This work was all done using the small columns, so the next step was to determine how to use this information to predict large-scale results. This is the central topic of the following chapter.

9.4 Conclusions

Due to the heterogeneity of the IgY molecule, it is not possible to obtain complete binding, even after the application of only one bed volume of sample. For this reason, it has been suggested that ion-exchange chromatography is unlikely to prove feasible for IgY separation (Higgins, 1976). However, the breakthrough analysis studied here shows that under the right conditions of pH and flowrate, it is possible to bind a significant quantity of the molecule. Since not all subclasses may be desired, the fact that a sub-fraction of the IgY does not bind in cation exchange chromatography may actually be desireable. The simple empirical model used to fit the experimental curves is useful in estimating the recovery at various sample loadings and flowrates.

Figure 9.32: Recovery as a Function of Loading for 3 Different Flowrates



Chapter 10

Scale-up of the Separation Process

10.1 Introduction

This chapter deals with efforts to use the results from the small-scale experiments carried out in Chapter 9 to predict the performance of a pilot-scale column. The bed volume of the column was increased by a factor of 500 (from the 3 ml columns to a pilot-scale 1.5 litre column, 100 cm^2 cross-sectional area and 15 cm bed height). The pilot-scale work was carried out in both batch and column mode in order to compare these two techniques.

As well, a test of the pressure drop across the pilot-scale column as a function of the superficial velocity of the mobile phase was done to determine whether or not there would be any danger of bed compaction at high flowrates.

10.2 Materials and Methods

10.2.1 Flow Testing a Column of HC-2

A pressure gauge was connected just before the inlet to the column in order to measure the pressure drop at various flowrates. The column was equilibrated with 0.2 M PB pH 5.0, and the same buffer pumped through the column at various flowrates using a peristaltic pump. The flowrate was varied stepwise from 30 ml/min to 500 ml/min. Once the pressure drop across the column had stabilized, a reading was taken and the flowrate

then increased to the next level.

10.2.2 Batch Separation Using HC-2

Approximately 1.0 kg of HC-2 cation exchanger, previously used in the 1.5 litre column experiments, was poured into a 4 litre flask and allowed to equilibrate with 0.2 M phosphate buffer (pH 5.0). In order to ensure proper mixing and contact of the buffer with the exchanger particles, an 8 cm long magnetic stirring bar was added, and the flask placed on a Fisher Model 11-500-73H stirring/hot plate, with the stir control set on maximum. It was necessary to stir manually at first in order to fluidize the exchanger particles. After equilibration, the buffer was decanted, and the remaining buffer removed by filtration.

To accomplish this, the flask contents were poured into an empty 1.5 litre column and air was pumped through with a pressure drop across the column of approximately 17 kPa gauge until the excess aqueous buffer was removed. This was considered to have occurred when the flowrate leaving the column had dropped to less than 1 ml/min.

Washing buffer, 0.01 M phosphate buffer (pH 5.4), was then used to wash the exchanger back into the 4 litre flask. Mixing, decanting, and replacement of spent buffer with fresh continued until the ionic strength of the aqueous phase, after significant mixing, approached that of fresh buffer. The procedure outlined above for removal of buffer then repeated, and the exchanger was then ready for sample loading.

The equilibrated and washed exchanger was first mixed with 1.5 litres of diluted yolk supernatant resulting in a total volume of about 2200 ml. Following 50 minutes of agitation with the stir bar, the exchanger was allowed to settle for 10 minutes. A sample of the clear supernatant above the exchanger was taken, and the contents of the flask then poured into the 1.5 litre column for aqueous phase removal. Since a significant amount of the exchanger adhered to the sides of the flask, 200 ml of washing buffer was

used to help wash it into the column. A total of 1.6 litres of liquid was recovered from the exchanger.

The exchanger was then washed back into the 4 litre flask with 1.5 litres of 0.2 M phosphate beffer (pH 5.0, hereafter called elution buffer). After another 50 minutes of mixing and 10 minutes of settling, a sample of the supernatant was again taken, representing the eluted fraction. Buffer removal resulted in the collection of a total of 1.8 litres of eluted fraction. Following buffer removal, a saltwash (0.01 M phosphate buffer/0.5 M NaCl/pH 4.8) was used to remove any remaining protein and prepare the exchanger for equilibration. This completed one cycle of the batch process.

10.2.3 Column Separation Using HC-2

The automated chromatography system developed in Chapter 3 was used to control the separation of IgY on a 1.5 litre column (i.d. approximately 11.3 cm, bed height 15 cm) packed with HC-2 cation exchanger. A photograph of the system including the pilot scale column is shown in Figure 10.33. The breakthrough experiment was carried out under the same set of conditions used in Chapter 9. Samples were automatically collected at the column exit after every bed volume applied, and the IgY concentration for selected fractions determined by RID.

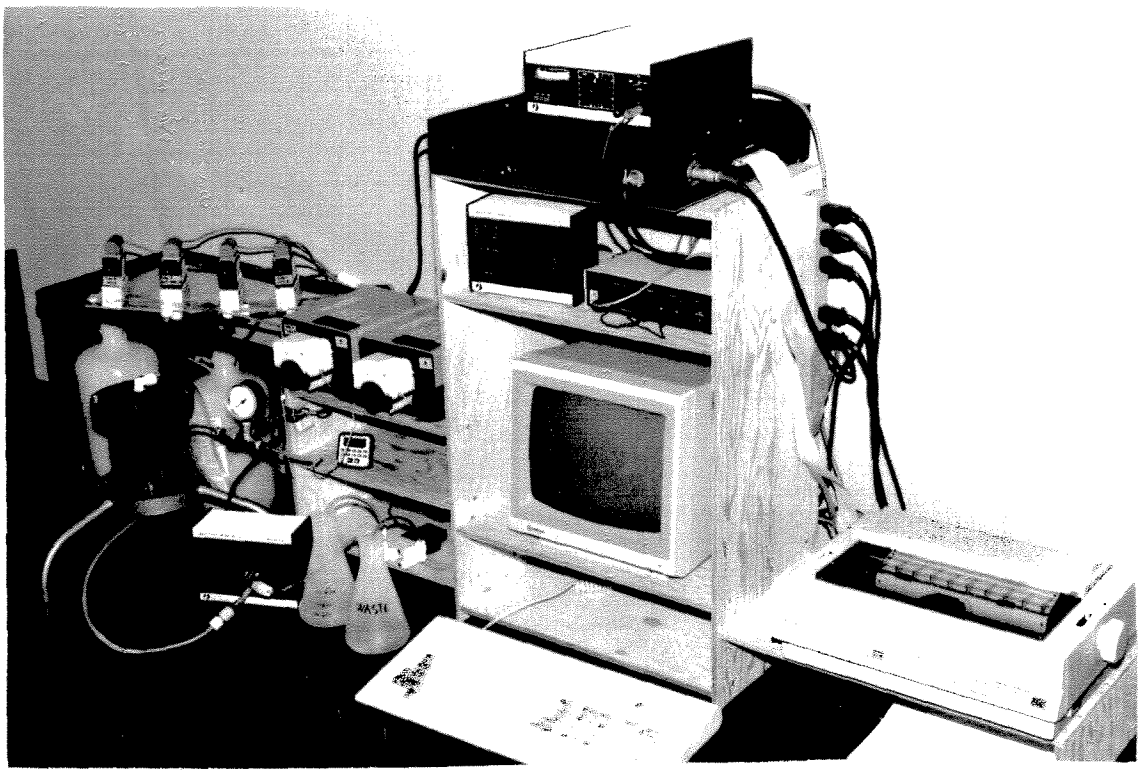
10.3 Results and Discussion

10.3.1 Flow Test Results

Many cellulose-based ion-exchangers are suitable only for laboratory work involving relatively low flowrates[1]. In order to determine the limiting flowrate for sample application

[1]For example, attempts to vary the flowrate in initial experiments carried out using DEAE-Sephacel resulted in some cases in column adapters being blown to their extreme positions by the build-up in

Figure 10.33: Automated Chromatography System Including Pilot Scale Column



and elution with HC-2, an experiment was carried out with a pilot-scale column and the pressure drop versus the superficial velocity plotted. Figure 10.34 shows the results of the flow test using a 1.5 litre column packed with HC-2. In this experiment the column was subjected to superficial velocities far beyond those generally recommended (ie: under 50 cm/h). At a pH of 5.0, the exchanger was able to withstand a superficial velocity of over 300 cm/h, at a pressure drop across the column of about 20 kPa, without irreversible compaction. This demonstrates that the exchanger has excellent flow properties.

10.3.2 Batch Separation

A batch experiment indicated that recovery and purity, 53 and 57% respectively, were lower than for the column experiments. To verify these values, the experiment was repeated and similar results were obtained. The lower recovery may be due to loss of IgY when removing the mobile phase, since a portion may be loosely bound to the exterior of the cellulose exchanger particles. In any case, the performance of the batch separation did not appear interesting enough to warrant further study.

10.3.3 Column Separation

The 1.5 litre column was equilibrated and washed with the usual buffers and then 31 equivalent bed volumes of yolk supernatant (approximately 46.7 litres) was applied at a flowrate of 30 ml/min. The dimensionless IgY concentration was calculated by dividing the IgY concentration determined for each sample by that of the yolk supernatant, resulting in values between 0 and 1. The resulting breakthrough curve is illustrated in Figure 10.35.

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pressure caused by compaction of the bed.

Figure 10.34: Pressure Drop vs. Superficial Velocity for a Pilot Scale HC-2 Column

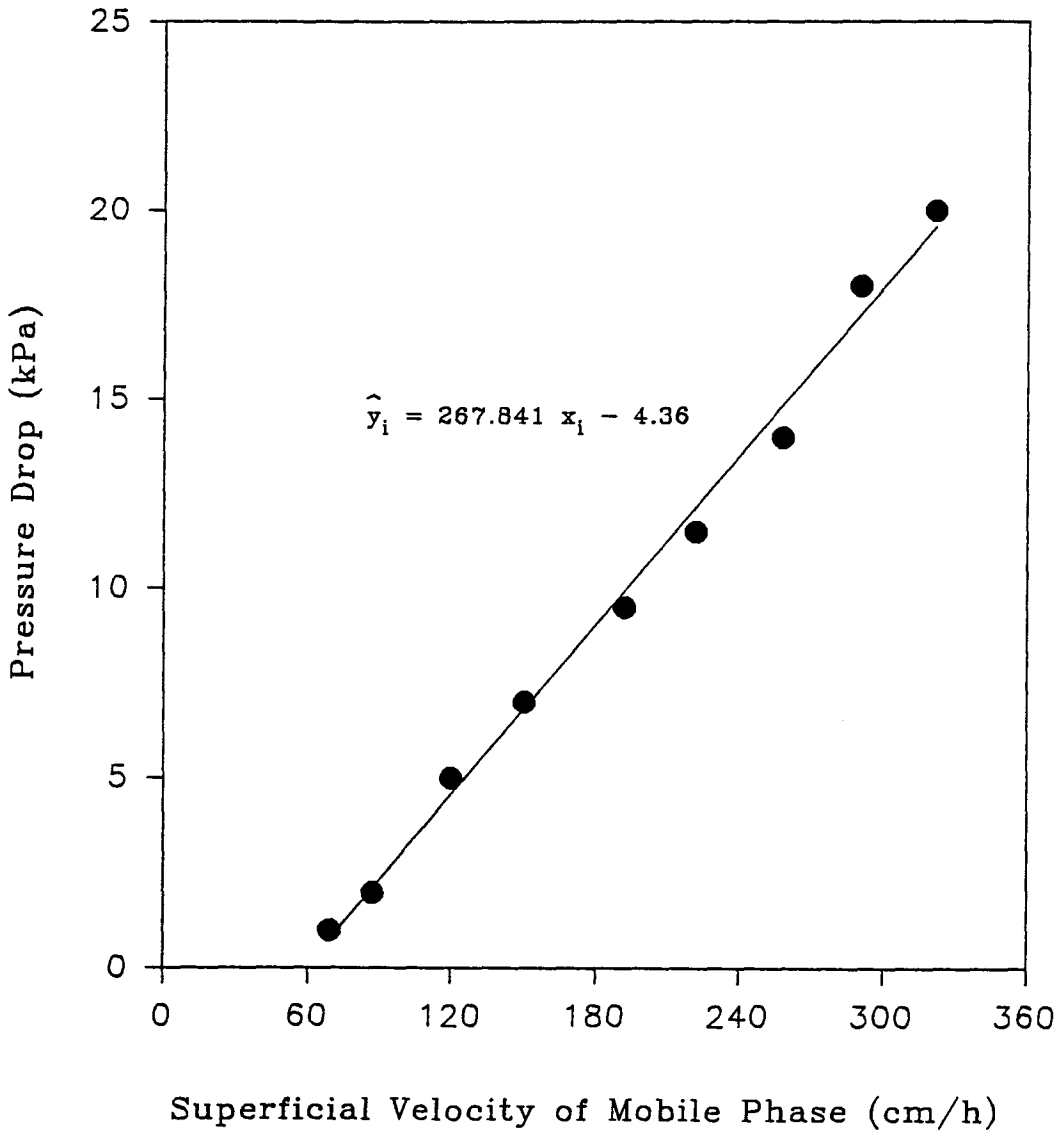
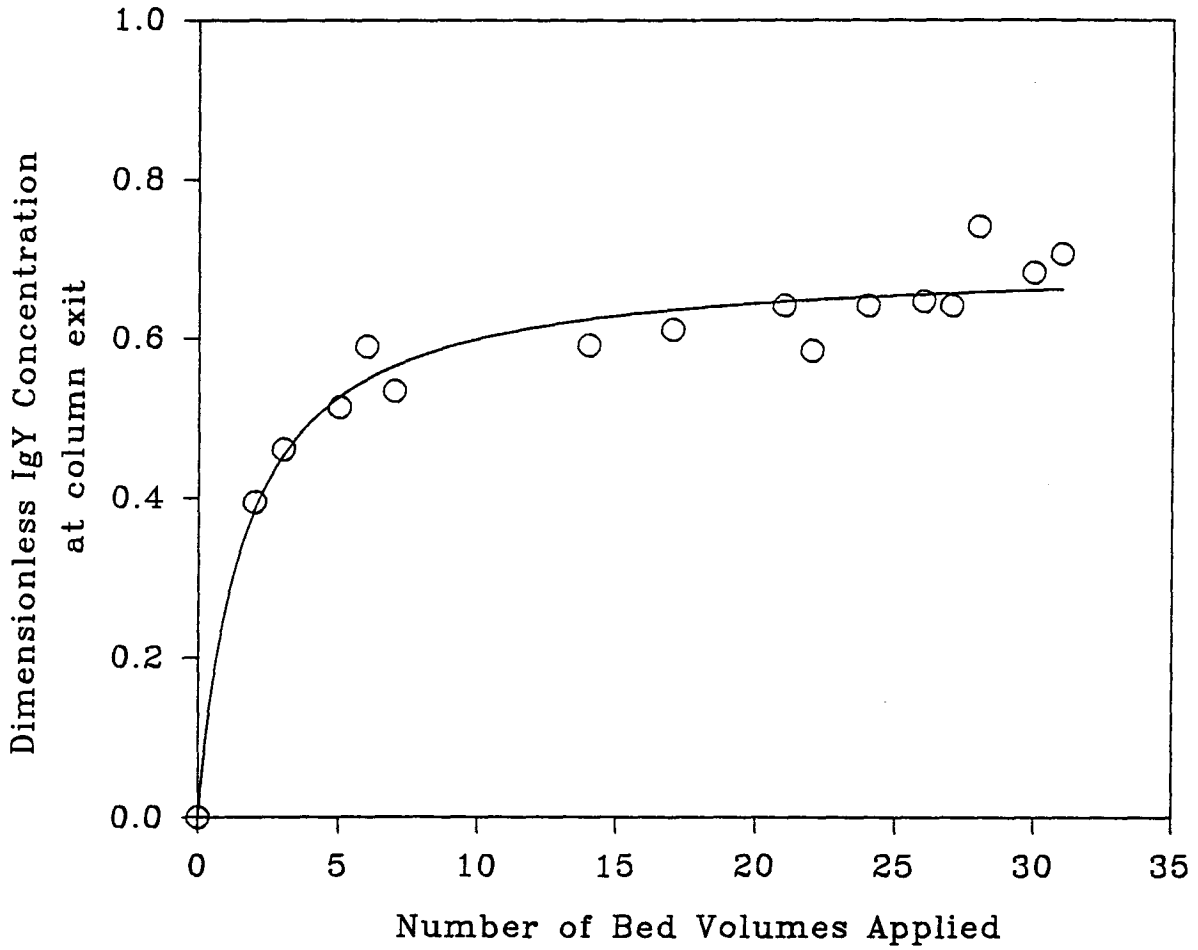


Figure 10.35: Breakthrough Curve for the Pilot Scale Column



The values of the two fitted parameters and their standard errors where determined to be:

Parameter	Value	Standard Error
a	0.696	0.017
b	1.603	0.267

Since the shape of the breakthrough curve is a function of the flowrate, the parameters cannot be equated directly to those of an adsorption isotherm which represents equilibrium binding conditions. However the parameter 'a' does indicate that based on data from the first 31 bed volumes applied approximately 30% of the IgY present in the supernatant is binding very strongly to the exchanger. An additional 10% or so binds strongly enough to remain on the column under the conditions and flowrate used in this experiment. Thus 20 to 30 bed volumes can be applied with a recovery of around 40% and purity of 60-63% according to HPLC analysis.

Figure 10.36 shows the fitted curve for the pilot scale run plotted together with the fitted breakthrough curve for the 12 ml column. Although both curves are rather steep during binding of the initial 5 bed volumes, once 25 to 30 bed volumes have been applied a greater percentage of the IgY is binding to the pilot scale column than to the smaller column, despite the fact that the diameter of the pilot scale column is 15 times that of the 12 ml column, and one would expect the velocity profile across the cross-section of the smaller column to be more even than in the large column. The only other difference between the two cases is the superficial velocity, which is 18 cm/h for the pilot scale column and 25 cm/h for the small column. Thus it would appear that the superficial velocity has such a great influence on the binding that it outweighs the effect of increasing

column diameter by a factor of 15.

As was shown in Figure 9.32, the recovery for the 3 ml columns is strongly dependent on the flowrate used to apply sample. If the recovery after the application of a specified number of bed volumes is plotted against the superficial velocity (rather than flowrate, so that columns of various dimensions can be compared), then the result is a plot such as shown in Figure 10.37a, which shows the points obtained by plotting recovery after 30 bed volumes of sample application. An exponential curve has been fitted to the data by first taking the logarithm of the superficial velocity and carrying out a linear regression as shown in Figure 10.37b.

This strong correlation between superficial velocity and extent of binding suggests that diffusion of IgY into the pore spaces of the adsorbent particles may be the limiting factor. A similar attempt to correlate recovery to the residence time of the sample in the column failed to yield a significant regression. For scale-up purposes then, it is possible to obtain a reasonable estimate of the recovery for a large column by using a small laboratory-scale column to prepare a plot of recovery as a function of superficial velocity for a given number of bed volumnes.

Improvements to Binding of IgY Once it became evident that recoveries of over 60% like those obtained for applications of 3 to 5 bed volumes could not be reproduced when binding closer to the capacity of the exchanger, a small scale experiment using the 15 cm column was repeated using supernatant adjusted with 0.1 N HCl to pH 5 instead of the usual pH 5.5. HPLC analysis indicates that a significant increase in binding of IgY occurs, but at the expense of increased impurity. One of the problems with using a lower pH is that α- and β-livetin bind more strongly. As well, ovalbumin has an isoelectric point of about 4.7 and may also be bound more strongly. Using HPLC analysis, the purity

Figure 10.36: Breakthrough Comparison for Pilot Scale and Small Scale Columns

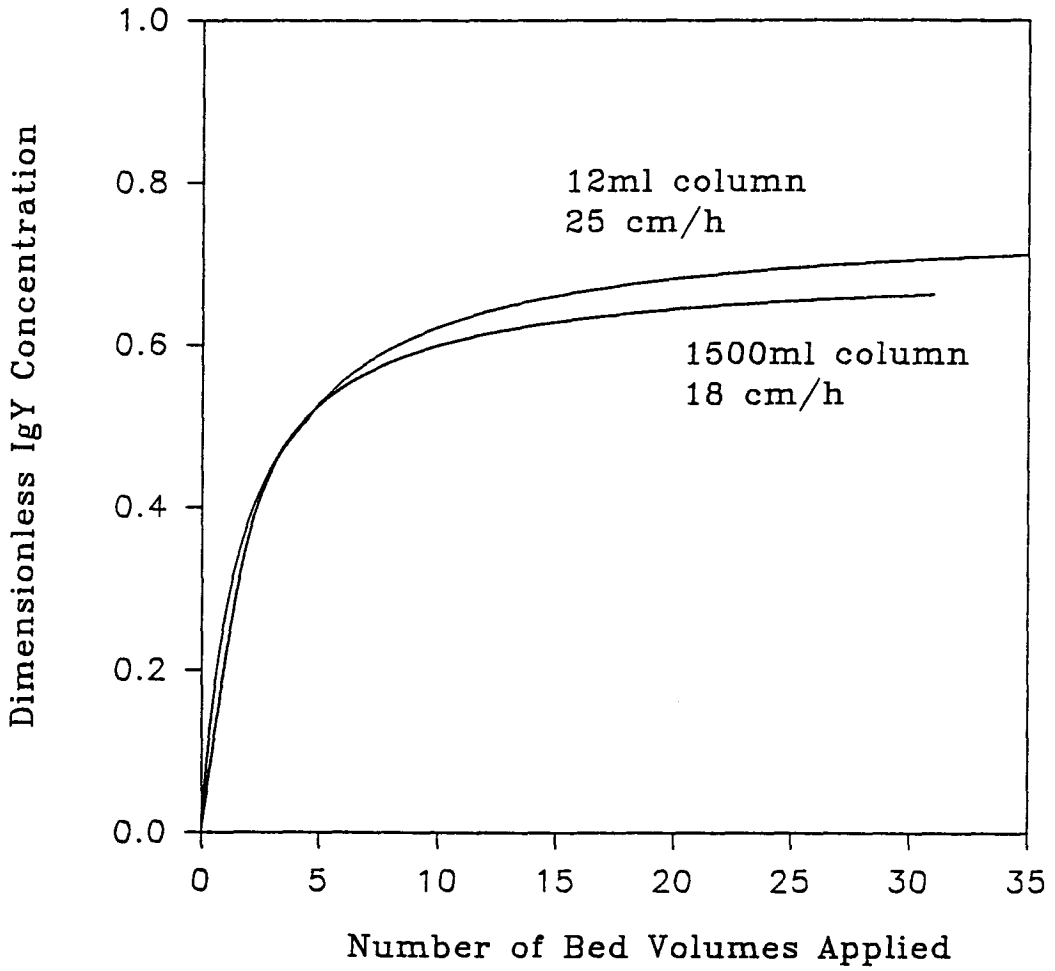


Figure 10.37: Recovery as a Function of Superficial Velocity for Several Experiments

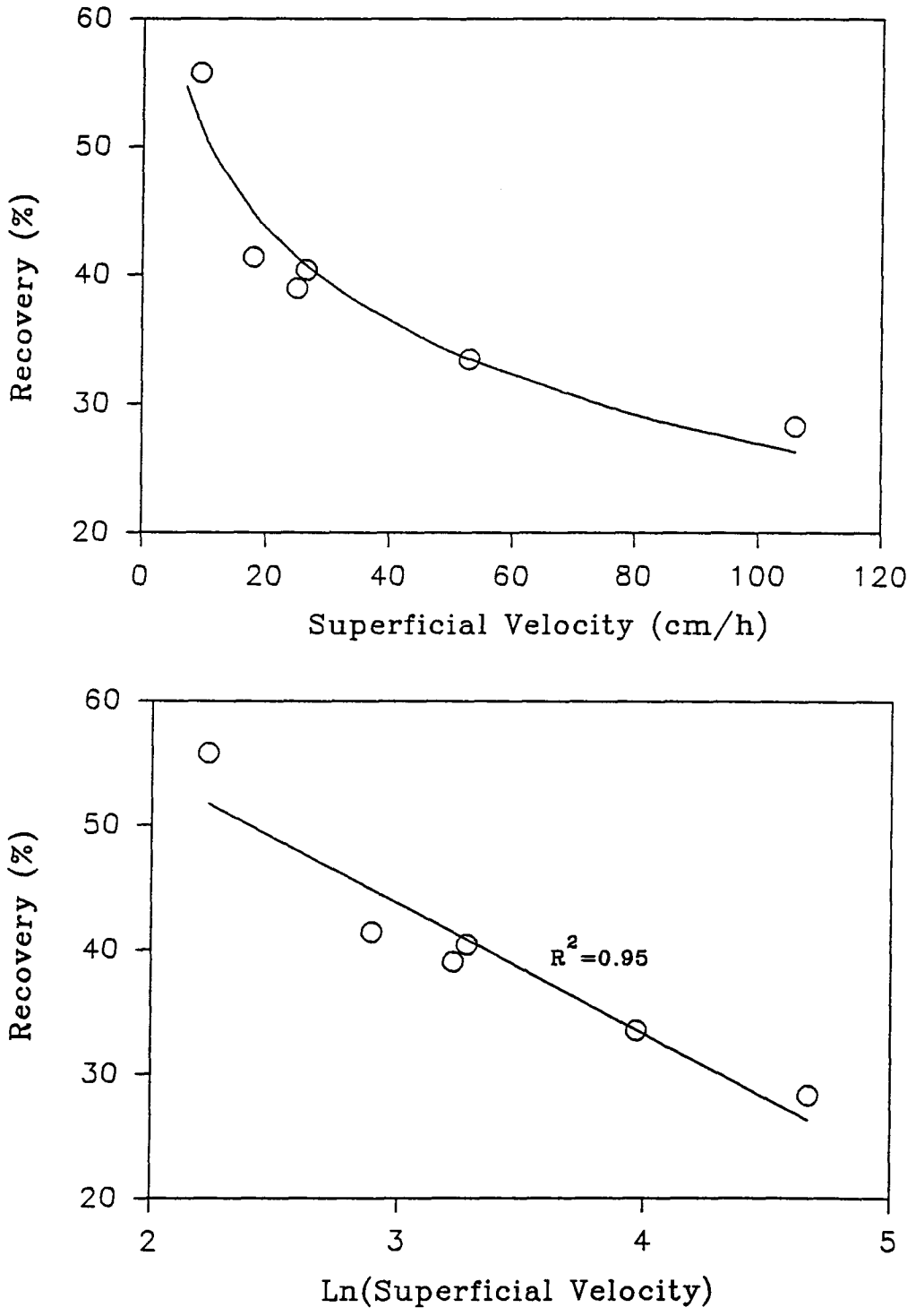


Table 10.13: Example Protocol for IgY Purification. Extraction was using distilled water and pH adjustment; separation using CEC; and purification using sodium sulphate precipitation.

	Column Separation		Batch Separation	
Step	IgY Cumulative Recovery (%)	IgY Purity (%)	IgY Cumulative Recovery (%)	IgY Purity (%)
1 (Extraction)	80*	18	80	18
2 (Separation)	51	61	42	57
3 (Purification	50	95	42	95

*Assuming two-step dilution/extraction is used, or recycling of the precipitated LSF in order to improve recovery.

dropped to around 42%, and the recovery increase to over 60%. Thus it is necessary as usual to trade purity in order to obtain better recovery, and the more important parameter is determined by the end use of the product.

Summary of the Process Although many combinations of the isolation methods descussed in this dissertation are possible, to complete the discussion of scale-up an example protocol is summarized in Table 10.13. This procedure would be relatively simple to scale up, including a small number of steps (3), and yet yield a relatively high purity product. A brief discussion of economic considerations is given in Appendix C.

10.4 Conclusions

The degree of binding of IgY to the cation exchanger is primarily affected by pH and the superficial velocity of the mobile phase. Even when scaling-up the process by a factor of 500 with respect to exchanger bed volume and volume of sample applied, it is possible to obtain a reasonable estimate of the recovery and breakthrough curve based on small-scale experiments. Column operation results in better binding and therefore recovery

of IgY than continuously stirred batch operation under the same conditions. Lower pH allows higher recovery of the molecule (probably due to stronger binding of additional subclasses), but at the expense of lower purity of the eluted fraction.

Chapter 11

Conclusions

As IgY is a heterogeneous polyclonal antibody with multiple isoeletric points, it is not possible, using ion-exchange chromatography, to completely isolate it from the other water soluble proteins in egg yolk. It is necessary to trade off purity for recovery, or vice versa, if the intact molecule is desired. Cation exchange chromatography is a potentially useful technique for the separation of IgY from the water soluble fraction of egg yolk, and has been shown to allow better recovery and purity than other ion-exchange methods. A simple yet efficient 3 stage process has been developed which is easily scaled-up for industrial application.

Since subclasses of IgY exist and it appears that approximately $\frac{1}{3}$ of the IgY present in the WSF does not bind to the column under the conditions used here, it is possible that the technique could be used to efficiently fractionate some of these subclasses. Only further study using egg yolk from immunized birds that can be analyzed using immunoassays such as ELISA's would allow clarification of this hypothesis.

The major contaminant remaining after cation exchange chromatography of the WSF is α-livetin. This protein may be effectively separated from IgY by one-step salt precipitation with Na_2SO_4, yielding a final product with a purity of at least 95%.

HPLC gel filtration can be used effectively to determine IgY concentration and purity in relatively pure (greater than 50%) samples, and can also provide a rapid, rough estimate of molecular weight. The automated system assembled for this study is a practical

means of speeding up the testing of exchangers under various experimental conditions.

The key parameters in determining the extent of binding of IgY in cation exchange chromatography are the pH, ionic strength, and superficial velocity of the mobile phase. Breakthrough curve analysis of laboratory-scale columns has been shown to provide a practical method of estimating the performance of a much larger column.

Chapter 12

Recommendations

The LSF precipitated in the extraction stage of the process is substantial in volume and further work on its utilization would be desireable before the process is used at an industrial scale. Waste disposal is becoming a major concern and it would be prefereable not to waste the valuable proteins still present in the LSF.

An interesting extension of this work would be to study the effect of dilution in the extraction stage using previously extracted WSF so as to reduce water useage and increase IgY concentration in the supernatant to be applied to the chromatography column. Another potential variation would be to use the sample application fraction from chromatography to dilute yolk in the extraction stage.

Further study of ultrafiltration for purification of IgY from the eluate of the chromatography step would be valuable. It is possible that under certain conditions of salt concentration and pH the α-livetin might pass through a 100 kDa membrane more readily. Also, a larger membrane (perhaps 150 kDa NMWC) would likely allow α-livetin to pass while still retaining the majority of the IgY. Although ultrafiltration is generally more energy intensive than either sedimentation or centrifugation, it would allow continuous processing in the third stage of the process and is less labour intensive than batch centrifugation.

Detailed study of the binding of IgY subclasses using yolks of hens immunized against specific antigens would provide better insight into the potential of cation exchange as a technique for separation of IgY from the WSF. As well, further work is needed on

the aggregation of IgY in aqueous solution, such as the eluate from the cation exchange column, in order to determine whether or not such aggregation is reversible. Optimization of the cation exchange portion of the process would also be beneficial before attempting to use it at a large scale.

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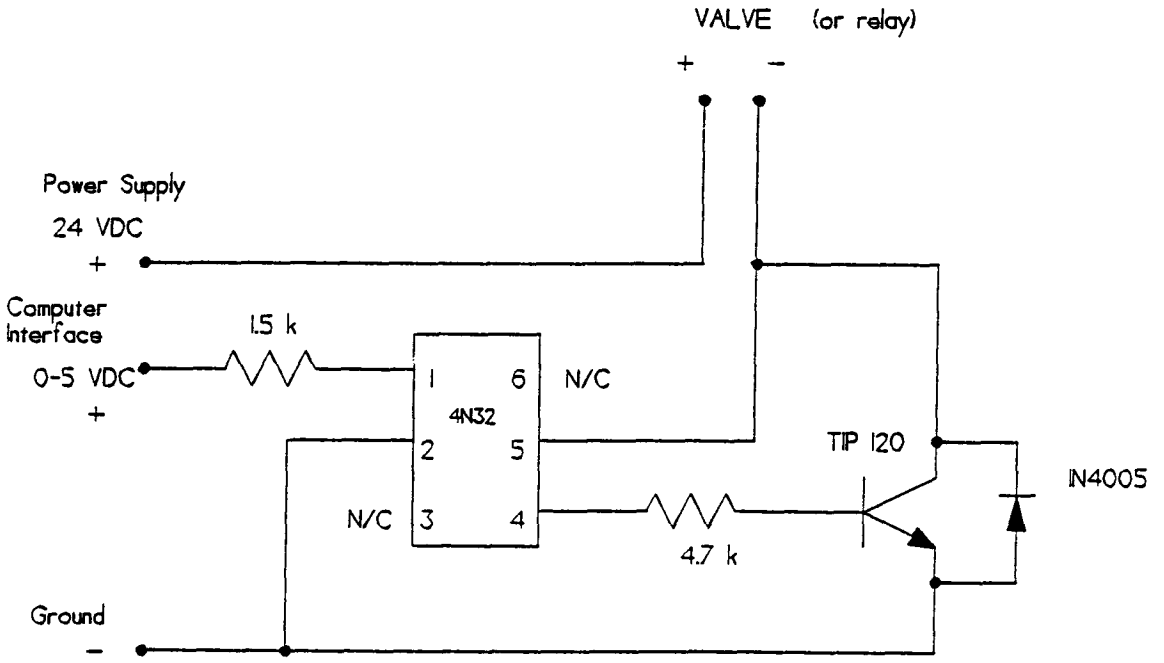
Appendix A

Schematic Diagram of ChemResearch/Solenoid Valve Interface[1]

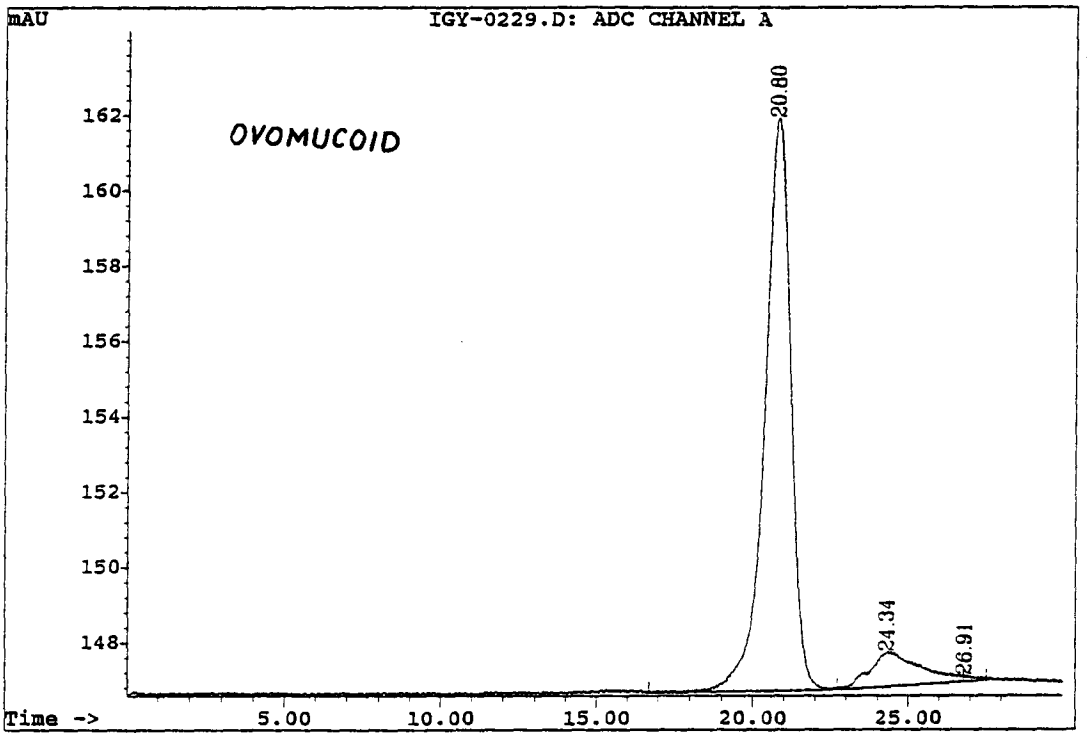


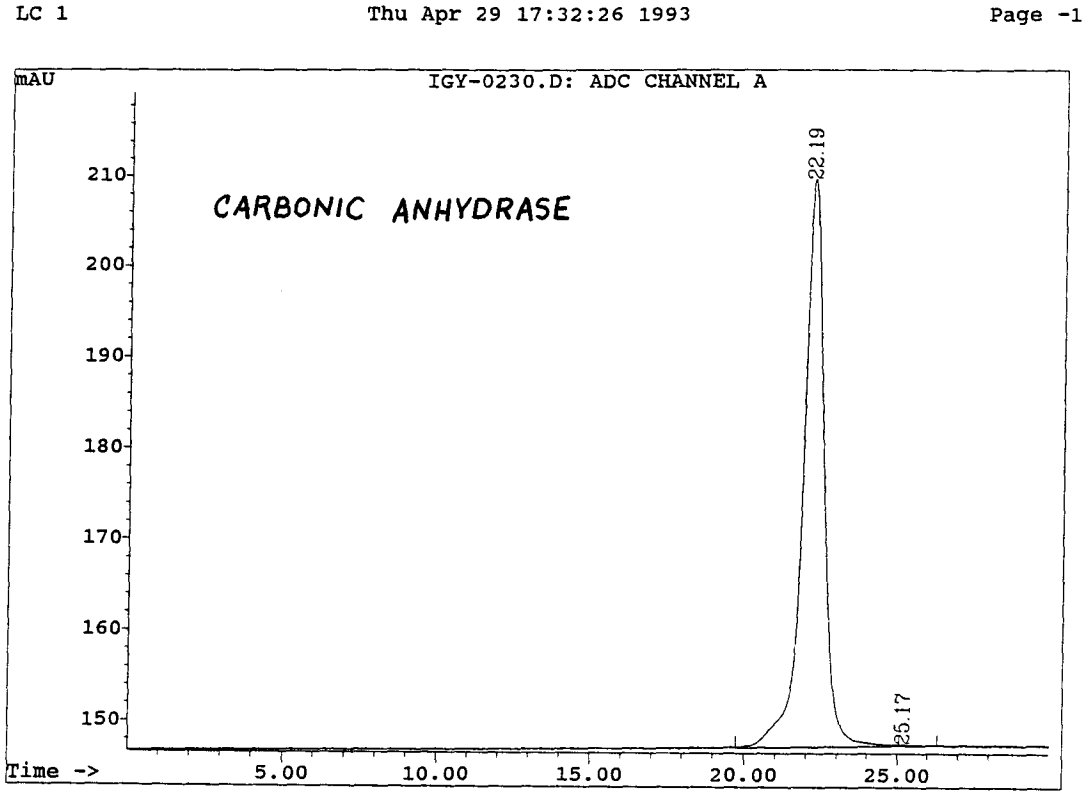
Schematic Diagram showing electrical connections between ChemResearch Interface and Solenoid Valve

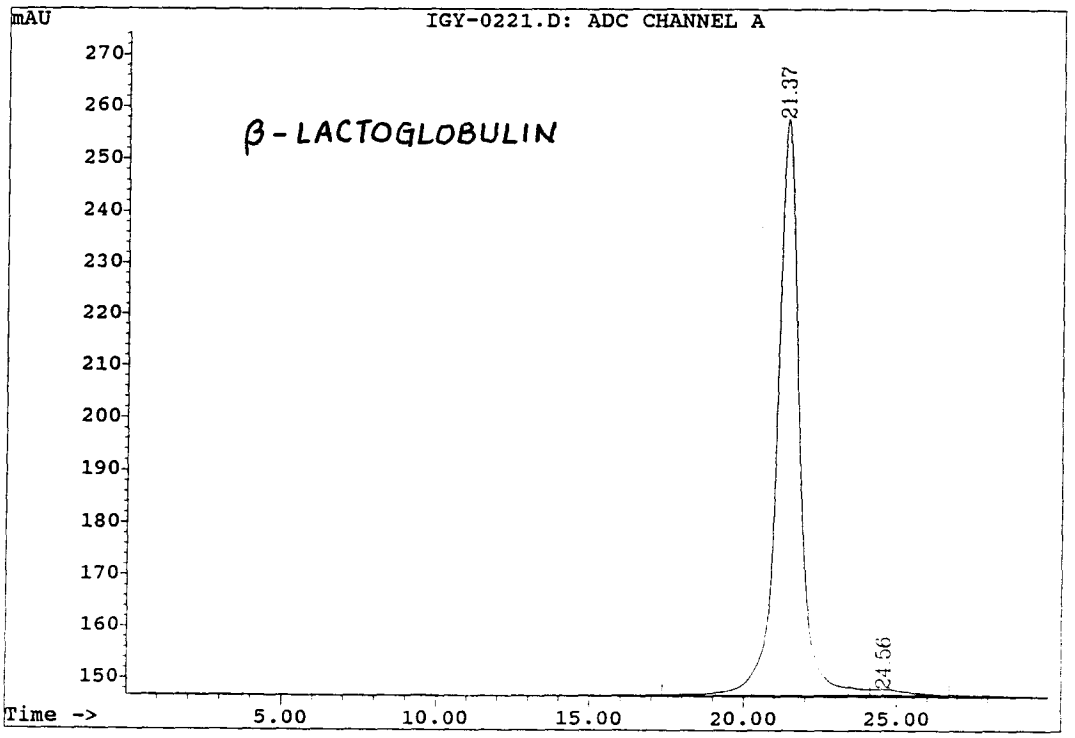
[1]First described in: March, A.C., Computer automation of a novel ion-exchange process for the simultaneous recovery of lysozyme and avidin from chicken egg albumen, Masters Thesis, 1988, UBC, Vancouver, Canada

Appendix B

Sample Chromatograms of Molecular Weight Markers




LC 1
Thu Apr 29 17:32:26 1993
Page -1-
mAU
IGY-0230.D: ADC CHANNEL A
CARBONIC ANHYDRASE
22.19
25.17
210
200
190
180
170
160
150
Time ->
5.00
10.00
15.00
20.00
25.00


LC 1
Thu Apr 29 17:34:19 1993
Page -1-
mAU
IGY-0221.D: ADC CHANNEL A
β-LACTOGLOBULIN
21.37
24.56
270
260
250
240
230
220
210
200
190
180
170
160
150
Time ->
5.00
10.00
15.00
20.00
25.00

Appendix C

Economics of IgY Purification

From an economic standpoint, separations of biological molecules are significant in that they account for 30 to 50% of bio-processing costs - possibly the largest discrete segment in the manufacture of biomolecules (DePalma, A., 1993). The following is a brief discussion of some of the costs that would influence the feasibility of large-scale production.

The economic analysis of the purification of IgY is dependent on a number of factors including raw material, equipment, operating and labour costs. As well, the market (or lack thereof) for the purified product cannot be neglected, since the most technologically sound and efficient process is of no use if the end product is not in demand. However, the market demand for IgY of various purities is uncertain at present, and until it has been approved by organizations such a the U.S. Food and Drug Administartion for use in food or medicines, it is unlikely that there will be a large enough demand to make large-scale processing feasible in North America.

Based on a market fax[1] the cost for a tanker load of liquid unpasteurized yolk was running at about 41.5 cents US per pound in mid 1992. Assuming a density of separated yolk of 1.0 mg/ml, a US/Canadian exchange rate of 1.28[2] and an IgY concentration of 8 mg/ml, the raw material cost would be approximately $0.15 Canadian per gram of IgY. With an overall recovery for the purification process of 50%, the material cost of finished product would be $0.30 per gram.

The estimation of equipment and operating costs requires a detailed calculation that

[1] Urner Barry Publications, dated June 23, 1992.
[2] The approximate rate as of the end of 1992.

is beyond the scope of this work. However, the equipment required would be similar to that found in most food processing plants (stainless steel tanks of various dimensions, centrifugal and/or positive displacement pumps, filters, etc.) and if IgY were to be an additional product for a company that was already producing several other food products (the most likely case), then this equipment might be shared. This would help to offset the cost somewhat. A significant operating cost could be attrition of the exchange media. Company literature from Gibco CEL, New Zealand, suggests that their exchangers have a low attrition rate, and in the case of whey protein recovery, an exchanger based on the same solid matrix as HC-2 (S-2) has been used repeatedly more than 18,000 times (Smith et al, 1986). However such claims would have to be verified for use with HC-2 under the conditions of separation of IgY. Utilities costs should be reasonably low for the process developed in this work since energy intensive operations have been avoided.

Labour is likely to represent another major cost in the separation and purification of IgY, and based on the experience of a local biotechnology firm, would probably account for a larger percentage of the overall costs involved than equipment and operating costs.

If IgY were to be prepared on a large scale (ie. metric tonne quantities per year) for use in a food product such as infant formula, then a purity of 40 to 50% might be quite acceptable[3]. This would significantly reduce the cost of separation as the salt precipitation step could be avoided resulting in substantial savings in chemical costs and labour. For the highly purified product, the costs would be somewhat higher, but could easily be offset by the much higher price generally paid for products of >99% purity.

[3]The major contaminant, α-livetin, would have to be studied with regards to infant formula to verify that it too would not cause an allergic response in infants.