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**THE SIZE EXCLUSION CHROMATOGRAPHY OF ANIMAL GLUES IN ZINC
ELECTROLYTES**

by

Carl J. Weatherell, B.Sc.

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of

the requirements for the degree of

Master of Science

Department of Chemistry

Carleton University

Ottawa, Ontario

June 14, 1994

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THE SIZE EXCLUSION CHROMATOGRAPHY OF ANIMAL GLUES IN ZINC ELECTROLYTES

submitted by Carl J. Weatherell, B.Sc.
in partial fulfillment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chairman, Department of Chemistry

Carlton University

June 14, 1994

ABSTRACT

The interactions of collagen, Croda glue and VM gelatin, which are organic additives employed in the base metals industry, with a polymeric and a derivatized silica stationary phase in size exclusion chromatography (SEC) are examined by a 2^3 factorial experimental design. Main factor coefficients indicate the presence of ion exclusion effects for all protein solutes on both stationary phases. Hydrophobic adsorption of collagen and Croda glue is also evident on the polymeric phase. Identical mechanisms of separation of collagen and Croda glue on both stationary phases are suggested by principal component analysis (PCA). Anomalous behaviour of VM gelatin is suggested by both main factor coefficients and principal components. In addition, determination of average SEC stationary phase pore size appears possible through PCA analysis of SEC data from the two different stationary phases.

A solvent extraction protocol to separate the animal glues from zinc electrolytes has been developed. The protocol along with optimum eluting conditions are applied to a simulated zinc electrolysis cell to determine the degradation rates of two animal glues in zinc electrolysis. Large molecular weight species ($M_r \approx 96,000$) are present after 2 hours of electrolysis. Croda glue and VM gelatin both show first order degradation kinetics with respect to glue concentration. Degradation rate constants of $0.0215 \cdot \text{min}^{-1}$ and $0.0082 \cdot \text{min}^{-1}$ were determined for Croda glue and VM gelatin respectively. The active component is tentatively identified as the α conformation of collagen.

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GLOSSARY

b_i	regression coefficients representing factor effects
BioSil	silica based stationary phase employed in this study
$[D]$	quantitative data matrix
$[D]'$	qualitative data matrix
$[D]^T$	transpose of the qualitative data matrix
F_{calc}	critical value of the F statistic
F_{crit}	calculated value of the F statistic
I	ionic strength (M)
$[I]$	identity matrix
k	first order reaction constant (min^{-1})
K_{sec}	partition coefficient for size exclusion chromatography
$K_{sec, sum}$	total weighted value of K_{sec} for a chromatogram
λ	eigenvalue matrix
N	chromatographic efficiency
N_c	normalization constant
N_{sum}	total weighted value of N for a chromatogram
$[PC]$	principal component matrix
PCA	principal component analysis
PC_i	principal component vector
PLS	partial least squares
r_i	responses constituting $[R]$
$[R]$	response matrix
$[R]^T$	transpose of the response matrix
SDS	sodium dodecyl sulphate
t_{crit}	critical value of the t statistic
TSKGel	polymeric stationary phase employed in this study

[V]	eigenvector matrix
v_{nm}	eigenvectors composing [V]
x_i	original factors of the response matrix
[X]	qualitative regression matrix
[Z]	covariance matrix of [R]

CHAPTER 1 INTRODUCTION

1.1 Glue Additives in Zinc Electrowinning

Metallic zinc has been marketed commercially for almost 200 years. Approximately 6 million tonnes per year of metallic zinc, valued at nearly \$20 billion is produced worldwide¹. Greater than 80% of this worldwide zinc production is accomplished by the electrowinning of zinc from acidic sulphate solutions. These solutions are produced by an industrial process which includes (as shown in Appendix A): concentrating zinc bearing ores via flotation, roasting the resulting concentrates to convert ZnS to ZnO, leaching the ZnO phase in sulphuric acid, removing iron from the leach solutions as jarosites, and finally removing Cu, Ni and Co by cementation with zinc dust. Although conditions may vary between refineries, zinc electrowinning cells are operated at 38-40 °C, with electric current densities of 400-540 A·m⁻² and efficiencies of 86%-94%.

Zinc electrolytes typically contain 1.5-2.0 M H₂SO₄, 125 g/L ZnSO₄ and low concentrations (0.01-10 mg/L) of Co, Cu, Cd, Ge, Ni, Cl⁻ and F⁻.² Each of these elements adversely affects the zinc electrowinning process through alteration of the hydrogen overpotential^{3,4}. The net result is a decrease in electric current efficiency and hence an increase in production costs. To offset the deleterious effects of these elements, animal glues, gelatins and other natural organic additives have been employed for decades as leveling agents in the electrodeposition of zinc from acidic sulphate solutions. Animal glues are added at a rate of 0.05 kg/tonne of Zn, yielding concentrations of 1-30 mg/L in the electrolyte. The fundamental effect of these additives is to provide a smooth Zn metal deposit with a preferred, compact crystal orientation at a high current efficiency⁵⁻⁹. Otherwise, irregularities and nodulation in the metal deposits result in short circuits,

causing elevated electrical power consumption, entrapment of impurities in the Zn metal and subsequently manual handling of the cathode sheets^{8,10,11}.

The effects of glue and gelatin additives on the current efficiency of zinc electrolysis is well documented^{2,4,6,7,12}. However, the exact mechanism and the molecular species responsible for causing these effects have yet to be determined. In aqueous solution of $\text{pH} < 1$, the protonated glue molecules are positively charged and will attach to negatively charged micro-dendritic formations on the cathode^{8,12-18} because the charge density of the micro-dendrites is larger than that of the surrounding cathode metal. Zinc then deposits preferentially in the valleys surrounding the glue-enclosed dendrites resulting in a decrease in the size of the dendrites. The charge density of the dendrites is therefore decreased, causing desorption of the glue molecules from their surfaces. This process yields a compact zinc deposit of preferred morphology.

A number of theoretical interpretations of the effects of glues on zinc electrolysis have been advanced^{5,12,16,20}. Unfortunately, these theories are based on assumptions or minimal information with regards to the chemical nature of the glue additives themselves. Indeed, the active organic species in zinc electrowinning has yet to be identified. The study of the mechanism of metal electrodeposition in the presence of animal glues has been carried out primarily through electrochemical studies on synthetic electrolytes or by structural characterization of the cathode after electrolysis⁵. Methods to control animal glue addition rates in zinc refineries are based on similar techniques. Overpotential measurements are routinely used to assess the impact of glue additives in zinc electrolytes^{4,7,24}. Unfortunately, the concentrations of other species (most notably Ge and Sb) also affect the overpotential measurement. Moreover, correlation of the overpotential measurements with historical current efficiency data in a refinery is necessary to establish the activity of glue in the electrolysis cell and thus to estimate proper addition requirements⁷. One Canadian zinc refinery is currently developing an expert system to facilitate the interpretation of overpotential measurements²⁴. Another method to establish

the activity of glue in zinc electrolysis cells involves cathodic deposition of zinc onto aluminum rods from the operating and other synthetic zinc solutions containing various concentrations of impurities and additives. Plant operators may simply examine and compare, visually, the results of numerous deposition experiments to determine the appropriate glue addition to follow.

Elucidation of the mechanism of degradation of animal glues, and a reliable method to measure the prevalent and thus active molecular species in zinc electrolytes are prerequisites to the elaboration of any formal theories as to the mechanism controlling their effects on current efficiency or zinc crystal orientation. Some electrolyte remediation processes rely upon current efficiency as a measure of the effectiveness of the electrolyte treatment method. Current efficiency is affected by numerous factors including temperature, current density and the concentrations of major, minor and trace elements in the electrolyte. Therefore, it is not a suitable method to accurately quantify a single species in an electrolyte. Degradation of the high molecular weight animal glues into shorter, smaller molecular weight species is also thought to be responsible for the deactivation of these species in base metal electrolytes^{12,19-21}. Since the isoelectric point of animal glues and gelatins is near pH 5^{19,22,23}, the assumption that glue molecules are positively charged at pH<1 is valid. However, little experimental evidence is available to support the remainder of the theory. To date, only one study has attempted to characterize animal glues and their degradation products in a base metal electrolyte¹⁹. In this study ultra-filtration followed by SEC was used to determine the kinetics of degradation of several animal glues in synthetic copper electrolytes. Unfortunately, the adsorption of these glues to the polymeric SEC stationary phase by ion exchange or hydrophobic interaction²⁵ was omitted. Thus, the reported molecular weights of these glues are questionable. Obviously there exists a need to develop a rapid, facile method to determine the concentrations of active organic components in zinc electrolytes.

1.2 Size Exclusion Chromatography

Size exclusion chromatography (SEC) techniques are commonly employed to analyze and purify biomolecules, such as glues and gelatins, according to their molecular size or molecular weight. The molecular weights of biomolecules with symmetrical configurations (i.e., globular proteins) can be determined, in the absence of secondary retention, since a direct correlation between the molecular size and the molecular weight of symmetrical molecules exists²⁶. Similar relationships also exist for molecules which assume asymmetrical conformations such as random coil and rod-like conformations if appropriate calibration standards are employed. However, none of these relationships can be applied to the determination of the molecular masses of species in denaturing eluents containing sodium dodecyl sulphate (SDS)²⁷⁻²⁹. Globular protein calibration standards have been shown ineffective to determine the molecular weights of collagen molecules and secondary structures which assume a rod-like configuration in the native state^{30,31}. Rod-like proteins show retention times less than spherical molecules of equal viscosity radius, R_{η} ³². Furthermore, the accurate measurement of apparent molecular weight for non-globular proteins is complicated by electrostatic and hydrophobic interactions of these solutes with the stationary phase³³⁻³⁹. The nature and extent of these interactions depend largely upon the type of stationary phase and elution conditions employed. Accurate molecular weight determinations using calibration curves with molecular weight markers require a thorough understanding of the relative magnitude of ion exclusion processes and hydrophobic interactions.

Porous silica stationary phases contain large numbers of silanol groups which may interact with solutes via ion exclusion, ion exchange or hydrogen bonding. Derivatization of silica stationary phases to effectively mask residual silanol groups on the surface of the stationary phase does not deter ionic interactions due to their long-range nature⁴⁰. Polymeric stationary phases are composed of hydrophilic, cross-linked polyether gels

containing hydroxyl groups and a few residual carboxyl groups^{36,40}. Ion exclusion effects are therefore also expected with this type of stationary phase. Several theoretical approaches to account for electrostatic interactions have been attempted^{38,41-43}, yet problems with their universal application still exist. Polymeric stationary phases are also known to exhibit tendencies towards hydrophobic adsorption^{30,42}. Irreversible adsorption of hydrophobic, cationic biopolymers has been documented³⁶.

Essentially, the determination and understanding of non-ideal SEC effects is still the greatest deterrent to the development of an accurate theory for SEC^{32,44}. Analysis of zinc electrolytes for animal glues has yet to be reported but the analysis of simulated copper electrolyte by SEC illustrates a rapid degradation of Croda glue (an animal glue common to the base metals refining industry) from a molecular weight of 491,000 to a species of molecular weight near 10,000 within 50 minutes¹⁹. Animal glues and gelatins are derivatives of Type I collagen that have molecular weights between 96,000 and 288,000^{31,38}. The discrepancy between the measured molecular weight of Croda glue and the known molecular weight of collagen is attributed to non-ideal size exclusion effects. Type I collagen molecules display cationic character below pH 4-5^{19,22,23}. Since the efficient separation of cationic polyions on conventional silica based stationary phases has yet to be reported^{36,39} denaturing conditions must be considered. Aside from producing anionic protein complexes, denaturing agents such as SDS promote the formation of stable molecular structures that resemble random coil and globular configurations⁴⁵⁻⁴⁸. Denaturing conditions also increase resolution and in some cases the accuracy of the determination of M_r ^{49,50}. Clearly, a careful evaluation of potential solute-stationary phase interactions must be undertaken before accurate measurement of M_r for uncharacterized biomolecules is attempted. The accurate evaluation of secondary retention effects on SEC stationary phases requires the development of an original method that does not make any *a priori* theoretical assumptions.

A flow diagram of the present M.Sc. thesis research is illustrated in Fig. 1. The focus of this work is to develop a facile method that employs SEC to analyze animal glues in zinc refining electrolytes. Inherent in the development of an accurate SEC method is knowledge of the factors controlling the secondary retention effects (e.g., ion exclusion, hydrophobic interactions) in SEC. Firstly, secondary retention effects on two different stationary phases will be examined through a chemometric approach. A combination of factorial experimental design (FED) and principal component analysis (PCA) will be applied to determine the nature and extent of secondary effects on the SEC of denatured animal glues.

Secondly, based on the results of the FED/PCA study, appropriate SEC operating conditions will be applied to yield maximum chemical information on a simulated electrowinning cell. Kinetic data describing the degradation of 2 commonly used animal glues, Croda glue and Vieille-Montagne (VM) gelatin, will be determined. Finally, major glue species present in Zn electrowinning cells will be identified.

Figure 1. Flow chart for present work

CHAPTER 2 FACTORIAL EXPERIMENTAL DESIGN (FED) AND PRINCIPAL COMPONENT ANALYSIS (PCA)

2.1 Introduction

Variables or responses used to describe chromatographic separations are often not independently controllable and rely upon numerous, alterable separation conditions⁵¹. The number of experiments to efficiently characterize a chromatographic separation is thus enormous. The classical approach to experimentation is to determine the experimental conditions, by examining measured responses. The FED approach deliberately alters all experimental conditions to observe the responses produced⁵². FED is a useful tool to obtain a maximum amount of information from a minimum number of experiments⁵³⁻⁵⁶, thus optimizing the efficiency of the experiment^{53,57,58}. Also, factor-factor interactions, which occur in most chemical systems⁵⁹⁻⁶¹ but are typically ignored when designing SEC and other chromatographic separation strategies⁵⁴⁻⁵⁶, are readily determined. FED yields coefficients which can be used to judge which of the factors incorporated into a model are important within the scope of the proposed model (i.e., the range of factors and responses considered). It may also be used to determine the magnitude and extent of the effects of experimental factors on the measured responses⁵³⁻⁵⁵. Mechanistic information may be gleaned from FED data to advance the theory of chromatography in general.

The responses obtained from any chemical system are the result of the interactions between different experimental variables on the system under investigation. Principal components result from natural associations among variables which produce new responses that can be expressed as linear combinations of the original variables⁶². Usually the specific interactions and their contributions to the measured responses are not immediately apparent. PCA decomposes the response data into two separate data sets, namely a data set of the interactions and a data set of their respective significance on the

measured responses. It reduces the dimensions of a multivariate data set while preserving the original data structure and grouping factors according to their influence on a given chemical system. The factors which control the measured responses, their influence on a chemical system and the similarities or differences may then be determined. PCA makes no *a priori* assumptions about the mechanistic origin of the data, thereby eliminating erroneous interpretation of them due to incorrect assumptions.

In this study an FED approach will be applied to the SEC of industrial glues and gelatins under denaturing conditions. A Type I collagen of similar composition to these additives will also be examined as a control. A three-factor two-level FED will be employed in an effort to gauge the magnitude of adsorption and exclusion effects and any first-order interaction effects⁵⁹ on a polymeric stationary phase. The FED will also be applied to a silica based stationary phase which provides a different mechanism of macromolecular separation⁶³. In SEC, where numerous types of solute-stationary phase interactions may lead to mixed modes of separations, FED should clarify the nature and extent of these interactions. A comparison of the results for the two stationary phases should give a comparative indication of the type and extent of any effects, other than size exclusion, influencing the separation of collagen based protein-SDS complexes by the two different stationary phases. PCA will next be applied to the FED data as a method to classify the different types of secondary retention effects, their magnitudes and relative control on the elution characteristics of the two stationary phases. PCA will also reveal the origin of unique factor-factor interactions.

2.2 Theory

2.2.1 FED

Factors considered for the FED are: pH, methanol concentration ($[\text{CH}_3\text{OH}]$) and ionic strength (I). Adjustment of pH and I are traditionally used to control ion exchange and ion exclusion effects. An elevated I may also induce hydrophobic adsorption of protein solutes on the stationary phase if the stationary phase is sufficiently hydrophobic. Organic modifiers such as CH_3OH will reduce the hydrophobic adsorption of protein solutes. The factor levels selected for this study are shown in Table I. The concentration of SDS is not examined as a factor since literature data have shown that protein-SDS binding ratios do not change significantly in the ionic strength range 0.05-0.5 M^{46,64}. Moreover, the conformation of the collagen-SDS complexes are not appreciably influenced by pH at the SDS concentration employed in this study⁶⁵. Thus, any measured responses as a function of I and pH must be correlated to secondary solute-stationary phase effects.

Table I. Factorial experimental design parameters

Factor	Low	High
pH	4	7
$[\text{CH}_3\text{OH}]$	0% (v/v)	15% (v/v)
I	0.10 M	0.40 M

Generally, a linear model incorporating interaction effects between factors x_1 , x_2 and x_3 may be described by:

$$r = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

In this study x_1 is the pH, x_2 is $[\text{CH}_3\text{OH}]$, x_3 is I, the b_i 's are the regression coefficients, and r is the response. Responses to be considered for the model are partition coefficient (K_{sec}) and the number of theoretical plates (N). Volumetric flowrates, measured by weighing fractions of the eluent after specific time intervals, were constant to within 1% of the setting. Hence, K_{sec} is calculated with retention times rather than elution volumes according to:

$$K_{\text{sec}} = \frac{t_e - t_o}{t_i - t_o} \quad (2)$$

where t_e , t_o and t_i are practically the elution times of the analyte, blue dextran and the nitrate anion respectively. Blue dextran and nitrate are commonly employed as markers to determine the void volume and the total column volume respectively. The values of N are calculated by using the formula:

$$N = 16 \left(\frac{t_e}{w} \right)^2 \quad (3)$$

Values of w , peak width at the base, are calculated using:

$$w = \frac{2A}{h} \quad (4)$$

where h = peak height and A = peak area.

Total K_{sec} ($K_{\text{sec,sum}}$) and total N (N_{sum}) values for chromatograms with multiple peaks are determined by weighting calculated values for each peak by its relative abundance expressed as a percentage of the total peak area. For a chromatogram containing 2 peaks with calculated K_{sec} values of 7.50 and 9.25 and area percentages of

90% and 10% respectively, a calculated $K_{\text{sec,sum}}$ of 7.68 would result ($=0.90 \cdot 7.50 + 0.10 \cdot 9.25$). A greater weight in the total statistics calculation is therefore given to the peak with the higher relative abundance. Since SEC peaks are generally wide with low values of N , area measurements give greater accuracy than height when calculating relative abundances.

A FED calculation yields regression coefficients that can be used to judge which of the factors incorporated into the model are important within the experimental ranges considered. It can also determine the extent of the effects of the factors on the measured responses. Since a quantitative, predictive model is not being pursued here, a qualitative approach will be employed. The original factor matrix represented by $[D]$ can be transformed to a qualitative matrix $[D]'$. In this qualitative matrix $[D]'$, low values for each factor are represented by -1 and high factor values by 1. The method of coding is analogous to normalization in that each of the individual factors is allotted an equal weight in the regression calculation regardless of their original units^{66,67}. In the case of SEC where solute-stationary phase interactions (ion exclusion, ion exchange and hydrophobic adsorption) may be present, a qualitative model allows one to determine which of these effects are present under the experimental conditions^{53,64}. Also, a measure of the relative importance of each effect to the calculated responses is possible.

	pH	[CH ₃ OH]	I			x ₁	x ₂	x ₃
[D]=	6.9	15	0.4	⇒	[D]'=	1	1	1
	7.2	15	0.1			1	1	-1
	6.7	0	0.4			1	-1	1
	7.0	0	0.1			1	-1	-1
	4.5	15	0.4			-1	1	1
	4.7	15	0.1			-1	1	-1
	4.2	0	0.4			-1	-1	1
	4.4	0	0.1			-1	-1	-1
	6.9	15	0.4			1	1	1
	7.2	15	0.1			1	1	-1
	6.7	0	0.4			1	-1	1
	7.0	0	0.1			1	-1	-1
	4.5	15	0.4			-1	1	1
	4.7	15	0.1			-1	1	-1
	4.2	0	0.4			-1	-1	1
	4.4	0	0.1			-1	-1	-1

The complete qualitative regression matrix, originating from Eq. 1, is represented as [X]. The number of rows defines the individual experiments. In Eq. 1, the regression coefficients b_1 , b_2 and b_3 describe the effects of the main factors pH, [CH₃OH] and I respectively. The regression coefficients b_{12} , b_{13} and b_{23} represent the binary pH-[CH₃OH], pH-I and [CH₃OH]-I interactions respectively. The b_{123} coefficient accounts for tertiary interactions between all three main factors, pH-[CH₃OH]-I. Interpretation of the coefficients in terms of solute-stationary phase effects is subjected to bias due to the differing magnitudes of the calculated responses, $K_{\text{sec,sum}}$ and N_{sum} . The response data for N_{sum} are therefore normalized by multiplying each measured response, r_i , by a normalization constant⁶⁶, $N_c = (\sum r_i^2)^{-0.5}$. A different normalization constant is computed for each set of N_{sum} responses derived for each solute on one stationary phase. Thus, in this study 12 different normalization constants were calculated

for N_{sum} for the three protein-SDS solutes on the two different stationary phases. Normalized responses for N_{sum} are consequently in the same range as the values of normalized $K_{\text{sec,sum}}$ (i.e. $0 \geq r_i \geq 1$).

$[X] =$

x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
1	1	1	1	1	1	1
1	1	-1	1	-1	-1	-1
1	-1	1	-1	1	-1	-1
1	-1	-1	-1	-1	1	1
-1	1	1	-1	-1	1	-1
-1	1	-1	-1	1	-1	1
-1	-1	1	1	-1	-1	1
-1	-1	-1	1	1	1	-1
1	1	1	1	1	1	1
1	1	-1	1	-1	-1	-1
1	-1	1	-1	1	-1	-1
1	-1	-1	-1	-1	1	1
-1	1	1	-1	-1	1	-1
-1	1	-1	-1	1	-1	1
-1	-1	1	1	-1	-1	1
-1	-1	-1	1	1	1	-1

Parameters of Eq. 1 determined by the LINEST function of Microsoft EXCEL 4.0 include: individual b_i coefficients, F statistics for the regression, number of degrees of freedom and variance of the regression. The LINEST function employs a least-squares algorithm for regression analysis. The significance of each calculated coefficient is determined by comparing the ratio of the individual regression coefficients to their respective error values with the critical value ($t_{\text{crit}}=1.86$)⁶⁸ of the one-sided t-test at the 95% confidence interval with v_2 degrees of freedom ($v_2=n-k-1$), where n =number of experiments=16, k =number of factors in the regression analysis=7). Calculated ratios smaller than the critical value are considered insignificant and will not be included with the

other regression results. One sided F-tests are applied to regression equations to determine the significance of the proposed model using a 95% confidence interval with v_1 degrees of freedom ($v_1=k$) in the numerator and v_2 degrees of freedom in the denominator. Calculated values of F smaller than the critical value of ($F_{crit}=3.73$)⁶⁸ are considered insignificant and this implies that the measured responses are not adequately described by Eq. 1.

2.2.2 PCA

The theory of PCA techniques have been detailed elsewhere^{61,66}. Here, a synopsis of the technique and the relevant mathematical manipulations will be presented, for the mathematically inclined reader. Others may continue reading from page 18.

PCA decomposes the original response matrix, [R], into two distinct matrices, the row or eigenvector matrix and the column or eigenvalue matrix. The principal eigenvectors are a mutually orthogonal coordinate system that describes the original data as a series of linear combinations of the original factors. These linear equations may assume the form:

$$PC_1 = v_{11}x_1 + v_{12}x_2 + v_{13}x_3 + \dots + v_{1m}x_m \quad (5)$$

$$PC_2 = v_{21}x_1 + v_{22}x_2 + v_{23}x_3 + \dots + v_{2m}x_m \quad (6)$$

$$\vdots$$

$$PC_n = v_{n1}x_1 + v_{n2}x_2 + v_{n3}x_3 + \dots + v_{nm}x_m \quad (7)$$

where the PC_n 's and x_m 's are the principal components and original factors of [R], respectively and the coefficients, v_{nm} , constitute the eigenvectors. In matrix notation the above relationship may be written as:

$$[PC] = [V] \cdot [x] \quad (8)$$

where the matrices of the principal components, the eigenvectors and the original variables are written as:

$$[PC] = \begin{bmatrix} pc_1 \\ pc_2 \\ \vdots \\ pc_m \end{bmatrix} \quad [x] = \begin{bmatrix} x_1 \\ x_2 \\ \vdots \\ x_m \end{bmatrix}$$

and

$$[V] = \begin{bmatrix} v_{11} & v_{12} & \dots & v_{1m} \\ v_{21} & v_{22} & \dots & v_{2m} \\ \vdots & & & \\ \vdots & & & \\ v_{n1} & v_{n2} & \dots & v_{nm} \end{bmatrix}$$

Since the PC's are mapping the original variables onto a new 2-dimensional coordinate system, the eigenvectors must be orthogonal and observe the following equation:

$$[V] \cdot [V]^T = [I] \quad (9)$$

where $[V]^T$ is the transpose matrix of $[V]$ and $[I]$ is a diagonalized, identity matrix defined as:

$$[I] = \begin{bmatrix} 1 & 0 & \dots & 0 & 0 \\ 0 & 1 & & 0 & 0 \\ \vdots & & & & \\ \vdots & & & & \\ 0 & 0 & \dots & 0 & 1 \end{bmatrix}$$

Calculation of the eigenvector matrix $[V]$, to satisfy the conditions in Eq. 9, is accomplished through decomposition of the covariance matrix of $[R]$, $[Z]$, such that Eq. 10 is satisfied:

$$[Z] \cdot [V] = \lambda \cdot [V] \quad (10)$$

Here $[Z]$ is calculated by:

$$[Z] = [R]^T \cdot [R] \quad (11)$$

and λ is the eigenvalue matrix of dimensions $n \times 1$.

The eigenvalues in λ characterize the variances associated with the respective eigenvectors. Eigenvectors are consecutively calculated to give a maximum variance λ_r , in each step. Thus, the first eigenvector accounts for the largest amount of variance and is the best one-dimensional model of the response data. To calculate the first eigenvector $[V]_1 = [v_{11}, v_{12}, \dots, v_{1m}]$ and the associated eigenvalue λ_1 , Eq. 10 is re-written as:

$$[Z] \cdot [V]_1 = \lambda_1 \cdot [V]_1 \quad (12)$$

As a first approximation, normalized values of $[V]_1$ are arbitrarily chosen. These values are then multiplied by the covariance matrix $[Z]$, according to eq. 12. The resulting vector is then normalized to yield the first estimate of $[V]_1$. The normalization constant, calculated by $N = (\sum c_{ik}^2)^{-0.5}$, is approximately equal to λ_1 . The improved approximation to $[V]_1$ is inserted into Eq. 12 to yield a further improved approximation to $[V]_1$ and λ_1 . This iterative procedure is continued until the elements of $[V]_1$ converge to constant

values and Eq. 12 is obeyed. To calculate $[V]_2$ the first residual matrix, $[S]_1$, is used in place of $[Z]$ in Eq. 12, where $[S]_1$ is defined by:

$$[S]_1 = [Z] - \lambda_1 \cdot [V]_1 \cdot [V]_1^T \quad (13)$$

where $[V]_1^T$ is the transpose of $[V]_1$. The iterative technique described above is applied to $[V]_2$ until convergence of the $[V]_2$ values is obtained. Supplementary eigenvectors and eigenvalues are determined using the appropriate residual matrices and applying Eq. 13 again in the iterative fashion described.

The magnitudes of the eigenvalues, λ_r , dictate the relative importance of the corresponding eigenvectors for describing the original response data. Since there are n original factors, n eigenvalues and n eigenvectors of dimension m will be determined. However, only p eigenvectors are required to adequately describe the response data since the majority of the variance in the data may be explicated by a few eigenvectors. There are no concrete rules to reduce the number of eigenvectors but the relationship:

$$\frac{\sum_{r=1}^p \lambda_r}{\sum_{r=1}^n \lambda_r} \geq 0.8 \quad (14)$$

is often used.

In the present work, eigenvectors are calculated for the two responses, $K_{\text{sec,sum}}$ and N_{sum} , using the response matrix:

$$[R] = \begin{bmatrix} x_{1,1} & x_{1,2} & x_{1,3} \\ x_{2,1} & x_{2,2} & x_{2,3} \\ x_{3,1} & x_{3,2} & x_{3,3} \\ x_{4,1} & x_{4,2} & x_{4,3} \\ x_{5,1} & x_{5,2} & x_{5,3} \\ x_{6,1} & x_{6,2} & x_{6,3} \\ x_{7,1} & x_{7,2} & x_{7,3} \\ x_{8,1} & x_{8,2} & x_{8,3} \\ x_{9,1} & x_{9,2} & x_{9,3} \\ x_{10,1} & x_{10,2} & x_{10,3} \\ x_{11,1} & x_{11,2} & x_{11,3} \\ x_{12,1} & x_{12,2} & x_{12,3} \\ x_{13,1} & x_{13,2} & x_{13,3} \\ x_{14,1} & x_{14,2} & x_{14,3} \\ x_{15,1} & x_{15,2} & x_{15,3} \\ x_{16,1} & x_{16,2} & x_{16,3} \end{bmatrix}$$

Here in PCA, the components $x_{i,1}$, $x_{i,2}$ and $x_{i,3}$ represent the responses (either $K_{sec,sum}$ or N_{sum}) for collagen, Croda glue and VM gelatin respectively while the subscript 'i' refers to the experiment number. Recall on p. 13 that eight different eluents are used in duplicate experiments to yield a total of 16 experiments. Hence, $x_{12,2}$ represents the response for Croda glue from experiment 12. Separate covariance matrices $[Z]$ for the two responses are calculated by Eq. 11 which are then applied to Eq. 12 to determine the eigenvectors and eigenvalues for each response matrix $[R]$. Two principal eigenvectors are chosen using Eq. 14 as a guide to ensure that the chosen eigenvectors adequately represent the data set. Subsequently, the principal components (PC's) are calculated from the original response data. To correlate the PC's and the experimental conditions, a partial least squares (PLS) linear regression is performed between each PC vector and the qualitative regression matrix $[X]$ from the FED. The qualitative matrix $[X]$ is used in the

PLS regression to overcome the difficulty with the various units contained in the otherwise quantitative matrix. Calculated regression coefficients are then used to determine the influence of the experimental conditions on the PC's. The type and extent of secondary retention or single factor effects and the origin of factor-factor interaction effects may then be ascertained.

Interpretation of PCA data is facilitated by a two-dimensional plot such as the one illustrated in Fig. 2. Clusters of points may be interpreted as similar effects of the PC's on the measured response. Thus, the factors represented by the cluster of $\square$ points have similar effects on the measured response. Similarly, the factors represented in each of the other clusters (Δ , $\diamond$ and $\circ$) also influence the measured response although through different means. The positioning of clusters usually indicates a difference in magnitude of the individual influences or a different mechanism for influencing the measured response. Since the first PC, PC_1 , contains the largest amount of variance of the response data, it may be related to the magnitude of the individual factor effects on the response. On the other hand, PC_2 may classify the different mechanisms by which the factors influence the measured response. The exact relationship between the PC's and the response depends upon the system under investigation, the factors themselves and the measured response. However, a cluster near the origin always signifies a minimal if not negligible influence of the factors in that cluster on the measured response, such as those $\diamond$ points in the figure.

Figure 2. A typical 2-dimensional PCA plot

2.3 Experimental

2.3.1 Reagents

Croda glue was obtained from the Inco Copper Refinery, Copper Cliff, Ontario while the Vieille-Montagne (VM) gelatin was donated by the Centre Technologie Noranda Ltée., Pointe-Claire, Québec. The industrial grade reagents were used without further purification. Type I collagen was obtained from Sigma Chemicals (St. Louis, Missouri). Sodium chloride, sodium monohydrogen phosphate and sodium dihydrogen phosphate were obtained from BDH, (Toronto, Ontario). Sodium dodecyl sulphate (SDS) for preparation of 0.10 M stock solutions was purchased from Regis Chemical (Morton Grove, Illinois). Methanol was high purity grade (Burdick and Jackson, Pointe-Claire, Québec). All eluents were prepared with distilled deionized water buffered with phosphate solutions at an ionic strength of 0.05 M, filtered (Millipore 0.45 μm), degassed and stored under helium.

2.3.2 Apparatus

A Dionex chromatography system (Sunnyvale, California), used for all experiments, consisted of an APM analytical pump, an LCM-I chromatography module and a VDM-II variable wavelength UV-visible detector. The injection valve was equipped with a 100 μL sample loop. Data was collected with the Dionex chromatographic interface module (CIM-I) using the AI-450 software (Version 3.31) with a custom-built IBM compatible 386 host computer. Although the software calculated the efficiency, N , the reported values were inconsistent with values calculated manually and were therefore not used. A BioSil SEC-250 (BioRad Laboratories, Mississauga, Ontario) silica-based size exclusion column (30 cm x 7.8 mm i.d.) and a guard column (5 cm x 7.8 mm i.d.) were used for a derivatized silica stationary phase. A TosoHaas TSKGel 4000PW_{xL} (Supelco, Oakville, Ontario) column (30 cm x 7.8 mm i.d.) was employed as a polymeric

stationary phase. Both phases contained 10 μm particles. Manufacturer-recommended constant eluent flow rates of 1.0 mL/min and 0.8 mL/min were used for the silica and polymeric stationary phases, respectively. The VDM-II detector was operated at 210 nm.

All statistical calculations were performed using Microsoft EXCEL 4.0 (Redmond, Washington). A matrix diagonalization routine written as part of a dynamic link library for Microsoft EXCEL 4.0 was used for all PCA analysis⁶⁹. The LINEST function of Microsoft EXCEL was used to perform PLS linear regression analysis.

2.3.3 Procedure

Blue dextran and the NO_3^- anion were used to determine the void volume and the total column volume, respectively. Stock solutions of Croda glue and VM gelatin were prepared by stirring 0.100 $\pm$ 0.001 g at 80°C for 20 min in water followed by cooling at ambient temperature for 2 hr. The solutions were then bulked up to 100 mL to give a concentration of 1.0 g/L. Fresh stock solutions were made every 3 days and were stored at 4°C when not in use. A stock solution of Type I collagen was prepared by adding 1-2 mg to 8 mL of the eluent. The solution was heated for 15 min at 100°C to aid dissolution and denaturation, cooled to ambient temperature and bulked to 10 mL with the eluent. All experiments were performed in duplicate to give an estimate of the experimental error.

2.4 Results and Discussion

2.4.1 Factorial Experimental Design

2.4.1.1 $K_{sec,sum}$

Chromatograms obtained for collagen, Croda glue and VM gelatin for each stationary phase under the eight different experimental conditions are shown in Appendices B, C and D respectively.

A complete listing of calculated main factor and factor-factor interaction coefficients can be seen in Appendix E. Here, regression coefficients (b_0 , b_1 , b_2 and b_3) for the main factors of $K_{sec,sum}$ for collagen, Croda glue and VM gelatin are shown in Table II. The factors corresponding to each b_i coefficient are shown in the top row. The polymeric phase is represented by TSKGel and the derivatized silica phase by BioSil. Calculated F values (F_{calc}) greater than F_{crit} ($=3.73$) indicate the significance of the regression coefficients for describing $K_{sec,sum}$ in terms of the three factors and their interactions for each solute on a given stationary phase. The larger F_{calc} , the more significant the three factors and their interactions are.

Table II **Calculated regression coefficients for $K_{sec,sum}$**

			<div>pH CH₃OH I</div>			
Sample	Column	F_{calc}	b_0	b_1	b_2	b_3
Collagen	TSKGel	13860	0.574	-0.153	-0.054	0.147
	BioSil	268	0.202	-0.071	0.048	0.060
Croda glue	TSKGel	101	0.616	-0.038	-0.037	0.127
	BioSil	15	0.256	-0.033	0.038	0.041
VM gelatin	TSKGel	61	0.795	-0.013	0.022	0.044
	BioSil	69	0.552	0.016	0.042	0.031

Elution times for nitrate, which determined the total column volume, exhibit relative standard deviations (RSD) of 2.9% and 2.6% on the polymeric and derivatized silica phases, respectively, for the 16 different experiments using 8 distinct eluents. These RSD values are within experimental errors and suggest that electrostatic interactions between NO_3^- and the stationary phases are minimal under the experimental conditions. This lack of ionic exclusion effect for the NO_3^- anion is not surprising. Previous work has shown that, under conditions where ion exclusion is normally present ($\text{pH}=7$ and $I=0.1\text{ M}$), the citrate anion yields a K_{sec} of 0.9 on a silica-based stationary phase similar to that employed in this study⁷⁰. Also, the above RSD for NO_3^- is calculated over pH and I ranges where ion-exclusion effects are reduced or possibly eliminated.

Although the pK_a of some silanols on the surface of silica stationary phases may be as low as 3, a pK_a of 7 is generally regarded to be a good estimate for all silanols. Therefore, at $\text{pH}\geq 7$ the surface of the derivatized silica phase will exhibit a net negative charge due to deprotonation of residual, non-derivatized, silanol groups. Similarly, deprotonation of carboxyl groups on the polymeric stationary phase will lead to a net negative charge on this phase at $\text{pH}\geq 7$. The protein-SDS complexes possess a net negative charge at $\text{pH}\geq 7$ ⁶⁵. Thus, repulsive forces would exist between the negatively charged solute and both stationary phases when the eluent pH is neutral or basic. This ion exclusion effect yields an increased rate of elution and therefore a reduction of $K_{\text{sec,sum}}$. The linear model contains a b_1 term to account for the pH effects. Negative b_1 values are observed in Table II with both stationary phases for most of the solutes investigated, suggesting ion exclusion effects were present. The magnitudes of the b_1 coefficients for collagen and Croda glue on the polymeric column are all larger than those for the silica based column. This may suggest a greater influence of pH on the separation process on the polymeric stationary phase and hence a greater degree of ion exclusion at higher pH. The lack of a negative effect for VM gelatin on the silica column (BioSil) suggests that the VM-SDS gelatin complex does not possess a net negative charge up to pH 7.2. A

diagram representing the ion exclusion effect is shown in Fig. 3. In this scenario three distinct phases are considered. The first phase is the flowing eluent, the second phase is the solid matrix of the silica or polymeric stationary phase and the third phase is the aqueous phase that is occluded within the pores of the stationary phase. Under conditions where the surface of the stationary phase exhibits a net negative charge (i.e., $\text{pH} \geq 7$), negatively charged solutes may be partially or totally excluded from the pores due to electrostatic repulsion.

Organic modifiers such as methanol typically reduce hydrophobic adsorption of protein solutes onto the stationary phase, effectively reducing retention times of these solutes. In accordance with Eq. 2, a decreased $K_{\text{sec,sum}}$ would result leading to negative b_2 coefficients in the linear model given by Eq. 1. The negative b_2 coefficients in Table II observed for collagen and Croda glue on the polymeric phase (TSKGel) can be attributed to hydrophobic adsorption. The chromatograms for eluents 1 and 3 in Fig. B3 (in Appendix B), C3 (in Appendix C) and D3, (in Appendix D) all show decreased retention times for eluent 1 which contains CH_3OH . This indicates hydrophobic adsorption of the protein-SDS complexes onto the stationary phase. Hydrophobic adsorption of proteins has previously been observed with TSKGel PW stationary phases^{28,40,71}. In contrast, the influence of hydrophobic adsorption is not observed on the silica based stationary phase, as evidenced by the positive b_2 coefficients in Table II. In fact the opposite trend can be seen for eluents 1 and 3 in Fig. B1 (in Appendix B), C1 (in Appendix C) and D1, (in Appendix D). A plausible explanation may be the hydrophobic adsorption of SDS from the eluent onto the silica stationary phase^{72,73}. Adsorption of SDS and similar molecules either positively or negatively charged are routinely employed for the separation of charged species through dynamic ion-pairing mechanisms. The resulting repulsion of the negatively charged protein-SDS complex with the anionic SDS layer adsorbed onto the stationary phase in the absence of CH_3OH yields decreased residence time in the column. Desorption of SDS from the stationary phase

with methanol addition would eliminate these secondary exclusion effects and increase $K_{\text{sec,sum}}$, leading to positive b_2 coefficients. Although the polymeric stationary phase also adsorbs SDS, hydrophobic adsorption of the protein-SDS complexes probably dominates.

Ion exclusion effects are typically counteracted by increasing the ionic strength of the eluent. The critical micelle concentration of SDS also decreases, from approximately 8 mM to 0.5 mM when the ionic strength changes from 0.0 M to 0.4 M⁷⁴. However, the binding ratio of SDS/collagen remains constant at 1.4 g SDS/g protein⁴⁶. Stationary phases manifesting ion exclusion effects are expected to show an increase in $K_{\text{sec,sum}}$ with an increase in ionic strength. This is mainly due to a reduction in ionic exclusion effects and partly to a diminution of the thickness of the electrical double layer at the surface of the protein-SDS complexes at high ionic strength^{37,43}. This reduces the effective size of the macromolecule and hence size exclusion, shifting $K_{\text{sec,sum}}$ to larger values. The positive b_3 terms in Table II for all solutes on both stationary phases strongly suggest that ion exclusion effects are counter-acted and hence long retention times are resulted with eluents of high ionic strengths. The relative contribution of ion exclusion and the thickness of the double layer surrounding the protein-SDS complexes is, however, not immediately apparent. Increased I also induces the hydrophobic effect causing adsorption of protein-SDS solutes to the stationary phase. This is evident in Fig. B3, C3, and D3 if the chromatograms represented by Eluent 3 and Eluent 4 are compared. Eluent 3 is at an I of 0.4M while eluent 4 is at an I of 0.1 M. The chromatograms for eluent 3 all show smaller peak areas for each solute when compared to eluent 4 which again suggest incomplete elution of the protein-SDS complexes due to hydrophobic adsorption onto the stationary phase at the higher I.

Figure 3. Schematic illustration of ion exclusion effect

2.4.1.2 N_{sum}

Column efficiency, N , in chromatography is dictated by numerous factors, including the particle size of the stationary phase, mass transfer effects and diffusion effects. The diffusion coefficient, D_p , of the solute within the pore network of the stationary phase is directly proportional to $N^{30,44,75,76}$ and is determined by the Stokes radius of the solute and by secondary retention effects. For large molecular mass solutes, D_p will be small leading to low values of N . Where ion exclusion occurs, D_p is also reduced since the solutes are partially or fully excluded from entering the pore network.

Calculated main factor regression coefficients of N_{sum} for: collagen, Croda glue and VM gelatin on the two stationary phases are summarized in Table III. A complete listing of both main factor and factor-factor interaction coefficients can be seen in Appendix F. Negative b_1 coefficients are observed in Table III for all solutes on both stationary phases indicating a decrease in N_{sum} with increasing pH. Under high pH conditions where ion exclusion is present, protein solutes may be either partially or totally excluded from the pores of the stationary phase.

Table III. Calculated regression coefficients for N_{sum} .

Sample	Column	F_{calc}	b_0	pH	[CH ₃ OH]	I
				b_1	b_2	b_3
Collagen	TSKGel	208	0.151	-0.095	-0.017	-0.042
	BioSil	67	0.243	-0.021	-0.013	-0.015
Croda glue	TSKGel	4365	0.197	-0.050	0.009	-0.077
	BioSil	133	0.233	-0.044	-0.043	-0.062
VM gelatin	TSKGel	1312	0.205	-0.048	0.039	-0.051
	BioSil	212	0.220	-0.068	-0.073	-0.044

This leads to greater resistance to diffusion of the solutes through the pores of the stationary phase and hence decreased D_p , ultimately yielding a lower value of N_{sum} .

Similarly, b_2 coefficients are positive for Croda glue and VM gelatin on the polymeric phase and negative for all solutes on the silica phase. For the polymeric phase, desorption of Croda glue and VM gelatin by added CH_3OH is obvious by their reduced peak areas in the chromatograms shown in Fig. C3, C4, D3 and D4. Collagen is also desorbed by added methanol, as seen in Fig. B3 and B4, yet its N_{sum} coefficient is negative. This may be a mathematical effect whereby the peaks obtained under hydrophobic adsorption conditions have unusually narrow peak widths at longer retention times, thus yielding higher values of N_{sum} . Hydrophobic adsorption of the protein-SDS complexes onto the silica-based stationary phase was not apparent. Earlier in this thesis, adsorption of SDS from the eluent onto the silica stationary phase was suggested from the data for $K_{sec,sum}$. Adsorption of SDS would cause an increase in ionic exclusion due to repulsion of the $-SO_3^-$ moiety with the negatively charged protein-SDS complexes. As detailed earlier (p 29) this ultimately leads to a lower value of N_{sum} . Addition of methanol should alleviate these electrostatic effects and yield positive b_2 coefficients which is not observed for the silica stationary phase in Table III. A physical change in the geometry of the protein-SDS complexes due to methanol in the eluent must be responsible. The larger molecular size would hinder diffusion into the smaller pores of the stationary phase leading to diminished values of D_p and ultimately reduced values of N_{sum} . A diminution of column efficiency with increased Stokes radius has been reported previously^{44,75,76}. Consequently the negative b_2 coefficients for N_{sum} are the result of an expansion of the protein-SDS complexes induced by methanol in the eluent. Expansion of the protein-SDS complex may occur due to the exchange of CH_3OH for the H_2O molecules bound to the complex. With a larger size and greater hydrophobicity bound CH_3OH molecules would induce expansion of the complex yielding a net increase in radius and hence a decrease in N .

Calculated b_3 terms in Table III are negative for all solutes on both stationary phases. Chromatograms for collagen, Croda glue and VM gelatin using eluent 1 and eluent 2 on the polymeric stationary phase (Fig. B3, C3 and D3) all reveal a shift toward longer retention times at higher values of I. Although this is indicative of both hydrophobic adsorption and ionic exclusion, for the polymeric phase hydrophobic adsorption is likely responsible. Chromatograms for collagen, Croda glue and VM gelatin using eluent 1 and eluent 2 on the silica stationary phase (in Fig. B1, C1 and D1) also reveal a shift toward longer retention times at higher values of I. Since siliceous stationary phases are known to exhibit greater ionic exclusion effects than polymeric phases⁴² and in light of the experimental evidence displayed thus far, the negative b_3 terms observed for the silica based stationary phase result from ionic exclusion effects.

4.1.3 Optimum Elution Conditions

Qualitative or quantitative FED data are typically used to optimize a chromatographic separation process for a desired system^{53,58}. Substitution of b_i coefficients, determined for each solute on both stationary phases, into Eq. 1 yields the data shown in Table IV.

Table IV Maximum values of N_{sum} and the associated experimental conditions

Solute	Column	Maximum* Calculated N_{sum}	Conditions		
			pH	[CH ₃ OH]	I
Collagen	TSKGel	0.583	-1	-1	-1
	BioSil	0.327	-1	-1	-1
Croda glue	TSKGel	0.379	1	-1	-1
	BioSil	0.353	-1	-1	-1
VM gelatin	TSKGel	0.375	-1	-1	-1
	BioSil	0.474	-1	-1	-1

*Note: these are the maximum normalized values.

The polymeric stationary phase affords higher N_{sum} values and hence better separation efficiencies than does the silica phase for collagen and Croda glue while the silica stationary phase gives a higher value of N_{sum} for VM gelatin. Maximum values of N_{sum} are all realized at low values of [CH₃OH] (0%) and I (0.1 M) for all solutes on both stationary phases. With the exception of Croda Glue on the polymeric stationary phase, maximum N_{sum} on both stationary phases appears at pH=4 for all solutes.

Experiments for determining the fate of Croda glue and VM gelatin in acidic simulated zinc electrolyte solutions will incorporate the conditions shown above for each solute to achieve optimum efficiency. In other words, all separations will use the TSKGel polymeric stationary phase; eluents composed of pH=7, pH=4 and I=0.1 M will be used for Croda glue and VM gelatin solutes, respectively, at [CH₃OH] and I=0.1 M.

2.4.2 Principal Component Analysis

2.4.2.1 $K_{\text{sec,sum}}$

The eigenvectors determined for $K_{\text{sec,sum}}$ on the polymeric-based stationary phase are:

$$PC_1 = 0.5112x_1 + 0.5372x_2 + 0.6708x_3 \quad \lambda_1 = 22.2371 \quad (15)$$

$$PC_2 = 0.8246x_1 - 0.0865x_2 - 0.5591x_3 \quad \lambda_2 = 0.5343 \quad (16)$$

$$PC_3 = -0.2423x_1 + 0.8390x_2 - 0.4872x_3 \quad \lambda_3 = 0.1145 \quad (17)$$

where x_1 , x_2 and x_3 refer to the $K_{\text{sec,sum}}$ values for collagen, Croda glue and VM gelatin respectively. Eigenvalues, λ_i , for each eigenvector are shown at the end of each equation. Substitution of the eigenvalues from Eq. 15 and 16 into Eq. 14 reveals that these two eigenvectors account for 99.50% of the total variance in the original $K_{\text{sec,sum}}$ data matrix. Therefore, the eigenvectors PC_1 and PC_2 are applied to the original data to calculate PC's for $K_{\text{sec,sum}}$ for the polymeric stationary phase. Since PC_1 accounts for the majority of the variance it is assigned as the abscissa. PC_2 is then assigned the ordinate for the vector plot of the two PC's.

The following eigenvectors and associated eigenvalues were calculated for the silica based stationary phase:

$$PC_1 = 0.3314x_1 + 0.4069x_2 + 0.8512x_3 \quad \lambda_1 = 6.7416 \quad (18)$$

$$PC_2 = 0.8135x_1 + 0.3337x_2 - 0.4762x_3 \quad \lambda_2 = 0.1873 \quad (19)$$

$$PC_3 = -0.4778x_1 + 0.8503x_2 - 0.2204x_3 \quad \lambda_3 = 0.0143 \quad (20)$$

The principal components PC₁ and PC₂ account for 99.79% of the experimental variance for $K_{\text{sec,sum}}$ for the silica stationary phase and are used to calculate the corresponding PC's.

Summary statistics for the PLS regression of the PC's and the qualitative regression matrix, [X] (see p. 14), are shown in Table V.

Table V Summary statistics for the PLS linear regression of the principal components of $K_{\text{sec,sum}}$

Stationary Phase	Vector	σ^2	F _{calc}	Degrees of Freedom
TSKGel	PC ₁	0.9975	452	8
TSKGel	PC ₂	0.9986	808	8
BioSil	PC ₁	0.9878	93	8
BioSil	PC ₂	0.9921	144	8

The variances, σ^2 , of the regressions all approach 1 while the values of calculated F statistics, F_{calc}, are all greater than F_{crit} (=3.73). This implies the significance of the regression coefficients for describing $K_{\text{sec,sum}}$ in terms of the main factor and factor-factor interaction effects. The TSKGel polymeric stationary phase exhibits a better fit to the experimental data than does the BioSil silica based stationary phase.

The b_i coefficients for Eq. 1 determined through PLS regression of the PC's and the qualitative regression matrix [X], for $K_{\text{sec,sum}}$, are represented graphically in Fig. 4 for the polymeric and silica stationary phases. Generally, b_i coefficients in close proximity to each other induce similar effects on the measured response^{57,62}. Clustering of b_i values on the PCA plots indicate similar effects of the corresponding factors on the measured response. Principal components near the origin denote a minor influence of the variable on the measured response^{57,62}. The eigenvectors for PC₁ in Eq. 15 and 16 show that collagen and Croda glue contribute almost equally to the PC₁ vector while VM

gelatin lends a higher contribution to PC_1 on both stationary phases. A corresponding trend is not observed for PC_2 . On the polymeric phase, the collagen solute contributes a large positive component to PC_2 while Croda glue and VM gelatin lend negatively to PC_2 . The PC_2 vector of the silica stationary phase contains a large positive collagen component, a somewhat smaller Croda glue component and a negative VM gelatin component. Since PC_1 is the best one dimensional model of the data and it contains over 95% of the variance for both stationary phases it may be used to link characteristics of the protein-SDS solutes. The similarities of the collagen and Croda glue components of the PC_1 vector suggest that these two species behave very similarly on both stationary phases and hence are probably very similar in molecular structure.

The FED calculations for the silica stationary phase yield negative b_1 coefficients and positive b_2 and b_3 coefficients. Similarly, the FED calculations yielded negative b_1 and b_2 coefficients and positive b_3 coefficients for the polymeric stationary phase. The PC plot of $K_{sec,sum}$ in Fig. 4 shows corresponding tendencies for the b_i coefficients calculated by PCA. The b_i coefficients which are positive when calculated by the FED method appear at positive values of the PC_1 axis, when calculated by the PCA method. Similarly, those b_i coefficients which are negative when calculated by the FED method have negative PC_1 components when calculated by the PCA method. This suggests that the first principal component, PC_1 , describes the magnitude and direction of the influence of a factor on $K_{sec,sum}$.

The main factor effects described by b_1 , b_2 and b_3 have been discussed in detail in the preceding section 2.4.1.1 in terms of their origin and significance toward non-ideal effects in SEC. Factor-factor interaction effects however, are more difficult to describe individually as they may result from either a combined effect of two or three, individual factors involved. Or they may exert their own unique, synergistic type of effect on the measured response. PCA plots are useful in this regard as they may assist in the determination of the origin of the interaction effects. Typically, clusters of PC's represent factors which exert a similar influence on the measured response. Thus, the clustering of b_2 , b_3 and b_{23} for the silica phase (Δ) and b_{12} for the polymeric stationary phase ($\square$) in Fig. 4 indicates that these factors and factor-factor interactions all exert a similar degree of influence on $K_{sec,sum}$. Since the interaction coefficient, b_{23} , is in close proximity to the individual factors, this interaction effect is likely a result of the combined effect of the two individual components. Similarly, the b_{123} interaction coefficient for the TSKGel polymeric stationary phase lies near the main factor coefficient b_2 and is probably influenced primarily by this main factor. In contrast, the b_{12} and b_{13} coefficients for the BioSil silica stationary phase are not proximate to any of their respective main factor coefficients. This signifies that the combination of these main factors yields unique effects on the measured response⁷⁷. In a like manner, the b_{23} coefficient for the TSKGel phase also exerts a unique effect on $K_{sec,sum}$. Lastly, the b_{123} coefficient for the BioSil silica stationary phase and the b_{13} coefficient for the TSKGel polymeric stationary phase are near the origin and therefore do not appreciably influence $K_{sec,sum}$.

Each of the aforementioned coefficients describes the physico-chemical effects controlling the chromatographic properties of the protein-SDS complexes on the two stationary phases. Ideally, SEC is controlled only by the physical dimensions of the solutes and the pores of the stationary phase particles. Equation 1 on p. 10 contains a single term not specifically related to any factor. Examination of this b_0 term reveals that it may be the descriptor for physical properties of the stationary phase or more specifically

the pore volume of the stationary phase. The stationary phases employed in this study each contained 10 μm particles with different pore sizes. The BioSil SEC-250 silica stationary phase has an average pore diameter of 250 $\text{\AA}$, and the TSKGel 4000PW_{xL} polymeric stationary phase has an approximate pore diameter of 500 $\text{\AA}$. The ratio of pore diameters for these two phases is 0.50=BioSil/TSKGel. Similarly, a ratio of the PC₁ component of the b_0 coefficients for $K_{\text{sec, sum}}$ determined on these stationary phases is 0.55=TSKGel/BioSil. This inverse ratio was used for the pore volume because K_{sec} is inversely related to the pore volume. Further experimental work is obviously required to confirm these new findings.

4.2.2 N_{sum}

The eigenvectors determined for N_{sum} on the polymeric and silica based stationary phases are shown as Eq. 21-23 and 24-26 respectively. The associated eigenvalues are shown at the end of each equation.

$$PC_1 = 0.5526x_1 + 0.5861x_2 + 0.5925x_3 \quad \lambda_1 = 2.7475 \quad (21)$$

$$PC_2 = 0.8300x_1 - 0.4514x_2 - 0.3276x_3 \quad \lambda_2 = 0.2334 \quad (22)$$

$$PC_3 = 0.0754x_1 + 0.6729x_2 - 0.7359x_3 \quad \lambda_3 = 0.0192 \quad (23)$$

Here x_1 , x_2 and x_3 refer to the N_{sum} values for collagen, Croda glue and VM gelatin respectively. The first two eigenvectors, Eq. 21 and 22, account for 99.36% of the total variance in the original N_{sum} response data matrix, as determined by Eq. 14 on p. 18. Therefore, Eq. 21 and 22 are applied to the original N_{sum} data to calculate the PC₁ and PC₂ for N_{sum} for the polymeric stationary phase. For the silica based stationary phase:

$$PC_1 = 0.5753x_1 + 0.5815x_2 + 0.5753x_3 \quad \lambda_1 = 2.8782 \quad (24)$$

$$PC_2 = -0.7073x_1 + 0.0003x_2 + 0.7069x_3 \quad \lambda_2 = 0.0813 \quad (25)$$

$$PC_3 = -0.4109x_1 + 0.8136x_2 - 0.4115x_3 \quad \lambda_3 = 0.0404 \quad (26)$$

Similarly, 98.65% of the total variance is accounted for by PC_1 and PC_2 . Eq. 24 and 25 are then used to calculate PC's for the silica-based stationary phase from raw N_{sum} data for the three glues. A PLS linear regression using the qualitative data matrix $[X]$ (see p. 14) and the PC's is applied to derive the b_i coefficients for Eq. 1. These b_i coefficients, which describe the effects of pH, $[CH_3OH]$ and I on the elution properties (N_{sum}) of the three glues on the two stationary phases for the polymeric and silica stationary phases, are shown in Fig. 5. Summary statistics for the PLS linear regression are shown in Table VI.

Table VI Summary statistics for the PLS linear regression of the principal components of N_{sum} .

Stationary Phase	Vector	σ^2	F_{calc}	Degrees of Freedom
TSKGel	PC_1	0.9999	12117	8
TSKGel	PC_2	0.9982	637	8
BioSil	PC_1	0.9979	542	8
BioSil	PC_2	0.9763	47	8

Figure 5. Principal components of N_{mm} for the polymeric ($\square$) and silica (Δ) stationary phases

The variance σ^2 of the regressions all approach 1 while the values of F_{calc} are all greater than F_{crit} ($=3.73$). This implies that the linear model represented by Eq. 1 on p. 10 adequately describes the effect of eluent composition on the retention of the three protein-SDS solutes by SEC on the two stationary phases. The TSKGel phase generally exhibits a better fit to the data than does the BioSil stationary phase as evidenced by its larger σ^2 and F_{calc} values.

The eigenvectors of N_{sum} for PC_1 in Eq. 21 and 24 show that each protein solute makes an equal contribution to PC_1 on both stationary phases. A corresponding trend is not observed for PC_2 in Eq. 22 and 25. On the polymeric phase, the collagen solute contributes a large positive component to PC_2 while Croda glue and VM gelatin lend negatively to PC_2 in Eq. 22. The opposite is evident for the PC_2 vector of the silica stationary phase. Here, collagen contributes negatively to PC_2 while VM gelatin contributes positively and Croda glue makes only a minor positive contribution in Eq. 25.

The main factor regression coefficients b_1 and b_3 for N_{sum} (see Table III on p. 29) are all negative when calculated by the FED method. The corresponding PC_1 components of the b_1 and b_3 coefficients are also negative when calculated by the PCA method (see Fig. 5). Those factors which exert a negative influence on N_{sum} will be situated at negative values of PC_1 on the PC plot in Fig. 5. Similarly those factors which influence N_{sum} in a positive manner will contain positive PC_1 components. The second principal component, PC_2 , may then account for the different mechanisms by which the factors or their interactions affect N_{sum} .

Three clusters of data are immediately obvious upon inspection of Fig. 5. The first cluster contains all of the main factor coefficients (b_1, b_2, b_3) for the BioSil stationary phase (Δ) plus the b_1 coefficient for the TSKGel stationary phase ($\square$). Each of these coefficients has been previously linked (see p. 29) to ion exclusion effects through FED analysis. Thus, the cluster signifies that these factors exert similar ion exclusion effects on N_{sum} . The second cluster contains the b_2 term for the TSKGel stationary phase and the

interaction coefficients b_{13} and b_{123} for the BioSil stationary phase. The TSKGel b_2 term is associated with hydrophobic adsorption effects. The net effect of the interaction of pH-I and pH-[CH₃OH]-I on N_{sum} for the silica phase is then similar in magnitude to this hydrophobic effect on the TSKGel phase.

The b_{12} and b_{23} terms describing the BioSil phase both contain components which are related to ion exclusion (pH, I). Both also contain a common element of [CH₃OH]. These are all related to ion exclusion effects on the BioSil stationary phase (see section 2.4.1.2) and thus their clustering is not surprising.

The b_3 coefficient for the polymeric stationary phase is linked to the hydrophobic adsorption of the protein-SDS solutes while the b_3 coefficient for the silica stationary phase describes ionic exclusion effects. These opposite effects are readily observed by the location of this coefficient in opposite quadrants for the two stationary phases.

One of the most important observations of the PC plot in Fig. 5 is that most binary or tertiary factor-factor interaction coefficients for the polymeric stationary phase are not clustered near the main factor coefficients from which they are derived. This is a strong indication that the factors represented by these interaction coefficients affect N_{sum} in unique fashions that are not correlated to the main factors⁵⁷. Thus, the b_{12} , b_{23} and b_{123} coefficients for this phase all result from unique interaction due to the combination of the individual factors. This further indicates that unique separations may be possible with this stationary phase.

The first principal component, PC₁, contains the majority of the variance of the data set and represents the best one dimensional model of the system under investigation. Collagen, Croda glue and VM gelatin each rendered almost identical contributions to PC₁ for the polymeric and silica stationary phases for N_{sum} . Thus, although each solute demonstrates moderately different behaviour under diverse operating conditions, the PC₁ eigenvectors suggest that they are indeed very similar. A two dimensional PCA study⁷⁸ has shown that the three solutes Type I collagen, Croda glue and VM gelatin are almost

identical with respect to their interactions with both polymeric and silica based stationary phases by SEC.

2.5 Conclusions

Both the FED and PCA data illustrate major differences in the mechanism responsible for the separation of the three protein-SDS solutes on the TSKGel polymeric and the BioSil silica phases. The polymeric stationary phase shows ion exclusion properties similar to the silica stationary phase. The hydrophobicity of this former phase makes possible unique separations of the solutes by merely altering the pH and the ionic strength of the eluent. As ionic strength plays a significant role in the separation of protein-SDS complexes, eluents of relatively high ionic strength (0.4 M) should be avoided to prevent hydrophobic adsorption of the solutes onto the stationary phase. Similarly, these effects may be countered by added CH_3OH in the eluent. In comparison, the BioSil silica stationary phase showed no significant impact from $[\text{CH}_3\text{OH}]$ in the eluent, other than a possible increase in the size of the protein-SDS complexes due to the replacement of H_2O by CH_3OH in its internal structure.

Croda glue and collagen exhibit maximum separation efficiencies on the TSKGel polymeric stationary phase while VM gelatin yield maximum separation efficiency on the BioSil silica stationary phase. Maximum values of N_{sum} are all realized at low values of $[\text{CH}_3\text{OH}]$ (0%) and I (0.1 M) for all solutes on both stationary phases. With the exception of Croda Glue on the polymeric stationary phase, maximum N_{sum} on both stationary phases appears at $\text{pH}=4$ for all solutes.

The application of qualitative FED provides a robust chemometric approach to map the various secondary retention effects in SEC. The utility of the FED/PLS approach to map a SEC system has been demonstrated to yield the maximum amount of information with a minimum number of experiments, while providing statistical validation of the optimum experimental conditions (within the framework of the FED). A rigorous quantitative model incorporating higher-order effects may reveal further information on

SEC separation mechanisms. Such a model, however, requires additional factor levels in a 3^3 FED design.

The PCA analysis provides an insight into the mechanism of SEC separation of the three solutes on different stationary phases. The significance of factor-factor interactions on the separation was clearly demonstrated by the PC plots of both $K_{\text{sec,sum}}$ and N_{sum} . In both cases, unique contributions to the separation were demonstrated by the interaction of the various factors. Thus, what may not be apparent with a rigorous FED/PLS analysis is readily observed by PCA/PLS analysis of the raw data. Also, new information related to the pore volumes of the stationary phases may be deduced from PCA results.

CHAPTER 3 PRE-SEPARATION OF ANIMAL GLUES FROM SYNTHETIC ZINC ELECTROLYTES

3.1 Introduction

A rapid, facile method to analyze glue addition reagents in authentic zinc electrolytes requires that sample pre-treatment is minimal. Direct injection of these electrolytes without extensive sample work up is preferred. However, zinc electrolytes typically contain up to 2 M H_2SO_4 and 125 g/L ZnSO_4 . Separation by SEC employing silica-based stationary phases is thus not possible without some form of sample pre-separation. Direct injection of zinc electrolytes bearing animal glues may be possible with polymeric stationary phases as they are considerably more stable to extremes of pH, ionic strength, and organic modifiers. Direct injection of a sample containing 100 mg/L Croda glue in 1.8 M H_2SO_4 , neutralized to pH 7 with NaOH, onto the polymeric phase illustrates poor resolution of the glue from the sulphate peak (Fig. 6). Methods to reduce the acidity of samples and remove excess ZnSO_4 are therefore required. Since the nominal concentration of animal glues in zinc electrolytes is generally in the range 1-30 mg/L, pre-concentration of the animal glues is also necessary. Ultra-filtration is typically used to separate macromolecules from small-molecular-weight species. Unfortunately, sample processing is unacceptably lengthy requiring approximately 1 hour per sample¹⁹. Also, adsorption of macromolecules onto the ultra-filtration membrane cannot be ruled out. Alternative methods to remove animal glues from synthetic zinc electrolytes will therefore be examined.

Figure 6. Chromatogram of 100 mg/L Croda glue in 1.8 M H_3SO_4 injected onto the TSKGel 4000PW_{XL} stationary phase

3.1.1 Pre-separation by Chromatographic Methods

Solid phase extraction (SPE) is rapidly becoming a routine method to remove analytes from a harsh matrix which may interfere with the final analysis. At the same time, the analyte may be concentrated by one or two orders of magnitude to bring the analyte concentration in the final sample to the working range of the analytical technique. Although SPE is commonly employed for numerous inorganic, organic, protein and biopolymer species, removal of collagen from aqueous solution has not yet been reported in the literature. SPE offers two distinct advantages over traditional solvent extraction techniques; namely, no organic solvents are required and sample preparation time is typically on the order of a few minutes. The separation of proteins from aqueous solution may be performed by four chromatographic techniques:

- size exclusion
- cation exchange
- anion exchange
- hydrophobic interaction

Unfortunately, there are also numerous drawbacks to the application of these methods to the extraction of collagen from zinc electrolytes. Size exclusion methods, more commonly referred to as de-salting, tend to dilute the concentration of protein in the collected eluent. Strong multi-point attachment of biopolymers onto cation or anion exchange chromatographic supports would require an eluent of high ionic strength and extreme pH for their complete removal. Therefore, although the sample may be pre-concentrated, the final sample matrix would still be at an undesirably extreme pH and high ionic strength. Initial attempts to remove animal glue solutes from synthetic zinc electrolytes (1.8 M H_2SO_4 , 125 g/L ZnSO_4) using a BioRad (Mississauga, Ontario) MacroPrep HIC C1 hydrophobic interaction stationary phase failed due to irreversible adsorption of the animal glues.

3.1.2 Pre-separation by Solvent Extraction

Solvent extraction (SX) is normally not the preferred method for sample pre-concentration since large volumes (10-500 mL) of solvent are typically required. Also, SX techniques generally demand a greater amount of sample handling than SPE thus increasing the potential for error in the overall procedure. However, considering that ultra-filtration methods require up to one hour to process a single sample¹⁹ and SPE methods are not applicable and that previous work has extracted glues from copper electrolytes into chlorinated organic solvents⁷⁹, SX methods present the only alternative. A method to quantitatively remove soluble and hydrophobic proteins from dilute protein solutions in the presence of salts and detergents via SX has been reported⁸⁰. In aqueous solutions of pH<1, protein-SDS complexes are more hydrophobic than in neutral solutions due to protonation of $-\text{SO}_3^-$ groups. Extraction of these complexes into polar organic phases is hence feasible and also practical since the SEC protocol can separate protein-SDS complexes.

3.2 Experimental

3.2.1 Reagents

Zinc sulphate was purchased from BDH (Toronto, Ontario). A synthetic electrolyte solution was prepared by dissolving 123 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 56 mL H_2SO_4 (18 M) in 500 mL of water to give final concentrations of 2.0 M H_2SO_4 and 137 g/L ZnSO_4 . A 9 mL aliquot of this synthetic electrolyte diluted to a volume of 10 mL yields a solution of 125 g/L Zn and 1.8 M H_2SO_4 .

Chloroform, acetone and methanol were high purity grade (Burdick and Jackson, Pointe-Claire, Québec). Sulphuric acid was obtained from Baker (Toronto, Ontario). A protein assay kit obtained from BioRad (Mississauga, Ontario) was used to determine

protein concentrations in the extracts. De-ionized water was used for all aqueous solutions.

3.2.2 Apparatus

A Phillips, Pye Unicam PU 8610 UV/VIS spectrophotometer (Montreal, Québec) with matching 1.0 cm cells was used to determine protein concentrations.

3.2.3 Procedure

3.2.3.1 Literature Protocol⁸⁰

A 1 mL aliquot of the Croda glue or VM gelatin solution was added to a 9 mL aliquot of the synthetic electrolyte solution in a 125 mL separatory funnel. After stirring the solution, 40 mL of CH_3OH was added. The solution was then shaken for 30 s and a 10 mL aliquot of CHCl_3 added. After shaking for a further 30 s, 30 mL of H_2O was added. The solution was shaken and the phases were allowed to separate. The lid to the separatory funnel was then removed and rinsed with 1.0 mL of CHCl_3 . After draining the organic phase into a 25 mL volumetric flask, an additional 1 mL aliquot of CHCl_3 was then added to the separatory funnel as a rinse. A total of 3 rinses with CHCl_3 was employed. The organic phase was drained after each addition of CHCl_3 into the 25 mL volumetric flask. The flask was then sealed with a septum and the CHCl_3 removed under vacuum. All dry samples were stored at -4°C until ready for analysis. Blank solutions were prepared by substituting the 1.0 mL aliquot of glue or gelatin solution with a 1.0 mL aliquot of H_2O . These blanks were then treated in an identical fashion as the synthetic electrolyte samples. All extractions were performed in triplicate.

3.2.3.2 Solvent extraction protocol

A 1 mL aliquot of the Croda glue or VM gelatin solution was added to a 9 mL aliquot of the synthetic electrolyte solution in a 60 mL separatory funnel and the solution shaken. A 350 μ L aliquot of SDS stock solution (100 mM) was then added and the solution again stirred. Chloroform (2 mL) and acetone (1 mL) were then added sequentially and the mixture shaken for 0.5-3 min (acetone was used to facilitate phase separation). The lid to the separatory funnel was then removed and rinsed with 1.0 mL of CHCl_3 . After draining the organic phase into a 10 mL volumetric flask, additional CHCl_3 was then added to the separatory funnel either as a rinse (1 mL) or a second extraction step (2 mL). The organic phase was drained after each addition of CHCl_3 into the 10 mL volumetric flask. The flask was then sealed with a septum and the CHCl_3 removed under vacuum. Blank solutions were prepared by substituting the 1.0 mL aliquot of glue or gelatin solution with a 1.0 mL aliquot of H_2O . These blanks were then treated in an identical fashion as the synthetic electrolyte samples. All extractions were performed in triplicate.

3.2.3.3 Total Protein Analysis

Samples prepared via the two protocols described above were re-dissolved in 10 mL of solution containing NaCl and a phosphate buffer ($I=0.15$ M, $\text{pH}=7$). In some cases the samples were heated at 60 $^{\circ}\text{C}$ for 10 minutes to aid dissolution. These re-constituted extract samples were cooled to ambient temperature before protein analysis. A summary of the BioRad protein assay protocol is as follows. A working reagent A' was prepared by adding 20 μ L of reagent S to each 1.0 mL of reagent A required. A 200 μ L aliquot of the re-constituted extract was transferred to a clean, dry test tube and 100 μ L of working reagent A' added. A 800 μ L aliquot of reagent B was then added to each test tube, the solution stirred vigorously and the sample left to develop for 15

minutes. Blank solutions were prepared by treating a 200 μ L aliquot of the SEC eluent in a similar manner.

Standard solutions were prepared by adding a 100 μ L aliquot of the 1.0 g/L stock Croda glue or VM gelatin solution to a separate test tube followed by a 1.0 mL aliquot of the blank solution (total volume 1.1 mL). A 200 μ L aliquot of this solution was then treated for the protein assay as described above.

Protein concentrations of extract samples were also determined from the absorbance at 750 nm of the standard solution. This method has a lower detection limit of 5 mg/L total protein and can tolerate SDS concentrations up to 10%.

3.3 Results and Discussion

The literature protocol furnished low protein recoveries. Croda glue and VM gelatin gave 2.9% ($\pm 5.0\%$) and 3.2% ($\pm 3.9\%$) protein recoveries respectively from the synthetic zinc electrolyte. Also, this method reportedly yielded solid protein precipitates in the lower, organic phase which were not observed as was expected.

Using the SDS extraction protocol the optimum extraction time for Croda glue and VM gelatin from the synthetic electrolyte was 1 min as shown in Table VII.

Table VII. The effect of extraction time on protein recovery from synthetic zinc electrolytes

Extraction Time (min)	% Protein Recovery	
	Croda Glue	VM Gelatin
0.0	0.0	0.0
0.5	89.9 $\pm$ 10.8	80.5 $\pm$ 14.5
1.0	110.6 $\pm$ 12.1	92.9 $\pm$ 2.7
2.0	71.6 $\pm$ 7.9	29.9 $\pm$ 6.8
3.0	48.7 $\pm$ 2.8	33.6 $\pm$ 4.4

Virtually all protein can be extracted from the synthetic electrolyte. Both proteins, however, show a decrease in extraction after 1 min that may be due to preferential extraction of SDS after longer extraction times. Nevertheless, the objective requirement to achieve the maximum percent protein recovery within the shortest time is attained.

3.4 Conclusions

All subsequent experiments with simulated electrolysis cells employed a single 1 min extraction followed by three rinses with 1.0 mL aliquots of CHCl_3 for both Croda glue and VM gelatin.

CHAPTER 4 SEC ANALYSIS OF CRODA GLUE AND VIELLE-MONTAGNE GELATIN FROM A SIMULATED ZINC ELECTROLYSIS CELL

4.1 Introduction

Industrial electrowinning cells for zinc electrolysis operate at 38-40 °C with a current density of 400-540 A/m² and current efficiencies of 86%-94%. Anodes are usually made of lead while cathodes are aluminum. The electrolytes contain 1.5-2.0 M H₂SO₄, 125 g/L ZnSO₄ and low concentrations (0.01-10 mg/L) of Co, Cu, Cd, Ge, Ni, Cl⁻ and F⁻. Glue additives are employed at concentrations of 1-30 mg/L in solution and apparently reside in the electrolysis cell for approximately 45 minutes⁸².

There is currently little information regarding the kinetics of degradation of animal glues in zinc electrolytes. Previous work has shown a correlation between the molecular mass of the glue additives and their activity in copper electrorefining circuits^{12,19}. Similar theories have been assumed for zinc electrolysis. Unfortunately, the accurate determination of the molecular mass of glues in electrolytes, either copper or zinc, has yet to be reported. Furthermore, the active species in zinc electrolysis cells has yet to be identified or characterized. Thus, the majority of data relating the molecular weight or molecular structure of these additives to their activity are speculative and based on measurements of the effects of additives rather than their concentrations or molecular speciation. Recently, animal glues have been reported to decrease to 10% of their original concentration within 4 hours of industrial copper electrolysis⁸¹. It was argued that since industrial glues in sulphuric acid at 65 °C retain their activity for 5 hours, these glues should retain their activity for at least 20 hours at the lower temperatures used in zinc

electrolysis (38-40 °C). The accurate measurement of these additives in simulated and real electrolysis cells is obviously necessary to support further development of the theories related to their activity in zinc electrolysis.

The object of this final work is to apply the developed solvent extraction (SX) and SEC protocols to monitor the changes in concentrations of two glue species in synthetic zinc electrolytes. It also examines the measurement of the molecular masses of Croda glue and VM gelatin in neat aqueous solutions and in synthetic zinc electrolytes. Errors produced by conventional SEC calibration curves will be disclosed.

4.2 Experimental

4.2.1 Reagents

Molecular weight markers were purchased either from Sigma (St. Louis, MO) or BioRad (Mississauga, Ontario).

4.2.2 Apparatus

The electrolysis cell consisted of a water jacketed 600 mL beaker as shown in Fig. 7. A Plexiglas cover was used to hold the two Pt anodes, an Al cathode and a sampling pipette in place. The cathode had a surface area of 13 cm² exposed to the zinc electrolyte and the anodes were each positioned 3 cm from the cathode. A Haake D8 circulator ran water from a heated Haake L bath to the jacketed cell through tygon tubing. A constant temperature of 39.0 ± 0.1 °C was maintained for all experiments. The output current of a Hewlett Packard 6282A power supply was measured with a HP 3465A digital ammeter during electrolysis.

Figure 7. Electrolysis cell

4.2.3 Procedure

A solution containing 125 g/L ZnSO_4 and 1.8 M H_2SO_4 was prepared by first dissolving 110 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 300 mL H_2O followed by adding 50 mL of H_2SO_4 (18 M). The solution was cooled to room temperature and transferred to a 500 mL volumetric flask. A 50 mL aliquot of either a Croda glue or VM gelatin stock solution (1000 mg/L) was then added and the solution diluted to 500 mL. This simulated zinc electrolyte was placed in the electrolysis cell and allowed to equilibrate to 39.0 ± 0.1 °C over approximately 15 min. A constant stirring rate of approximately 500 rpm was used to ensure a homogeneous and uniform solution. The first sample, labeled $t=0$, was taken immediately before the electrolysis was initiated. A constant electric current of 0.581 A ($=450 \text{ A} \cdot \text{m}^{-2}$) was then applied for 2 hr. Samples were withdrawn at regular 10-15 min intervals with a sampling pipette and transferred to a 60 mL separatory funnel. The SDS extraction (SX) protocol developed in section 3.3 was then utilized. After 120 min the power was turned off; the electrodes were removed and rinsed with distilled water.

Solutions of molecular weight markers were prepared by dissolving 10-30 mg of each protein in 10 mL of SEC eluent and heating the solution at 80 °C for 10 min to ensure complete denaturation. No reagent was added to reduce the protein -S-S- bonds since any changes in conformation (and hence elution volume) due to the lack of rotational freedom about these bonds would be minimal⁷⁸. Calibration curves were prepared for the polymeric (TSKGel 4000PW_{XL}) column only. Total column and void volumes were determined with nitrate and blue dextran, respectively. A constant flow rate of 0.8 mL/min and an injection loop volume of 100 μL were used for all experiments.

4.3 Results and Discussion

4.3.1 SEC of Glues in Zinc Electrolysis Cells

Chromatograms of Croda glue and VM gelatin standards in H_2O and in the electrolyte during 120 minutes of zinc electrolysis are shown in Fig. 8 and 9, respectively. It appears in Fig. 8 that H_2SO_4 itself has a dramatic affect on Croda glue by the rapid disappearance of the peaks at $K_{sec}=0.05$ and $K_{sec}=0.38$. This is probably due to the denaturation of the triple helical and β conformations into the α strands. After 60 minutes of electrolysis, a single major peak for the α strands is evident at $K_{sec}=0.60$. The chromatograms of VM gelatin in Fig. 9 show a similar major peak at $K_{sec}=0.60$. This strongly suggests that the active component for both Croda glue and VM gelatin are identical and are probably the α conformation of collagen.

Degradation curves for Croda glue and VM gelatin shown in Fig. 10 and 11, have been constructed using the areas of the $K_{sec}=0.60$ peaks. It is evident in Fig. 10 that the concentrations of both active compounds dropped to approximately 10% of the original concentrations after approximately 120 min in the zinc electrolysis cell. Croda glue showed an initial normalized concentration of 0.57 at $t=0$ min, which is below that obtained after only a few minutes of electrolysis. This may be due to the denaturation of the triple helix complex into the three smaller α sub-units as mentioned above. As the triple helix denatures producing the three α collagen species, the concentration of the glue component at $K_{sec}=0.60$ increases. This is readily seen in Fig 10 where at $t=0$ min the initial concentration of the Croda glue component is less than that at $t=1$ min. Monitoring of Croda glue activity in copper electrolytes by a galvanostatic method had revealed a similar increase in the activity of the glue during the first 30 minutes of electrolysis¹² followed by a steady decrease at longer times, up to 6 hours.

Figure 8. SEC chromatograms of Croda glue for different times during zinc electrolysis

Figure 9. SEC chromatograms of VM gelatin for different times during zinc electrolysis

Figure 10. Degradation curve for Croda glue during zinc electrolysis

Figure 11. Degradation curve for VM gelatin during zinc electrolysis

These two pieces of evidence suggest that this species at $K_{\text{sec}}=0.60$ may be the active glue species in zinc electrolysis.

A similar trend is not observed in Fig. 11 for VM gelatin since there was no evidence of the larger molecular weight (i.e. $K_{\text{sec}} < 0.60$) species at any time during the electrolysis. The concentration of VM gelatin at $K_{\text{sec}}=0.60$ decreased to 42% of the initial concentration within 1 minute of electrolysis. The lack of any triple helical species for VM gelatin does not have any appreciable effect on the rate of active component degradation in zinc electrolysis.

The kinetics of glue degradation can be described by first-order reaction kinetics with respect to glue concentration:¹²

$$\ln C = \ln C_0 - kt \quad (27)$$

where C is the concentration of glue at time t , C_0 is the initial concentration of glue at $t=0$, and k is the rate constant. First order reaction kinetic curves for the degradation of Croda glue and VM gelatin, shown in Fig. 12 and 13, have been constructed using the areas of the $K_{\text{sec}}=0.60$ peaks and initial glue solution concentrations of 100 mg/L. The calculated k values for Croda glue and VM gelatin are 0.0215 min^{-1} and 0.0082 min^{-1} respectively. Correlation coefficients of 0.99 and 0.70 were realized for the linear fits for Croda glue and VM gelatin respectively. In comparison, degradation of Croda glue in a simulated copper electrolyte¹² yielded $k=0.008 \text{ min}^{-1}$. This lower value of k for the copper electrolyte indicates that Croda glue degrades at a higher rate in zinc electrolysis than in copper electrolysis. The higher rate in turn explains why the glue in zinc electrolysis was observed to retain its activity for 2-3 hr only, instead of the 20 hr predicted in the Introduction on page 54, even though the temperature of 39°C was lower than the 65°C previously reported for the industrial copper electrolysis.

Figure 12. Kinetics of degradation for Croda glue during zinc electrolysis

Figure 13. Kinetics of degradation for VM gelatin during zinc electrolysis

4.3.2 Determination of the Molecular Mass of Croda Glue and VM Gelatin in Zinc Electrolysis Cells

The molecular weight markers, their masses in the natural and denatured states, and their K_{sec} values are shown in Table VIII. A calibration curve constructed with these markers is shown in Fig. 14.

Table VIII Standard calibration of K_{sec} for Croda glue and VM gelatin analysis.

Molecular Marker	Molecular Mass (kD)		log(Molecular Mass)	K_{sec}
	Natural State	Denatured		
Blue dextran	2,000,000	-----	6.30	0
Thyroglobulin	670,000	335,000	5.53	0.03
Bovine serum albumin	66,000	66,000	4.82	0.39
λ globulin	158,000	50,000	4.70	0.22
Ovalbumin	44,000	44,000	4.64	0.36
Alcohol dehydrogenase	150,000	36,200	4.56	0.38
Carbonic anhydrase	29,000	29,000	4.46	0.40
Apoferitin	443,000	18,500	4.27	0.50
Equine myoglobin	17,000	17,000	4.23	0.52
Cyanocobalamin	1,350	1,350	3.13	1.57
Nitrate	62	-----	1.79	1
Croda Glue	262,033*	-----	5.42*	0.026
Croda Glue	34,058*	-----	4.53*	0.40
Croda Glue	15,492*	-----	4.19*	0.60
VM Gelatin	13,825*	-----	4.14*	0.60

* values calculated from calibration data.

The K_{sec} for cyanocobalamin of 1.57 illustrates the hydrophobic adsorption characteristics of the polymeric stationary phase and was not included in the calibration plot. The slope of the linear calibration curve was -2.38 with a correlation coefficient of 0.93.

Figure 14. Calibration curve for the determination of molecular masses of Croda glue and VM gelatin

Calculated molecular masses previously reported for Croda glue species and VM gelatin range from 13.8 kD to 262 kD. Considering that these additives are similar in structure to Type I collagen, their molecular masses should be similar to that of collagen. In the natural state Type I collagen is a trimeric structure with a molecular mass of 288 kD^{22,31}. In the denatured state the molecular mass is 192 kD for the dimeric β conformation or 96 kD for the monomeric α conformation^{30,38}. The molecular masses determined experimentally for Croda glue and VM gelatin shown in Table VIII are relatively low probably due to an error in the calibration of the polymeric column with the selected molecular weight markers. The mechanism of SEC depends upon the conformation of the protein as it diffuses through the pores of the stationary phase. If the conformations of the proteins employed for calibration and that of the unknown are not similar, inaccurate molecular mass determinations will result. Note that, the FED and PCA data in Chapter 2 have both revealed the existence of ionic and hydrophobic retention mechanisms for Croda glue and VM gelatin on the polymeric stationary phase. Thus, accurate molecular mass determinations are difficult if not impossible to achieve. In fact, errors associated with the determination of the molecular mass of Type I collagen on silica based stationary phases as high as 40% have been reported³⁰.

Nevertheless, comparison of SEC chromatograms of extracts of Croda glue and VM gelatin from zinc electrolytes with that of a denatured sample of Type I collagen standard, as shown in Fig. 15, suggests that the prevalent active species in synthetic zinc electrolyte is the monomeric or α form of collagen. As reported previously, the molecular mass of this protein is 96 kD, yielding errors of 84% and 86% for the determination of the molecular weight of Croda glue and VM gelatin respectively in Table VIII.

Figure 15. SEC chromatograms of Type I collagen, Croda glue and VM gelatin in their denatured states

4.4 Conclusions

This final study has shown that the industrial additives, Croda glue and VM gelatin, degrade to approximately 10% of their initial concentration after 2 hours of electrolysis when decomposition to smaller molecular weight species occurs. Calibration of the polymeric, TSKGel 4000PW_{XL}, SEC stationary phase with traditional molecular weight markers has yielded errors of 86% in the M_r determination for the two additives. The active glue species in zinc electrolysis is tentatively identified as the α conformation of collagen. Corroboration of SEC data with spectroscopic (FTIR, NMR) and chemical analysis (CHNO) is still required in future work.

CLAIMS TO ORIGINAL RESEARCH

1. Development of a protocol to determine elution characteristics, non-ideal effects and mechanistic behaviour in size exclusion chromatographic (SEC) separations.
2. Application of factorial experimental design (FED) to the optimization of chromatographic conditions for the SEC of biopolymers in denaturing eluents. Demonstration of the importance of factor-factor interactions in the design of SEC experimentation strategies.
3. Application of principal component analysis (PCA) to examine the elution characteristics of unknown, industrial biopolymers by denaturing SEC.
4. Development of a rapid, facile SDS extraction method for pre-separation, and potentially, pre-concentration of animal glues in zinc electrolytes.
5. Development of a facile SEC method for routine analysis of glue additives in zinc electrolytes.
6. Characterization of the degradation behaviour of glue additives during zinc electrolysis.
7. Chromatographic Identification of the active glue species during zinc electrolysis.

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APPENDIX A Simplified process flow diagram for the refining and electrowinning of zinc.

**APPENDIX B Chromatograms of Collagen for eight experimental conditions on
two stationary phases**

Eluent Number	Eluent Composition		
	pH	[CH ₃ OH] (% v/v)	I (M)
1	7	15	0.4
2	7	15	0.1
3	7	0	0.4
4	7	0	0.1
5	4	15	0.4
6	4	15	0.1
7	4	0	0.4
8	4	0	0.1

Figure B1. Chromatograms of Collagen eluted from the BioSil SEC-250 silica stationary phase at pH=7

2 of/de 2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
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PRECISIONSM RESOLUTION TARGETS

Figure B2. Chromatograms of Collagen eluted from the BioSil SEC-250 silica stationary phase at pH=4

Figure B3. Chromatograms of Collagen eluted from the TSKGel PW4000- μ L polymeric stationary phase at pH=7

Figure B4. Chromatograms of Collagen eluted from the TSKGel PW4000_{IL} polymeric stationary phase at pH=4

APPENDIX C Chromatograms of Croda Glue for Eight Experimental Conditions on Two Stationary Phases

Figure C1. Chromatograms of Croda glue eluted from the BioSil SEC-250 silica stationary phase at pH=7

Figure C2. Chromatograms of Croda glue eluted from the BioSil SEC-250 silica stationary phase at pH=4

Figure C3. Chromatograms of Croda glue eluted from the TSKGel PW4000_{IL} polymeric stationary phase at pH=7

Figure C4. Chromatograms of Croda glue eluted from the TSKGel PW4000_{HL} polymeric stationary phase at pH=4

APPENDIX D Chromatograms of VM Gelatin for Eight Experimental Conditions on Two Stationary Phases

Figure D1. Chromatograms of VM gelatin eluted from the BioSil SEC-250 silica stationary phase at pH=7

Figure D2. Chromatograms of VM gelatin eluted from the BioSil SEC-250 silica stationary phase at pH=4

Figure D3. Chromatograms of VM gelatin eluted from the TSKGel PW4000_{XL} polymeric stationary phase at pH=7

Figure D4. Chromatograms of VM gelatin eluted from the TSKGel PW4000_{XL} polymeric stationary phase at pH=4

APPENDIX E FED main factor and factor-factor interaction coefficients calculated for $K_{sec,Sum}$

Sample	Column	F _{calc}	pH			CH ₃ OH			pH-CH ₃ OH-I			pH-CH ₃ OH-I		
			b ₀	b ₁	b ₂	b ₃	b ₁₂	b ₁₃	b ₂₃	b ₁₂₃	b ₁₂₃	b ₁₂₃	b ₁₂₃	b ₁₂₃
Collagen	TSKGel	13860	0.231	-0.061	-0.022	0.059	0.019		0.011		-0.032			
	BioSil	268	0.217	-0.076	0.051	0.064	-0.022	-0.038	0.027		-0.015			
Croda	TSKGel	101	0.242	-0.015	-0.014	0.050		-0.010	-0.032					
	BioSil	15	0.239	-0.031	0.035	0.038	-0.033		0.014					
VM	TSKGel	61	0.249	-0.004	0.007	0.014			-0.018					
	BioSil	69	0.249	0.007	0.019	0.014								

APPENDIX F FED main factor and factor-factor interaction coefficients calculated for N_{sum}

Sample	Column	F_{calc}	pH			pH- CH ₃ OH			pH-I			CH ₃ OH- I			pH-CH ₃ OH-I		
			b_0	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{123}	b_0	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}
Collagen	TSKGel	208	0.151	-0.095	-0.017	-0.042	0.028	0.017	0.127	-0.106							
	BioSil	67	0.243	-0.021	-0.013	-0.015	-0.006	0.034	0.031	0.024							
Croda	TSKGel	4365	0.197	-0.050	0.009	-0.077	-0.052		0.111								
	BioSil	133	0.233	-0.044	-0.043	-0.062	-0.021	0.010	-0.008	0.010							
VM	TSKGel	1312	0.205	-0.048	0.039	-0.051	-0.026	0.009	0.115	-0.012							
	BioSil	212	0.220	-0.068	-0.073	-0.044	0.008	0.006	0.044	-0.017							

END

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FIN